RESEARCH ARTICLE

Hepatocyte nuclear factor 4α negatively regulates connective tissue growth factor during liver regeneration

Junmei Zhou1,2 | Xiaowei Sun1,3 | Lu Yang4 | Liqun Wang5 | Gai Ran1,6 | Jinhui Wang4 | Qi Cao7 | Lizi Wu8 | Andrew Bryant5 | Chen Ling1,6 | Liya Pi1

1Department of Pediatrics, University of Florida, Gainesville, FL, USA
2Institute of Cardiovascular Disease, Key Laboratory for Arteriosclerology of Hunan Province, University of South China, Hengyang, China
3Institute of Pathology, School of Basic Medical Sciences, Lanzhou University, Lanzhou, China
4Integrative Genomics Core, Beckman Research Institute of the City of Hope, Duarte, CA, USA
5Department of Medicine, University of Florida, Gainesville, FL, USA
6State Key Laboratory of Genetic Engineering, School of Life Sciences, Zhongshan Hospital, Fudan University, Shanghai, China
7Department of Diagnostic Radiology and Nuclear Medicine, University of Maryland School of Medicine, Baltimore, MD, USA
8Department of Microbiology & Molecular Genetics, College of Medicine, University of Florida, Gainesville, FL, USA

Correspondence
Chen Ling, State Key Laboratory of Genetic Engineering, School of Life Sciences, ZhongShan Hospital, Fudan University, Shanghai 200438, China. Email: lingchenchina@fudan.edu.cn
Liya Pi, Department of Pediatrics, University of Florida College of Medicine, 1200 S Newell Drive, Gainesville, FL 32610, USA. Email: lpi@peds.ufl.edu

Funding information
HHS | NIH | National Institute on Alcohol Abuse and Alcoholism (NIAAA), Grant/Award Number: KO1AA024174 and R01AA028035; Children Miracle Network Foundation; National Key Research and Development Program of China, Grant/Award Number: 2018YFA0109400; Shanghai Sailing Program, Grant/Award Number: 17YF1401300; Shanghai Eastern Scholarship, Grant/Award Number: TP2016004

Abstract
Liver regeneration after injury requires fine-tune regulation of connective tissue growth factor (Ctgf). It also involves dynamic expression of hepatocyte nuclear factor (Hnf)4α, Yes-associated protein (Yap), and transforming growth factor (Tgf)-β. The upstream inducers of Ctgf, such as Yap, etc, are well-known. However, the negative regulator of Ctgf remains unclear. Here, we investigated the Hnf4α regulation of Ctgf post-various types of liver injury. Both wild-type animals and animals contained siRNA-mediated Hnf4α knockdown and Cre-mediated Ctgf conditional deletion were used. We observed that Ctgf induction was associated with Hnf4α decline, nuclear Yap accumulation, and Tgf-β upregulation during early stage of liver regeneration. The Ctgf promoter contained an Hnf4α binding sequence that overlapped with the cis-regulatory element for Yap and Tgf-β. The upstream inducers of Ctgf, such as Yap, etc, are well-known. However, the negative regulator of Ctgf remains unclear. Here, we investigated the Hnf4α regulation of Ctgf post-various types of liver injury. Both wild-type animals and animals contained siRNA-mediated Hnf4α knockdown and Cre-mediated Ctgf conditional deletion were used. We observed that Ctgf induction was associated with Hnf4α decline, nuclear Yap accumulation, and Tgf-β upregulation during early stage of liver regeneration. The Ctgf promoter contained an Hnf4α binding sequence that overlapped with the cis-regulatory element for Yap and Tgf-β. Ctgf loss attenuated inflammation, hepatocyte proliferation, and collagen synthesis, whereas Hnf4α knockdown enhanced Ctgf induction and liver fibrogenesis. These findings provided a new mechanism about fine-tuned regulation of Ctgf through Hnf4α antagonism of Yap and Tgf-β activities to balance regenerative and fibrotic signals.

KEYWORDS
connective tissue growth factor (Ctgf), hepatocyte nuclear factor 4α (Hnf4α), liver injury, liver regeneration

Abbreviations: αSMA, α smooth muscle actin; CCl4, carbon tetrachloride; ChIP, chromatin immunoprecipitation; CD, cluster of differentiation; Ctgf, connective tissue growth factor; DNA, deoxyribonucleic acid; DR, direct repeat; GS, glutamine synthetase; HCC, hepatocellular carcinoma; Hnf4α, hepatocyte nuclear factor 4α; IHC, immunohistochemistry; IL1α, interleukin 1α; mRNA, messenger ribonucleic acid; PH, partial hepatectomy; PCR, polymerase chain reaction; SEAP, secreted alkaline phosphatase; siRNA, small interfering ribonucleic acid; TEAD, transcriptional enhanced associate domain; Tgf-β, transforming growth factor-β; Tnfr, tumor necrosis factor receptor; Yap, yes-associated protein.

Junmei Zhou and Xiaowei Sun are contributed equally to this work.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

© 2020 The Authors. The FASEB Journal published by Wiley Periodicals LLC on behalf of Federation of American Societies for Experimental Biology.
1 | INTRODUCTION

The liver is a critical metabolic and digestive organ. It exposes to exogenous and endogenous toxins daily, such as alcohol, viruses, etc. In addition, drug-induced liver injury is a leading cause of death worldwide and complicates various drug treatment. Fortunately, the liver possesses an extraordinary ability to regenerate. Liver regeneration is a process of compensatory hyperplasia with hepatocyte replication to restore parenchymal loss. During regeneration, immune cells are rapidly recruited. They induce hepatocyte priming before cell cycle re-entry, followed by waves of proliferation of parenchymal and nonparenchymal cells. At the end of liver regeneration, extracellular matrix is synthesized and deposited onto new tissues. These regenerative processes go awry in chronic liver diseases. Persistent insults cause chronic inflammation, severe hepatocyte damage, and sustained activation of myofibroblast cells. These cells produce excessive amounts of collagen leading to liver fibrosis. If left untreated, cirrhosis and liver cancer may eventually develop. To date, no reliable cue for fibrosis exists. Understanding molecular mechanism governing liver regeneration and liver fibrosis is pre-requisite for many therapeutic interventions that optimize regenerative outcome and avoid scar formation after liver injury.

Increased cell plasticity is a key feature in liver regeneration. For instances, hepatocytes downregulate the epithelial genes and undergo epithelial to mesenchymal transition after partial hepatectomy (PH). Cross-regulatory cascades driven by hepatocyte nuclear factor (Hnf)4α and the Hippo/Yes associated protein (Yap) pathway have been shown to control hepatocyte differentiation and dedifferentiation. Hnf4α globally maintains the hepatocyte differentiation and function via binding to promoter sequences of thousands of genes. Loss of Hnf4α has been found after liver injury or during hepatocellular carcinoma (HCC) development. Re-activation of this transcriptional factor is essential for termination of liver regeneration. In contrast, Yap is ordinarily inactive in cytoplasm via phosphorylation by the Hippo kinases that maintain quiescence in the liver. During liver injury, Yap is activated so that its nonphosphorylated form enters nuclei and binds to members of transcriptional enhanced associate domain (TEAD) family to turn on target genes. Connective tissue growth factor (Ctgf) of the Cyr61/CTGF/Nov protein family is a known Yap target. It promotes HCC through autocrine action. Ctgf protein is also profibrotic. It binds to transforming growth factor (Tgf)-β, leading to enhanced Tgf-β/Smad3 signaling. Overexpression of Ctgf in hepatocytes renders liver susceptibility to fibrogenesis stimuli. Although Ctgf upregulation after liver injury has been reported in experimental and human studies, its fine-tune regulation during liver regeneration still remains elusive. In this paper, we utilized multiple mouse models of liver regeneration and demonstrated Hnf4α antagonism of Yap activities via a novel cis-regulatory element in the Ctgf promoter.

2 | MATERIALS AND METHODS

2.1 | Generation of Ctgf conditional knockouts

All animal protocols were approved by the University of Florida Animal Care and Usage Committee and were conducted in compliance with their guidelines. Ctgf conditional knockouts (Ctgfk/k) were previously published. These mice carried two loxP sites flanking exon 4 of Ctgf (termed Ctgf) gene and one allele of the human ubiquitin C promoter (ubc)-Cre/ERT2 transgene. At 3-week-old age, Ctgfk/k mice carrying ubc-Cre/ERT2 were given IP injection of the tamoxifen suspension (75 mg/kg body weight) over 5 days and the resulting Ctgfk/k mice lost exon 4 of Ctgf in genotyping analysis using primers and PCR condition described previously. One month later, these mice were fed with the Lieber-DeCarli liquid diet (BioServ, Flemington, NJ) containing 1% ethanol for 2 days followed by 2% ethanol for 10 days. Carbon tetrachloride (CCl4, 1 μL/g body weight) was injected through IP at 1 day before the end of experiment.

2.2 | Hepatocyte damage to induce liver regeneration following PH or CCl4 intoxication in combination with or without moderate ethanol exposure

For surgical resection, wild-type mice (n = 35) were subjected to PH by excision of the median and left lateral liver lobes at their stem under aseptic conditions according to previous publication. For CCl4 intoxication in combination with or without ethanol feeding, wild-type or mutant mice (8-10 week old) were subjected to moderate ethanol feeding using the Lieber-DeCarli liquid diet (BioServ, Flemington, NJ) containing 1% ethanol for 2 days followed by 2% ethanol for the duration of the experiment based on previous publication. Isocaloric maltose was administered to a pair-fed cohort. After that, 2% ethanol was fed for the remaining experiments. An average of 13.1 mL of the 2% ethanol-containing diet was consumed per day. Pair-fed mice were given an isocaloric diet in which ethanol calories were substituted with calories from maltose dextrin. Pair-fed animals received a diet volume equivalent to that of their ethanol-fed experimental counterparts on the previous day to ensure equivalent calories were consumed between groups. No differences were seen in final body weight between pair and ethanol-fed mice at any experimental time point. Ethanol-fed (n = 35) or pair-fed (n = 35) mice received a...
single acute dose of CCl₄ (1 μL/g body weight) prediluted 1:3 in olive oil and administered via intraperitoneal (IP) injection.

2.3 | Knockdown of Hnf4α and generation of piLenti-siHnf4α viral vectors

For knockdown in human HNF4α gene in HepG2 cells, Stealth siRNA containing 25 bp double-stranded RNA oligonucleotides were obtained (ThermoFisher, Carlsbad, CA). The target sequence is 5’ CACAGUAUGACCGAGGCGCUU 3’ that corresponds to 1017-1041 bp of this gene (GenBank: NM_008261.2). The HNF4α siRNA and Stealth RNAi negative control duplexes (50 nM) were transfected into cells with Lipofectamine RNAiMAX transfection reagent (ThermoFisher). Two days later, cells were lysed for RNA and protein isolation. HNF4α transcript was amplified using primer pair 5’ CACGGGCAAAACACTACGGT 3’ (sense) and 5’ TTGACCTTCGAGTGCTGATCC 3’ (antisense) with standard conditions in RT-PCR analysis.

To knock down mouse Hnf4α gene, we obtained plasmids corresponding to piLenti-GFP scramble siRNA and piLenti-GFP siHnf4α #1-4 from Applied Biological Materials Inc., BC, Canada (Abm). The siRNA#1-4 contained 29-bp sequences targeting open reading frame of mouse Hnf4α gene (NM_008261.2) starting at 298, 562, 833, and 1124 bp, respectively. Mouse hepatoma Hepa1-6 cells (ATCC, Gaithersburg, MD) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and transfected with piLenti-GFP siHnf4α plasmids. Two days later, messenger RNAs (mRNAs) and proteins were extracted and analyzed by RT-PCR and Western blotting to determine their effects on Hnf4α protein expression. Hnf4α was normalized against reference gene (18S) and calculated in delta delta CT method with scramble.

High-titer viruses for piLenti-GFP siHnf4α #3 and piLenti-GFP scramble siRNA were produced after cotransfection of lentiviral backbone as well as packaging plasmids that contain constructs encoding Gag/Pol, Rev, VSVG genes, of lentiviral backbone as well as packaging plasmids that contain constructs encoding Gag/Pol, Rev, VSVG genes. The titers were obtained (ThermoFisher, Carlsbad, CA). The titer was indicated as infectious unit (IU)/mL. The titers were evaluated by titering standard dilution series from 1 to 10⁶ IU/mL. The titers were determined by the percentage of positive GFP cells observed under a fluorescence microscope. Two days after transfection, the culture supernatant was collected, filtered via 0.45-μm filter and concentrated using ultracentrifugation according to previous publication.¹⁹ Lentiviral particles at 1.0 × 10¹⁰ infectious unit (IU)/mL were delivered via tail vein injection into wild-type C57BL/6J mice. After the lentiviral injection, animals were subjected to moderate ethanol feeding using the Lieber-DeCarli liquid diet containing 1%-2% ethanol for as described above. Ten days later, a single acute dose of CCl₄ (1 μL/g body weight) was given. Livers were harvested 2 days after the chemical-induced liver injury.

2.4 | RNA isolation, RT-PCR, qRT-PCR, and RNA sequencing analysis

Total RNAs were extracted using RNeasy Mini kit (Qiagen, Valencia, CA). Total RNA isolation and cDNA synthesis were reported previously.²⁰ In brief, total RNA was incubated with RQ1 RNase-free DNase (Promega, Madison, WI) to remove genomic DNA. Template cDNA was synthesized using reverse transcriptase in Superscript III First-Strand Synthesis with 50 pmol random hexamer (Invitrogen). RT-PCR analysis was carried out using 0.5 μL of cDNAs templates, 0.2 μM of each set of primers (listed in Supplementary Table 1), and 1xREDExtract-N-Amp tissue PCR kit (Sigma, St. Louis, MO) with standard amplification conditions that consisted of thirty cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. The quantitative RT-PCR (qRT-PCR) analysis for Ctgf was performed in triplicate using SYBR Green PCR master mixer (Applied Biosystems, Foster City, CA) with the following primer set Ctgf: 5’ AGTGGAGCGCCTGTTCTAAG 3’ (sense) and 5’ GTCTTCACACTTGCGAGCC 3’ (antisense). These primers detected exon 3 deletion in Ctgf deficient livers. Amplified products were analyzed in ABI Prism 7900 HT Fast Real-Time (Applied Biosystems). All qRT-PCR experiments were performed in triplicate using cDNA sample from independent RNA sets and the relative amount of target mRNA was calculated using delta-delta CT method normalized against reference gene (18S) in each sample.

For RNA-sequencing, total RNAs were isolated from ethanol/CCl₄-treated livers of Ctgf⁻/⁻, Ctgf⁺/⁺, Yap1flox/flox, and Yap1⁻/⁻ mice using RNeasy Extraction Kit from Qiagen. RNA-sequencing was carried out using an Illumina HiSeq 2500 system following manufacturer’s protocols (Illumina Inc. San Diego, CA). In brief, cDNA synthesis and fragmentation were carried out with the 200 bp peak setting by Covaris S220 (Covaris Inc., Woburn, Massachusetts). End repair, 3’ end adenylation and the barcoded adapters (Illumina) were performed to the fragmented cDNA in prior to ligation with Kapa LT library preparation kit (Kapa Biosystems, Wilmington, MA). The prepared libraries were validated using a 2100 Bioanalyzer DNA High Sensitivity chip, and quantified by Qubit Fluorometric Quantitation (Waltham, MA). The library templates were prepared for the sequencing using cBot cluster generation system with HiSeq SR Cluster Kit V4 (Illumina). The sequencing run was performed in a single read mode of 51 cycles of read 1 and 7 cycles of index read using HiSeq 2500 platform with HiSeq SBS Kit V4 (Illumina). HiSeq Control Software (HCS) 2.2.38 and Real Time Analysis (RTA) 1.18.61 on the
2.5 | Histology and morphometry

Blood samples and liver tissue were collected under deep anesthesia. Trimmed liver tissues were fixed in 4% paraformaldehyde PBS solution. Histology and immunofluorescent staining were performed with standard protocols using the antibodies, dilutions, and retrieval conditions listed in Supplementary Table 2. In brief, 5 μm formalin fixed paraffin embedded sections were rehydrated, blocked with 3% H2O2 in methanol for 10 minutes, subjected to the required retrieval conditions and then sequentially blocked in avidin and biotin solutions for 15 minutes each. Primary antibodies for Yap, Hnf4α, and Ki67 were applied overnight at 4°C. Detection was carried out according to the manufacturer’s instructions using the ABC-Elite kit with ImmPACT DAB substrate (Vector Laboratories, Burlingame, CA). In addition, immunobistochemistry (IHC) for either Ctgf or CD11b in liver sections was detected using a VECTASTAIN ABC-AP kit and Vector Alkaline Phosphatase Red substrate (Vector Laboratories). The immunofluorescent staining for glutamine synthetase (GS) was carried out using the rabbit antibody listed in Supplementary Table 2. Alexa Fluor 488 or 594 conjugated donkey anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA) was used for detection. For an estimation of percent necrosis, paraffin-embedded liver sections were subjected to standard H&E staining. Images were captured with CellSens software using an Olympus BX 51 upright fluorescence microscope outfitted with an Olympus DP80 camera, Plan Fluorite objectives and an LED transmitted light source (Olympus). DAB stained areas were quantified from 10 random fields of images (200× magnification) using Image J software (http://rsb.info.nih.gov/ij/) and IHC profiler according to published methods.

2.6 | PCR-based ChIP assays

PCR-Based ChIP assays were performed in HEK293 cells using the SimpleChIP Plus Enzymatic Chromatin IP kit (Cell Signaling Inc, Danvers, MA) according to the manufacturer’s instructions. In brief, HEK293 cells carrying Myc-DDK fused Yap, Smad3, or Hnf4α were cross-linked with formaldehyde, neutralized in glycine, digested by micrococcal nuclease, and then sonicated. The resulting chromatin was pre-cleared with Protein A conjugated magnetic beads followed by incubating with magnetic beads and five micrograms of specific or control antibody overnight. Specific antibodies are anti-Yap (Cell Signaling), anti-Smad3 and anti-Hnf4α (Santa Cruz Technologies, Dallas, TX). The beads were then washed, and the chromatin was eluted in ChIP elution buffer, reverse-cross-linked at 65°C overnight, and treated with RNase and Protease K. The DNA was extracted, and 2 μL of DNA was used for each ChIP-qPCR experiment. Quantitative real time PCR was performed using SYBR Green PCR master mix (Applied Biosystems) according to standard amplification conditions with the following primer sets: for Yap, Smad3 or Hnf4α binding: 5’ ATATGAATCAGGAGTGGTGCGA 3′ (sense) and 5’ CAACTCACACCCGATTTGAC 3′ (antisense). The percentage of input was calculated using the Ct value of the input DNA and ChIP-DNA. The data were normalized to the value of the IgG control antibody.

2.7 | Western blotting and ELISA assays

Western blotting was performed as previously described, with modification. Total proteins were extracted from mouse livers or cultured cells in RIPA buffer containing complete Proteinase Inhibitor (Sigma). Nuclear fractions were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific). Nuclear fractions (10 μg) or total protein lysates (50 μg) were boiled in 1× Laemmlı buffer containing 5% β-mercaptoethanol, separated on 4%-12% Bis-Tris protein gels (Novex, Carlsbad, CA), and electro-transferred onto polyvinylidene difluoride (PVDF) membrane for immunoblotting. Primary antibodies used were mouse anti-Smad3 (Santa Cruz Technologies), rabbit anti-Yap (Cell Signaling), rabbit anti-Hnf4α (Santa Cruz Technologies), mouse anti-Actin (Abcam, Cambridge, MA), mouse anti-Myc (Thermo Scientific), rabbit anti-Ctgf (Abcam), rabbit anti-collagen type I (Abcam), and rabbit anti-GAPDH (Abcam). Detection was carried out using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz biotechnologies) and the ECL Plus kit (Amersham Biosciences, Piscataway, NJ).

Serum levels of Tnfα and IL1α were determined from liver samples using ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Absorbance at wavelength of 450 nm was measured using the Titertek Multiskan Plus MKII microplate reader.
2.8 | Cell lines

HEK293 and HepG2 cells were cultured in DMEM supplemented with 10% FBS, 100 mg of penicillin/ml, and 100U of streptomycin/ml. AML12 mouse hepatocytes were from ATCC Inc and cultured in DMEM/F12 media containing 10% FBS, 400 nM dexamethasone, 1x insulin, transferrin, and selenium (ITS). LX-2 human stellate cells were from Dr. Scott Friedman (Mount Sinai School of Medicine, New York) and cultured in DMEM with 2% FBS. All cells were maintained in a humidified 37°C incubator with 5% CO2.

2.9 | Secreted alkaline phosphatase assay

A reporter plasmid carrying secreted alkaline phosphatase (SEAP) under the control of human CTGF promoter was a kind gift from Dr. Andrew Leask (Western University, Canada). Plasmids carrying Myc-DDK tagged cDNAs for murine Yap (GeneBank accession# NM_001171147), and Hnf4α (NM_008261) were purchased (OriGene, Rockville, MD). These plasmids, empty pCMV6 vector or in combination (100 ng per well) were transfected into HEK293 or HepG2 cells in 24-well plates. To knockdown human Hnf4α, 50 nM Stealth RNA oligonucleotides or non-targeting scramble control was also transfected. One day after transfection, media were switched to conditioned media containing with or without Tgf-β1 (5 ng/mL). A pCMV-lacZ plasmid (Clontech) at 20 ng/well was transfected as internal control for normalization based on β-galactosidase activity in the co-transfected cells according to Leask et al.27 The SEAP activity was measured in conditioned medium 48 hours later using the Great EscApe SEAP Chemiluminescence kit (Clontech, Mountain View, CA) according to the manufacturer’s instructions. SEAP activities were measured in triplicate experiments and relative CTGF promoter activity was expressed as fold change in comparison to the normalized SEAP activities of vector controls.

2.10 | EMSA and site directed DNA mutagenesis in CTGF promoter

The following primers were biotinylated using Pierce Biotin 3’ End DNA labeling kit (ThermoFisher Scientific) for EMSA. 5’ ATGCTGAGGTGTTTTACACCCGAGTCT G3′ (sense) and 5’ TTGATCTGCCCTTGCACACTGACAT 3′ (antisense) were for wild-type human CTGF probes. 5’ CAGACGGAGGAAT GCTGATTTTCTTTTTTTCAGGAT CAATCCGTTG 3′ (sense) and 5’ ACACCGGATGATC CTGAAAAAAGAAAAATCAGCATT CCTCCGTCTG 3′ (antisense) were for the mutant human CTGF probe. A mobility shift reaction mixture was set up using LightShift Chemiluminescent EMSA kit (ThermoFisher Scientific). It contained crude nuclear extracts overexpressing Hnf4α or Yap:Myc proteins (2 mg), poly(dIdC) (1mg), 0.1 mg of sonicated denatured salmon sperm DNA, biotin-labeled wild-type or mutant probe (4 pmol). Cold probe (20 fmol) was added in some experiments to determine binding specificities of tested probes. Antibodies against Hnf4α and Myc epitope (1 μg) were also included in some reactions for super-shift assays. Complexes in reaction mixtures (20 μL) were separated in polyacrylamide gel and electro-transferred onto a nylon membrane for immunoblotting. The biotin end-labeled DNA probe was detected using streptavidin conjugated to horseradish peroxidase and chemiluminescent substrate according to manufacturer’s instructions in the EMSA kit.

For site directed DNA mutagenesis, a strategy was designed to replace “5′ GTCAAGGGGTCAGG 3′” in the putative HNF4α binding site of the human CTGF promoter with mutant sequences “5′ TAAAACAACTAGT 3′” using two sets of complementary primers: P1151 primer: 5′ ACCGAGGAAT GCTGAGTTTAAAACAACTAGT 3′; P1152 primer: 5′ AACTCACACCGATT GATactagtTTGTTTAAACTCAGCATTCCTCCGT 3′; P1153 primer: 5′ TAACTGGCTTCAGCAGAGCG CAGATAAAAATACTGTCCTTCTA 3′, and P1154 primer: 5′ TAGAAGGCAGTATTGGATCCGTG 3′. SpeI restriction enzyme site (underlined and lowercase) was introduced in the mutant CTGFp to facilitate verification of mutations after cloning. P1151 and P1153 primer pairs were used to generate a 3538 bp PCR product containing sequences for the mutated CTGF promoter driven SEAP using the Gibson Assembly Site Directed Mutagenesis kit (SGI-DNA). P1152 and P1154 primer pairs were also used to amplify a 2000 bp PCR product that corresponded to the rest sequences of the SEAP reporter using the same kit. KpnI and XhoI restriction enzyme sites at the 5′ and 3′ ends of the human CTGF promoter were designed for cloning to re-assemble a full SEAP reporter with the correct orientation that carried mutations in the putative HNF4α binding site. The resulting plasmid with mutations was transformed into NEB 5 alpha competent cells (New England Biolabs, Ipswich, MH). Mutations were verified by SpeI digestion and sequencing analysis.

2.11 | Statistical analysis

GraphPad Prism 6.0 (GraphPad Software) was used for statistical analysis. Statistical significance (P < .05-.0005) was evaluated using the Student’s t test and one-way analysis of variance (ANOVA).
3 | RESULTS

3.1 | Ctgf deficiency attenuates liver regeneration after ethanol/CCl4-induced injury

Moderate ethanol feeding has been shown to aggravate hepatocyte damage, potentiate hepatocyte proliferation, and enhance liver fibrogenesis after acute CCl4 intoxication. Indeed, ethanol/CCl4 co-treatment caused slower resolution of necrotic liver mass (Supplementary Figure 1A). This slower removal of hepatic necrosis paralleled greater ratios between liver and body weights at 24-96 hours post the chemical induced injury in ethanol-fed groups than pair-fed groups (Supplementary Figure 1B). Compared to single treatment with CCl4 alone, more fibrogenesis occurred after ethanol/CCl4 co-treatment as evidenced by increased expression of α smooth muscle actin (αSMA), and collagen type I (Supplementary Figure 1C). Higher levels of Cyclin D1, Tgf-β1, and most importantly, Ctgf, were also observed after ethanol/CCl4 co-treatment (Supplementary Figure 1D).

In order to determine the function of Ctgf during liver injury after ethanol/CCl4 co-treatment, we deleted this gene utilizing Ctgf\textsuperscript{lox/lox} carrying ubc-Cre/ERT2 that mediated exon4-deletion in a tamoxifen-inducible manners. Liver tissues were obtained from ethanol-fed Ctgf\textsuperscript{lox/lox} mice and their Ctgf\textsuperscript{+/-} littermates at 24 hours after CCl4 administration. Loss of Ctgf gene products was confirmed by RT-PCR (Figure 1A), Western blotting (Figure 1B) and IHC staining (Figure 1C). It was evident that injury-induced Ctgf expression was limited to periportal areas in Ctgf\textsuperscript{lox/lox} livers, whereas minimal Ctgf expression was detected in Ctgf\textsuperscript{+/-} livers (Figure 1C). RNA Seq was further carried out to identify differentially expressed genes and pathways between the damaged Ctgf\textsuperscript{lox/lox} and Ctgf\textsuperscript{+/-} livers. Ctgf deletion caused downregulation of genes in inflammatory cytokines, positive regulation of cell proliferation, and collagen fibril organization downstream of MAPK and PI3K-Akt signaling pathways from functional enrichment analysis (Table 1). In addition, decreased protein expressions of Cyclin D1 and collagen type I (Figure 1B) as well as reduced mRNA levels of Cend1, Col5a2, Coll1a1, and Colla2 (Supplementary Figure 2) were confirmed upon Ctgf deletion. Furthermore, tumor necrosis factor (Tnfα) and IL1α in the damaged Ctgf\textsuperscript{lox/lox} livers were lower at both mRNA and protein levels in comparison to those of Ctgf\textsuperscript{+/-} controls (Figure 1D,E). This attenuated inflammation was associated with reduced recruitment of cluster of differentiation (CD)11b\textsuperscript{+} cells (Figure 1F, upper). Concomitant decrease of hepatocyte proliferation was also evidenced by reduced number of proliferating hepatocytes in Ki67 staining (Figure 1F, lower). These observations indicated that Ctgf deficiency...

![Figure 1](image)

**Figure 1** | Ctgf deficiency is associated with reduced inflammation, hepatocyte proliferation, and collagen production after liver damage caused by ethanol/CCl4 co-treatment. Ctgf\textsuperscript{lox/lox} and Ctgf\textsuperscript{+/-} mice were subjected to ethanol/CCl4 co-treatment and were sacrificed at day 1 after CCl4 administration. (A-C) Ctgf loss was confirmed by qRT-PCR analysis (A), Western blotting (B) and IHC staining (C). Downregulated Tnfα and IL1α expression in the damaged Ctgf\textsuperscript{lox/lox} livers were examined by qRT-PCR analysis (D) and ELISA assays (E). (F) IHC staining showed that Ctgf deficiency reduced recruitment of CD11b\textsuperscript{+} macrophages and number of Ki67\textsuperscript{+} proliferating hepatocytes. Scale bar: 10 μm. Quantification was calculated from 10 random fields at 200X magnification based on staining of three different livers per group. Data are means ± SD (n = 3 per group). *P < .05 and **P < .005 (Student's t-test)
Hepatocyte nuclear factor (Hnf)4α, Yap, and Smad3 of the Tgf-β1 signal transduction pathway are all necessary for liver regeneration after injury. Thus, we compared their expression patterns following ethanol/CCl4 co-treatment. As shown in Figure 2A, hepatocytes re-entered cell cycle as indicated by increased amounts of Cyclin D1 post-injury. Yap and Smad3 were upregulated from 2 to 72 hours after the chemical induced injury. Coincidently, Ctgf protein was rapidly upregulated within the first 4 hours post-treatment and reduced to a basal level at 72 hours. In contrast, Hnf4α was decreased at 2-24 hours post the injury. The inductions of Yap and Ctgf proteins as well as loss of Hnf4α protein were further confirmed by IHC and immunofluorescence staining (Figure 2B). Interestingly, dual staining labeled Ctgf and Hnf4α proteins in the same populations of periportal hepatocytes (Figure 2C,D), implicating a potential regulation of Ctgf and Hnf4α expression. Similar results were observed during liver regeneration that was induced by CCl4 alone (Supplementary Figure 3A-C).

Another commonly used model of liver regeneration is PH. As shown in Figure 3A,B, Ctgf mRNA and protein were rapidly upregulated within the first 3 hours after PH while Tgf-β1 was induced up to 24 hours before the peak of hepatocyte proliferation as indicated by induction of Ccn1l mRNA and protein at 24-72 hours post injury. These changes were correlated with transient decline of Hnf4α at the first half hour post PH, and nuclear localization of Yap and Smad3 proteins at 0.5-24 hours after PH (Figure 3C,D). IHC staining showed that Hnf4α maintained parenchymal distribution, whereas Yap had nuclear localizations that spread from periportal to central parenchyma (Figure 3D,E). Particularly, Ctgf was located in Hnf4α+ periportal areas that were negative for the pericentral hepatocyte marker GS in the PH-treated livers (Figure 3D,F).

Hnf4α knockdown enhances Ctgf expression and sustains fibrogenic responses after liver injury

Hepatocyte nuclear factor (Hnf)4α is essential for termination of liver regeneration while loss of it is associated with hepatocyte proliferation and activation of Tgf-β signaling during liver injury.28 To determine whether Hnf4α regulates Ctgf during ethanol/CCl4 induced liver injury, we screened four siRNAs in piLentiviral vector and identified siHnf4α#3 with target murine sequences at 833-861bp (NM_008261.2) that caused more than 85% downregulation of Hnf4α expression in Hepa1-6 hepatoma cells (Figure 4A,B) and 75%-85% in mouse livers (Figure 4C,D). Several Hnf4α target genes, which encode sodium taurocholate cotransporting polypeptide (Slc10a1),29 UDP glucuronosyltransferase 2 family polypeptide B1 (Ugt2b1),30 and Cytochrome P450 member 7a1 (CYP7a1),31 were lower in the mouse livers that carried lentivirus expressing siHnf4α#3 than those containing scramble siRNA during ethanol/CCl4-induced liver injury (Supplementary Figure 4). This siRNA-mediated Hnf4α knockdown was also associated with enhanced expression of Ctgf, in addition to upregulation of αSMA and Collagen type I (Figure 4C,D). IHC and Sirius Red staining verified increased areas of αSMA positive cell population and elevated collagen deposition, respectively (Figure 4E).

3.2 Hnf4α expression is negatively correlated to Ctgf expression after liver injury

HNF4α conditional knockouts after ethanol/CCl4 induced injury.

Another commonly used model of liver regeneration is PH. As shown in Figure 3A,B, Ctgf mRNA and protein were rapidly upregulated within the first 3 hours after PH while Tgf-β1 was induced up to 24 hours before the peak of hepatocyte proliferation as indicated by induction of Ccn1l mRNA and protein at 24-72 hours post injury. These changes were correlated with transient decline of Hnf4α at the first half hour post PH, and nuclear localization of Yap and Smad3

| Term | Fold enrichment | P value | Genes |
|------|----------------|---------|-------|
| GO:0006954—inflammatory response | 7.3005490 | 7.76E-06 | Tnf, Il1a, Csf1r, Ifi202b, Nfkbi, P2rx7lxn, Anxa1, Tnfaip3, Nlrp3 |
| GO:0008284—positive regulation of cell proliferation | 4.1702029 | 0.001 | Ccn1l, Tnf, Enpp2, Jun, Camp, Ntrk2, Zfp703, Sox4, CSF1R |
| GO:0030199—collagen fibril organization | 25.757834 | 4.79E-04 | Colla2, Cola1, Lox, Cola5a2 |
| mmu04010:MAPK signaling pathway | 5.384794234 | 0.004 | Tnf, Jun, Ntrk2, Dusp10, Dusp8, IL1a |
| mmu04151:PI3K-Akt signaling pathway | 3.881347411 | 0.016 | Ccd1, Itgb7, Cola2, Cola1, Cola5a2, Tgfr |

Abbreviations: Anxa1: annexin A1; Camp: cathelicidin antimicrobial peptide; Ccdn1: cyclin D1; CSF1R: colony-stimulating factor 1 receptor; Cola2: collagen, type I alpha-2; Cola1: collagen, type I alpha-1; Cola5a2: collagen, type V alpha-2; Dusp10: dual specificity phosphatase 10; Dusp8: dual specificity phosphatase 8; Enpp2: ectonucleotide pyrophosphatase/phosphodiesterase 2; Ifi202b: interferon activated gene 202B; Il1a: interleukin 1α; Itgb7: integrin beta-7; Jun: v-Jun avian sarcoma virus 17 oncogene homolog; Lox: lysyl oxidase; Lxn: Latexin; Nlrp3: NLR family, pyrin domain-containing 3; Nfkbi: nuclear factor of kappa light chain gene enhancer in b cells inhibitor, zeta; Ntrk2: neurotrophic tyrosine kinase, receptor; P2rx7lxn: purinergic receptor p2x, ligand-gated ion channel, 7; Sox4: Sry-box 4; Tnf: tumor necrosis factor alpha; Tnfaip3: tumor necrosis factor alpha-induced protein3; Zfp703: zinc finger protein 703.
To test whether Hnf4α knockdown in mouse hepatocytes influenced hepatic stellate cell activation in vitro, we first examined the effects of siHnf4α#3 on Ctgf production in AML12 cells in absence or presence of Tgf-β1 stimulation. Lentivirus expressing siHnf4α#3 gave rise to very low levels of Hnf4α protein, but did not affect Ctgf production in AML12 hepatocytes without Tgf-β1 treatment (left panel, Supplementary Figure 5A). This could be due to low basal levels of Ctgf expression in normal hepatocytes. In contrast, stimulation by recombinant murine Tgf-β1 protein (2 ng/mL) significantly upregulated Ctgf in AML12 cells that were transduced with siHnf4α#3 lentivirus compared to scramble controls (right panel, Supplementary Figure 5A). These results implicated that loss of Hnf4α enhanced Ctgf induction by Tgf-β1 in mouse hepatocytes. Then we used conditioned media from the Tgf-β1-stimulated AML12 cells and treated LX-2 human stellate cells. The qRT-PCR analyses detected higher levels of αSMA and Collagen I transcripts in LX-2 cells that were exposed to conditioned media from AML12 cells carrying siHnf4α#3 than those of scramble controls (Supplementary Figure 5B). These results were in agreement with our in vivo results that Hnf4α knockdown increased Ctgf expression and liver fibrogenesis after ethanol/CCl4-induced liver injury in Figure 4.

3.4 The CTGF promoter contains an Hnf4α binding site that overlaps with cis-elements for Yap

The human CTGF promoter contains consensus sequences for Tgf-β, Smad3, and Yap/TEAD.32,33 Our computational annotation identified DNA sequence “GTCAAGGGGTCAGG” that resembles a variant direct repeat (DR) 2 for Hnf4α binding in both human and mouse promoters of the CTGF gene. As shown in Figure 5A, these putative Hnf4α binding sequences overlap with a known Tgf-β regulatory element downstream of cis-elements for Yap/TEAD and Smad3.8,32,33 Next, we generated plasmids expressing individual Myc tagged murine Hnf4α, Yap and Smad3 (Hnf4α:Myc, Yap:Myc, and Smad3:Myc) in Supplementary Figure 6A. PCR-based ChIP assays verified association of the CTGF promoter with Hnf4α:Myc, Yap:Myc, and Smad3:Myc proteins (Figure 5B). SEAP assays showed that the Hnf4α:Myc protein induced 1.91-fold increase of wild-type CTGF promoter than empty vector in HEK293 cells, whereas a mutant promoter that did not have this binding site lost the Hnf4α-induced activity (Figure 5C). Direct evidence about Hnf4α binding to the CTGF promoter was detected as shift bands in gel shift assays. In addition, the DNA/Hnf4α complexes...
**FIGURE 3** Ctgf upregulation is associated with transient decline of Hnf4α during early stage of liver regeneration following PH. Ctgf and key regulators for transcriptional reprogramming were examined by qRT-PCR analysis (A) and Western blotting (B and C) in PH-treated livers. Results in (A) were means ± SEM (n = 5 per group). *P < .05 and **P < .005 relative to tested genes of control group (0 hr). (D) IHC labeled Hnf4α, Yap, Ctgf, and the pericentral hepatocyte marker GS on PH treated livers. (E) Two sets of images are low and high magnifications of consecutive sections showing Ctgf in Hnf4α+ periportal hepatocytes. (F) The immunofluorescent staining confirmed Ctgf localization in Hnf4α+ periportal areas. Image were taken in the same areas for Ctgf (red) and Hnf4α (green). DAPI was stained for nucleus. Scale bar: 100 μm. hr, hour; PT, portal tract; CV, central vein
could be disturbed with excessive cold wild-type probe or a mutant probe (Figure 5D). Furthermore, this binding was specific since addition of Hnf4α antibody or in combination with Myc antibody could form “super-shifted” products with higher molecular weight (Figure 5D).

3.5 Hnf4α mediates fine-tuned regulation of Ctgf via antagonistic effects on Yap activity in vitro

Yap and Hnf4α can reciprocally repress each other in regulating gene expression, whereas Yap and Tgf-β are co-operative partners with synergistic effects on Ctgf expression. Hnf4α can directly interact with Yap/TEAD complexes and antagonize their activities leading to ctgf downregulation. In an effort to clarify regulatory mechanisms of Ctgf expression in vitro, we first downregulated HNF4α in HepG2 cells and tested its effects on Yap, Tgf-β, and Smad3 activities. Significant knockdown was achieved with 50 nM siHNF4α that targeted 1017-1041 bp of this gene (Supplementary Figure 6B). In comparison to scramble siRNA control, this siHNF4α treatment increased CTGF promoter activities after co-stimulation by Tgf-β1 protein (5 ng/mL), or co-transfection with Myc tagged plasmids for Yap, Smad3, or in combination (Figure 5E). Conversely, when we expressed the Hnf4α:Myc plasmid...
ZHOU et al.

in HEK293 cells, the activities of CTGF promoter that were stimulated by Tgf-β1, Yap, Smad3, or in combination became significantly lower than control cells that were transfected with empty vector (Figure 5F). These results indicated that Hnf4α could mediate fine-tuned regulation of the CTGF promoter via antagonism of Yap and the Tgf-β/Smad3 activities.

4 | DISCUSSION

Liver regeneration involves extensive cellular changes and coordinated extracellular remodeling. Alterations in extracellular matrix composition take place immediately after injury and guide reparative processes following PH, CCl4 or other drugs poisoning. Without proper microenvironments, hepatocytes are unable to regenerate in cirrhotic livers. Ctgf is a matricellular protein capable of regulating cell motility and mobility through binding to growth factors, receptors, and matrix proteins. The formation of Ctgf-enriched microenvironments represents a proliferative and profibrogenic mechanism because conditional knockouts showed downregulation of genes in collagen fibril organization, cell adhesion, cell proliferation, and cell migration after ethanol/CCl4 treatment. Our most recent studies showed that Ctgf knockouts exhibit defects in recruitment of CD11b+ inflammatory cells. CD11b+ macrophages are the main cellular source of pro-inflammatory cytokines during PH or CCl4 induced liver injury. CD11b is encoded by the integrin αM gene and Ctgf is able to bind integrin αMβ2 for monocyte recruitment. Thus, downregulation of pro-inflammatory genes in damaged Ctgf null livers could be explained by low efficiency of recruitment of CD11b+ macrophages in the absence of this pro-adhesion molecule.

Hnf4α is a master regulator for metabolic homeostasis in hepatocytes. Injury-induced stimuli disrupt homeostasis resulting in loss of Hnf4α protein. This study showed transient loss of Hnf4α protein in nuclear fractions at 0.5 to 1.5 hours after PH and prolonged loss of this protein for 2-3 days during CCl4-induced injury in absence or presence of moderate ethanol feeding. Hnf4α loss after PH should not be due to tissue necrosis since liver injury following surgical resection does not involve cell death and hepatic inflammation. In another model of liver regeneration that involves hepatocyte death after metabolism of carbon tetrachloride in central zones, Hnf4α loss in nuclei of peri-central hepatocytes took place as early as 2-4 hours post CCl4 intoxication, whereas necrosis was evident at later stages (24-72 hours) of the liver damages. Although Hnf4α loss might take place in necrotic tissues, we argued other
mechanisms that involve deregulation of this protein for hepatocyte priming during early stages of CCl₄-triggered liver regeneration. In fact, cellular localization and protein levels of nuclear receptors including Hnf4α can be regulated through post-translation modifications such as acetylation and phosphorylation.⁴¹,⁴²

Hnf4α antagonism of Yap and Tgf-β/Smad3 via cis-elements in Ctgf promoter represents a potential mechanism to balance liver regeneration and prevent liver fibrosis. A rapid induction of Ctgf was observed after PH, which could be due to the transient decline of Hnf4α. It was reported that Hnf4α resets transcriptional regulatory networks and represses pro-mitotic genes in hepatocytes within hours after PH.⁴³ More pronounced reprogramming occurred after CCl₄ toxicity according to sustained loss of Hnf4α. Furthermore, moderate ethanol pre-exposure potentiated this CCl₄-triggered reprogramming due to the prolonged loss of Hnf4α, increased nuclear accumulation of Yap, and elevated expression of Ctgf and fibrosis related genes. As shown in Figure 6, we proposed a model about Ctgf regulation by Hnf4α, Yap, and Tgf-β/Smad3 signaling during liver regeneration. Hepatocytes exit quiescence after injury and reset transcriptional programs regulated by Hnf4α and Yap for regeneration. Failure to regenerate causes scarring that overproduces profibrotic factors such as Ctgf and Tgf-β. We propose that Ctgf production is a result of transient reprogramming in regenerating livers characterized by Hnf4α decline in conjunction with activation of Yap and Tgf-β/Smad3 signaling. Hnf4α antagonism of Yap and Tgf-β/Smad3 activities can downregulate Ctgf after the completion of liver regeneration. Otherwise, profibrotic signals are sustained leading to overproduction of Ctgf protein that may potentiate Tgf-β actions and promote hepatic stellate cell activation during liver fibrosis

FIGURE 6 A model about Ctgf regulation by Hnf4α, Yap, and Tgf-β/Smad3 signaling during liver regeneration. Hepatocytes exit quiescence after injury and reset transcriptional programs regulated by Hnf4α and Yap for regeneration. Failure to regenerate causes scarring that overproduces profibrotic factors such as Ctgf and Tgf-β. We propose that Ctgf production is a result of transient reprogramming in regenerating livers characterized by Hnf4α decline in conjunction with activation of Yap and Tgf-β/Smad3 signaling. Hnf4α antagonism of Yap and Tgf-β/Smad3 activities can downregulate Ctgf after the completion of liver regeneration. Otherwise, profibrotic signals are sustained leading to overproduction of Ctgf protein that may potentiate Tgf-β actions and promote hepatic stellate cell activation during liver fibrosis

ACKNOWLEDGMENTS
We want to thank Drs. Bryon Petersen, Gregory Schultz, Edward Scott, and Arun Srivastava for helping establishment of mouse models and discussion about this manuscript. This study is supported by National Institutes of Health NIAAA KO1AA024174 and R01AA028035 grants and Children Miracle Research Foundation grant awarded to Dr. L Pi. Dr. Ling is supported by the National Key Research and Development Program of China (2018YFA0109400), the Shanghai Sailing Program (17YF1401300) and Shanghai Eastern Scholarship (TP2016004).
CONFLICT OF INTERESTS
The authors declare that there is no conflict of interest regarding publication of this manuscript.

AUTHOR CONTRIBUTIONS
L. Pi, C. Ling, L. Wu, and C. Qi designed research. L. Pi, and C. Ling established mouse models. J. Zhou, X. Sun, L. Yang, G. Ran, J. Wang, L. Wang, and L. Pi performed research and analyzed data. C. Ling and L. Pi wrote the manuscript. Final approval of the paper was done by all authors.

REFERENCES
1. Nwidu LL, Oboma YI. Telfairia occidentalis (Cucurbitaceae) pulp extract mitigates rifampicin-isoniazid-induced hepatotoxicity in an in vivo rat model of oxidative stress. J Integr Med. 2019;17:46-56.
2. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. J Hepatol. 2012;57:692-694.
3. Ling CQ, Fan J, Lin HS, et al. Clinical practice guidelines for ductular reaction and biliary fibrosis in mice. J Lipid Res. 2016;57:692-694.
4. Zhao B, Ye X, Yu J, et al. TEAD mediates YAP-dependent gene expression during the priming phase of liver regeneration. J Hepatol. 2018;69:359-367.
5. Huck I, Gunewardena S, Espanol-Suner R, Willenbring H, Apte U. Hepatocyte nuclear factor 4 alpha activation is essential for termination of liver regeneration in mice. Hepatology. 2019;70:666-681.
6. Patel SH, Camargo FD, Yilmali D. Hippo signaling in the liver regulates organ size, cell fate, and carcinogenesis. Gastroenterology. 2017;152:533-545.
7. Urutasun R, Latasa MU, Demartis MI, et al. Connective tissue growth factor autocrine in human hepatocellular carcinoma: oncogenic role and regulation by epidermal growth factor receptor/yes-associated protein-mediated activation. Hepatology. 2011;54:2149-2158.
8. Zhao B, Ye X, Yu J, et al. TEAD mediates YAP-dependent gene induction and growth control. Genes Dev. 2008;22:1962-1971.
9. Gressner OA, Gressner AM. Connective tissue growth factor: a fibrogenic master switch in fibrotic liver diseases. Liver Int. 2008;28:1065-1079.
10. Abreu JG, Ketpura NL, Reversade B, De Robertis EM. Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. Nat Cell Biol. 2002;4:599-604.
11. Tong Z, Chen R, Alt DS, Kemper S, Perbal B, Brigstock DR. Susceptibility to liver fibrosis in mice expressing a connective tissue growth factor transgene in hepatocytes. Hepatology. 2009;50:939-947.
12. Kuttipurathu L, Juskeviciute E, Dippold RP, Hoek JB, Vadigepalli R. A novel comparative pattern analysis approach identifies chronic alcohol mediated dysregulation of transcriptomic dynamics during liver regeneration. BMC Genom. 2016;17:260.
13. Su AI, Guidotti LG, Pezacki JP, Chisari FV, Schultz PG. Gene expression during the priming phase of liver regeneration after partial hepatectomy in mice. Proc Natl Acad Sci USA. 2002;99:11181-11186.
14. Pi L, Robinson PM, Jorgensen M, et al. Connective tissue growth factor and integrin alphavbeta6: a new pair of regulators critical for ductular reaction and biliary fibrosis in mice. Hepatology. 2015;61:678-691.
15. Pi L, Fu C, Lu Y, et al. Vascular endothelial cell-specific connective tissue growth factor (CTGF) is necessary for development of chronic hypoxia-induced pulmonary hypertension. Front Physiol. 2018;9:138.
16. Liu S, Shi-wen X, Abraham DJ, Leask A. CCN2 is required for bleomycin-induced skin fibrosis in mice. Arthritis Rheum. 2011;63:239-246.
17. Pi L, Oh SH, Shupe T, Petersen BE. Role of connective tissue growth factor in oval cell response during liver regeneration after 2-AAF/PHx in rats. Gastroenterology. 2005;128:2077-2088.
18. Roychowdhury S, Chiang DJ, Mandal P, et al. Inhibition of apoptosis protects mice from ethanol-mediated acceleration of early markers of CCl4 -induced fibrosis but not steatosis or inflammation. Alcohol Clin Exp Res. 2012;36:1139-1147.
19. Pi L, Shenoy AK, Liu J, et al. CCN2/CTGF regulates neovessel formation via targeting structurally conserved cysteine knot motifs in multiple angiogenic regulators. FASEB J. 2012;26:3365-3379.
20. Lin S, Liu Q, Lelyveld VS, Choe J, Szostak JW, Gregory R1. Mettl1/Wdr4-mediated m(7)G RNA methylation is required for normal mRNA translation and embryonic stem cell self-renewal and differentiation. Mol Cell. 2018;71:244-255:e245.
21. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013;29:15-21.
22. Anders S, Pyl PT, Huber W. HTSeq-a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31:166-169.
23. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26:139-140.
24. da Huang W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4:44-57.
25. Varghese F, Bukhari AB, Malhotra R, De A. IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. PLoS ONE. 2014;9:e96801.
26. Wu Y, Xie L, Wang M, et al. Mettl3-mediated m(6)A RNA methylation regulates the fate of bone marrow mesenchymal stem cells and osteoporosis. Nat Commun. 2018;9:4772.
27. Leask A, Sa S, Holmes A, Shiwen X, Black CM, Abraham DJ. The control of ccn2 (ctgf) gene expression in normal and scleroderma fibroblasts. Mol Pathol. 2001;54:180-183.
28. Deshpande KT, Liu S, McCracken JM, et al. Moderate (2%, v/v) ethanol feeding alters hepatic wound healing after acute carbon tetrachloride exposure in mice. Biomolecules. 2016;6:5.
29. Geier A, Martin IV, Dietrich CG, et al. Hepatocyte nuclear factor-4alpha is a central transactivator of the mouse Ntcp gene. Am J Physiol Gastrointest Liver Physiol. 2008;295:G226-G233.
30. Lu H, Gonzalez FJ, Klaassen C. Alterations in hepatic mRNA expression of phase II enzymes and xenobiotic transporters after targeted disruption of hepatocyte nuclear factor 4 alpha. Toxicol Sci. 2010;118:380-390.
31. Stroup D, Chiang JY, HNF4 and COUP-TFIIF interact to modulate transcription of the cholesterol 7alpha-hydroxylase gene (CYP7A1). J Lipid Res. 2000;41:1-11.
32. Grotendorst GR, Okochi H, Hayashi N. A novel transforming growth factor beta response element controls the expression of the connective tissue growth factor gene. Cell Growth Differ. 1996;7:469-480.
33. Fujii M, Toyoda T, Nakanishi H, et al. TGF-beta synergizes with defects in the Hippo pathway to stimulate human malignant mesothelioma growth. *J Exp Med*. 2012;209:479-494.

34. Cai WY, Lin LY, Hao H, et al. Yes-associated protein/TEA domain family member and hepatocyte nuclear factor 4-alpha (HNF4alpha) repress reciprocally to regulate hepatocarcinogenesis in rats and mice. *Hepatology*. 2017;65:1206-1221.

35. Klaas M, Kangur T, Viil J, et al. The alterations in the extracellular matrix composition guide the repair of damaged liver tissue. *Sci Rep*. 2016;6:27398.

36. Nwidu LL, Teme RE. Hot aqueous leaf extract of Lasianthera africana (Icacinaceae) attenuates rifampicin-isoniazid-induced hepatoxicity. *J Integr Med*. 2018;16:263-272.

37. Liu L, Yannam GR, Nishikawa T, et al. The microenvironment in hepatocyte regeneration and function in rats with advanced cirrhosis. *Hepatology*. 2012;55:1529-1539.

38. Sato A, Nakashima H, Nakashima M, et al. Involvement of the TNF and FasL produced by CD11b Kupffer cells/macrophages in CCl4-induced acute hepatic injury. *PLoS ONE*. 2014;9:e92515.

39. Nishiyama K, Nakashima H, Ikarashi M, et al. Mouse CD11b+Kupffer cells recruited from bone marrow accelerate liver regeneration after partial hepatectomy. *PLoS ONE*. 2015;10:e0136774.

40. Schober JM, Chen N, Grzeszkiewicz TM, et al. Identification of integrin alpha(M)/beta(2) as an adhesion receptor on peripheral blood monocytes for Cyr61 (CCN1) and connective tissue growth factor (CCN2): immediate-early gene products expressed in atherosclerotic lesions. *Blood*. 2002;99:4457-4465.

41. Soutoglou E, Katrakili N, Talianidis I. Acetylation regulates transcription factor activity at multiple levels. *Mol Cell*. 2000;5:745-751.

42. Sun K, Montana V, Chellappa K, et al. Phosphorylation of a conserved serine in the deoxyribonucleic acid binding domain of nuclear receptors alters intracellular localization. *Mol Endocrinol*. 2007;21:1297-1311.

43. Jiao H, Zhu Y, Lu S, Zheng Y, Chen H. An integrated approach for the identification of HNF4alpha-centered transcriptional regulatory networks during early liver regeneration. *Cell Physiol Biochem*. 2015;36:2317-2326.

44. Zheng M, Mitra RN, Filonov NA, Han Z. Nanoparticle-mediated rhodopsin cDNA but not intron-containing DNA delivery causes transgene silencing in a rhodopsin knockout model. *FASEB J*. 2016;30:1076-1086.

45. Mitra RN, Zheng M, Weiss ER, Han Z. Genomic form of rhodopsin DNA nanoparticles rescued autosomal dominant Retinitis pigmentosa in the P23H knock-in mouse model. *Biomaterials*. 2018;157:26-39.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

---

**How to cite this article:** Zhou J, Sun X, Yang L, et al. Hepatocyte nuclear factor 4α negatively regulates connective tissue growth factor during liver regeneration. *The FASEB Journal*. 2020;34:4970–4983. [https://doi.org/10.1096/fj.201902382R](https://doi.org/10.1096/fj.201902382R)