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Susceptibility to liver fibrosis in mice expressing a connective tissue growth factor transgene in hepatocytes

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Abstract

Connective tissue growth factor (CCN2) is a matricellular protein that is up-regulated in many fibrotic disorders and co-expressed with transforming growth factor beta. CCN2 promotes fibrogenesis and survival in activated hepatic stellate cells, and injured or fibrotic liver contains up-regulated levels of CCN2 which are produced by a variety of different cell types, including hepatocytes. To investigate CCN2 action *in vivo*, transgenic FVB mice were created in which the human CCN2 gene was placed under the control of the albumin enhancer promoter to elevate hepatocyte CCN2 levels. Production of hCCN2 mRNA and elevated CCN2 protein levels was demonstrated in transgenic livers, while levels of endogenous mouse CCN2 were comparable between transgenic and wild-type mice. Liver histology and liver function tests were unaffected in transgenic animals. However, after chronic administration of carbon tetrachloride (CCl₄), alpha-smooth muscle actin (α -SMA)-expressing cells and collagen deposition were increased as a function of the dosage of the hCCN2 transgene (i.e. *hccn2*^{+/+} > *hccn2*^{+/-} > *hccn2*^{-/-}). Moreover, CCl₄-induced serum hyaluronic acid, hepatic tissue levels of α -SMA or acid-soluble collagen, and mRNA expression of α -SMA, collagen α 1 (I), matrix metalloprotease-2, or tissue inhibitor of metalloprotease-1 were greater in transgenic mice than in wild-type mice. Transgenic mice also exhibited enhanced hepatic deposition of collagen two weeks after bile duct ligation **Conclusion:** Production of elevated CCN2 levels in hepatocytes of transgenic mice *in vivo* does not cause hepatic injury or fibrosis *per se* but renders the livers more susceptible to the injurious actions of other fibrotic stimuli. These studies support for a central role of CCN2 in hepatic fibrosis and demonstrate a role of the micro-environment in regulating the pro-fibrotic action of CCN2.

Keywords

matricellular; CCN2; CTGF; fibrogenesis; hepatic stellate cell

Connective tissue growth factor (CCN2) is 349-residue mosaic protein that participates in critical processes such as differentiation, development, tumor growth, angiogenesis, placentation, and wound healing (1). The actions of CCN2 are complex and reflect its

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function as a matricellular protein whereby it resides as a non-structural matrix- or cell-associated molecule that regulates cellular responsiveness to a myriad of extracellular signals and environmental cues (2). These actions are likely achieved, at least partly, through its engagement of specific integrins and heparan sulfate proteoglycans on the cell surface (2).

Fibrosis is the most common pathophysiological condition in which CCN2 has been implicated and fibrotic conditions in the liver, lung, kidney, heart and skin are all strongly associated with CCN2 overexpression (1). In the liver, CCN2 mRNA expression is elevated in patients suffering from chronic viral hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, cryptogenic, alcoholic liver disease, congenital hepatic fibrosis or non-alcoholic steatohepatitis (3). CCN2 expression is also elevated in fibrotic livers from rodents subjected to bile duct ligation or exposure to carbon tetrachloride (CCl₄) or N-nitrosodimethylamine (4-6). High hepatic levels of CCN2 are associated with its entry into the circulation, as demonstrated by higher serum CCN2 concentrations in patients with biliary atresia or chronic viral hepatitis compared to control patients, with increasing CCN2 concentrations correlating with the progression of liver fibrosis (7,8).

Hepatocyte-derived CCN2 likely contributes to fibrotic pathways in the liver, at least in part, via its paracrine stimulation of activated HSC in which fibrogenic and survival pathways are promoted by CCN2 (3,9). Hepatocyte expression of CCN2 is usually low but it is enhanced in response to liver injury or insult such as partial hepatectomy, drug-induced toxicity, ethanol metabolism, infection with hepatitis C virus, or cancer (10-13). This effect may be due in part to the inductive action of TGF- β which stimulates CCN2 expression in hepatocytes via activation of PI3K/p38MAPK and/or ALK5-dependent Smad 3 (14,15). In these studies, we have investigated the pro-fibrotic aspects of elevated hepatocyte CCN2 levels in transgenic mice in which the human CCN2 gene was expressed under the control of the albumin enhancer promoter.

Materials and Methods

Cloning of human CCN2 expression vector

A 6600bp SacI fragment of human CCN2 (hCCN2) genomic DNA (16) was amplified by PCR using forward primer 5'-CGTGCCAACCATGACCGA-3' and reverse primer 5'-CGGAATTCTCTAATGAGTTAATGTCTCTCACTCTCTG-3'. The resulting product was digested with SacII and EcoRI and ligated into the SacII / EcoRI sites of pTRE (Clontech, Mountain View CA) to produce "pTRE/hCCN2". To achieve expression by hepatocytes, the CMV promoter in pTRE/hCCN2 was replaced with the mouse albumin enhancer/promoter sequence in pBluescript (KS-) plasmid p2335A-1 (17). Briefly, a 2.3-kb SacI / Kpn fragment from p2335A-1 containing the mouse albumin enhancer/promoter was blunted by T4 DNA polymerase and ligated into the blunted XhoI/SacII sites of pTRE/hCCN2 to generate "pALB/hCCN2" (Figure 1A) which was confirmed by nucleotide sequencing.

pALB/CCN2-mediated CCN2 over-expression in hepatocytes in vitro

FL83B mouse hepatocytes (American Type Culture Collection, Manassas, VA) were grown in 6-well plates in Hams F-12 medium (Mediatech Inc, Manassas VA) containing 10% bovine serum. At ~80% confluency, the medium was replaced with 1ml fresh Hams F-12 medium containing 2% bovine serum and 15 μ l FuGene 6 transfection reagent (Roche Diagnostics Corp. Indianapolis, IN). Cells were then transfected with 2 μ g pALB/hCCN2 for 48 hours. RNA was isolated using RNA STAT-60 Total RNA Isolation Reagent (Tel Test Inc., Friendswood, TX). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed on 2 μ g total RNA using SuperScript II Reverse Transcriptase (Invitrogen) to

generate the cDNA. The conditions for the reverse transcriptase reaction were: 25°C for 10min, 42°C for 50min and 70°C for 15min followed by PCR using the following primers : hCCN2 (632bp): 5'-TCCCTGCATCTTCGGTGGTA-3' and 5'-CCTCGCCGTCAGGGCAC-3'; mouse CCN2 (mCCN2) (734bp): 5'-CCGCACTGCCCCGCC-3' and 5'-CCC GCAGAACTTAGCCC-3'; and β -actin (324bp): 5'-AGCTTGCTGTATTCCCCTCCATCGTG-3' and 5'-AATTCGGATGGCTACGTACATGGCTG-3'. PCR conditions were 2 mins at 94°C, 35 cycles at 94°C for 30 sec, annealing at 56°C for 40 sec, and 72°C for 1.5min, followed by final extension for 10 mins at 72°C. For the last 6 hours of the 48-hour transfection period, the cells were metabolically labeled with 100 μ Ci/ml [³⁵S]cysteine/methionine (Trans³⁵S-label, MP Biomedical, Irvine, CA) prior to lysis in immunoprecipitation buffer (50mM TrisHCl (pH7.4) containing 150mM NaCl, 1% NP- 40, 0.25% DOC, 1mM EDTA, and 0.1% SDS) and subsequent pull-down of 100 μ g total cellular protein with anti-CCN2 (18) or Western blot of 50 μ g protein using monoclonal anti- β -actin (clone AC-15; Sigma-Aldrich, St. Louis, MO).

Generation of pALB/CCN2 transgenic mice

Animal procedures were approved by the Institutional Animal Care and Use Committee of The Research Institute at Nationwide Children's Hospital (Columbus, OH).

A 7.1kb linearized fragment of NotI-digested pALB/hCCN2 (Fig.1A) was microinjected into single-cell FVB/n mouse eggs which were then implanted into pseudo-pregnant female FVB/n mice (The Jackson Laboratory, Bar Harbor, ME), yielding 30 offspring (3 litters) of which two (1 male, 1 female) were positive for ALB/hCCN2 DNA by PCR screening (data not shown). Using these as founders, two essentially identical transgenic lines ("ZY1", "ZY2") were established. Data reported here were obtained from the ZY2 line which has been maintained continuously for 60 months. All of the experiments described were performed on male mice between 4 and 28 weeks of age. The three genotypes (*hccn2*+/, *hccn2*+/-, *hccn2*-/-) required for this study were initially determined by screening using semi-quantitative PCR and Southern blot, and then confirmed by quantitative real-time PCR (Q-RT-PCR).

Carbon tetrachloride administration

Wild-type or transgenic male mice that were 4-6 weeks of age were injected i.m. once per day on 4 successive days each week for 5 weeks with either 30 μ l of vegetable oil or a mixture of 0.5 μ l CCl₄ (Sigma-Aldrich) in 29.5 μ l of vegetable oil. This dose of CCl₄ induced sub-maximal hepatic fibrosis in wild-type FVB mice as established from dose-response studies (data not shown). Upon sacrifice, blood was collected and individual liver lobes were tied and harvested either immediately and snap-frozen in liquid nitrogen for subsequent RNA or protein extraction, or after *in situ* perfusion using PBS followed by 4% paraformaldehyde (Sigma-Aldrich) for histological analysis of fixed tissue.

Ethanol administration

Wild-type FVB mice received ethanol (6g/kg) or saline i.p. at 0, 8 and 16 hours prior to sacrifice at 24 hours. A portion of the liver was fixed and processed for *in situ* hybridization (ISH) to detect mCCN2 mRNA, while hepatocytes were isolated from the remainder of the fresh unfixed liver tissue by *in situ* collagenase digestion (19) and subjected to fluorescence activated cell sorting (FACS) using anti-CCN2 antibody essentially as described (20).

Bile duct ligation (BDL)

BDL or sham operations were performed on 4-6 week *hCCN2*^{-/-} or *hCCN2*^{+/+} mice as described (21), after which the animals were maintained under normal housing conditions for 14 days. During this period, survival was >90% in all groups of mice. On Day 14 post-surgery, mice were sacrificed and their livers were processed for histological evaluation.

Semi-quantitative PCR

Three-week old mice were genotyped by PCR of DNA obtained from tail clippings. DNA was isolated by sequential protease K digestion and phenol-chloroform extraction. PCR was performed using transgene-specific primers, one of which appned the junction between the albumin enhancer/promoter and *hCCN2* (Figure 1A; P1: 5'-AGAGCGAGTCTTTCTGCACACA-3'; P2: 5'-GAGAGAATCACGACCCTGACTT-3'). PCR conditions were 2 min at 94°C, 35 cycles at 94°C for 30 sec, annealing at 55°C for 40 sec, and extension at 72°C for 1.5 mins, followed by 10 mins at 72°C. The 1044bp *hCCN2* PCR product was detected by ethidium bromide staining after agarose gel electrophoresis.

Southern Blot—Genomic DNA (5 µg) from mice tails was digested in a 40 µl final volume with 20U *Stu* I in Reaction buffer II (New England Biolabs, Ipswich, MA). DNA fragments were separated in a 0.7% agarose gel and transferred to Hybond N+ membrane (Amersham-Pharmacia, Piscataway, NJ). The membrane was probed with a previously described 1.1 kb human *CCN2* cDNA (18) that was labeled using Radprime labeling kit (Invitrogen).

Quantitative PCR (Q-PCR)

Q-PCR was performed with TaqMan-minor groove binder, primers, probes and TaqMan Universal Master Mix using an ABI Prism 7500 machine (all from Applied Biosystems Inc, Foster City, CA). Primers were designed using Primer Express Software (version 3.0; Applied Biosystems,). Genotypes were established by determination of the amount of *hCCN2* product relative to that of mouse glucagon after 40 cycles. Specific conditions were 50°C for 2min, 95°C for 10 min, 40 cycles of 95°C for 15 secs, and then 58°C for 1min. Each amplification reaction was performed in triplicate on 20 ng of genomic DNA. The number of transgene copies was established from *hCCN2* plasmid DNA standard curves. Sequences were: *hCCN2* forward primer: CATCCCCACCCCTCTCT; *hCCN2* reverse primer: CCGTACCACCGAAGATGCA; *hCCN2* probe: TCTCCAGCCAAAGAT; glucagon forward primer: CACAACATCTCGTGCCAGTCA; glucagon reverse primer: ATCTGCATGCAAAGCAATATAGCT; and glucagon probe: TGGGATGTACAATTTCAA.

Histology

Perfused livers were fixed with 4% paraformaldehyde for 24 hours and then embedded in paraffin. Sections of 5 µm thickness were cut and stained with hematoxylin and eosin (H and E). For immunohistochemical detection of alpha-smooth muscle actin (α -SMA), slides were incubated with monoclonal mouse anti- α -SMA IgG (Dako, Glostrup, Denmark) followed by development with UltraTek reagents and AEC chromogenic substrates (all from ScyTek Laboratories, Logan, UT) and hemotoxylin counterstain. *CCN2* immunohistochemistry was performed using affinity-purified rabbit anti-*CCN2* IgG as described (22). *CCN2 in situ* hybridization was performed with digoxigenin-labelled RNA sense or antisense probes generated from a pCRII vector containing m*CCN2* cDNA (23). Collagen was detected by staining sections with 0.1% Sirius Red (Sigma-Aldrich). Protein staining for α -SMA or collagen was quantified over ten 20× fields per mouse using ImagePro™ software (Media Cybernetics Inc, Bethesda, MD).

RT-PCR and Q-RT-PCR of liver RNA

RT-PCR of CCN2 mRNA isolated from frozen liver tissue was performed as described above for FL83B mouse hepatocytes. For Q-RT-PCR of mouse liver RNA, the reverse-transcription step was performed as described above for mouse FL83B cells. Real-time amplification was then performed with SYBR®Green PCR Master Mix reagent (Applied Biosystems) using an ABI Prism 7500 machine. The 20µl reaction volume contained 30ng cDNA, 10 µl 2× PCR Master mix, and 0.4µl of each primer. After initial denaturation at 95°C for 10min, 40 PCR cycles were run at 95°C for 15 secs, followed by 60°C for 1 min. The primers were: mouse β-actin: forward: 5'-TGTTACCAACTGGGACGACA-3', reverse: 5'-CTTTTCACGGTTGGCCTTAG-3'; mouse α-SMA: forward: 5'-GGCTCTGGGCTCTGTAAGG-3', reverse: 5'-CTCTTGCTCTGGGCTTCATC-3; mouse collagen α1 (I) : forward: 5'-CCAAGGGTAACAGCGGTGAA-3', reverse: 5'-CCTCGTTTTCTTCTTCTCCG-3', mouse TIMP-1: forward: 5'-GCATCTCTGGCATCTGGCATC-3', reverse: 5'-GCGGTTCTGGGACTTGTGGGC-3'; mouse MMP-2 : forward: 5'-TTCCCCCGCAAGCCCAAGTG-3', reverse: 5'-GAGAAAAGCGCAGCGGAGTGACG-3'; and mouse TGF-β1 : forward: 5'-GGTTCATGTCATGGATGGTGC-3', reverse: 5'-TGACGTCACTGGAGTTGTACGG-3'.

Western blotting and marker assays

Liver extracts were prepared by homogenization of 100mg tissue in 1ml immunoprecipitation buffer (see above). Clarified tissue supernatant containing 50 µg of total protein was subjected to SDS-PAGE and Western blotting using anti-CCN2 (18), anti-β-actin (see above), or monoclonal anti-α-SMA (clone 1A4; Sigma-Aldrich). Intensities of immunoreactive bands were quantified using image analysis software (Scion Corp, Frederick, MD). Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by the Mouse Phenotyping Shared Resource (Ohio State University Comprehensive Cancer Center, Columbus OH). Serum hyaluronic acid (HA) levels were determined using a commercial kit (Corgenix Inc, Broomfield, CO). Acid-soluble hepatic collagen was determined by Sircol™ assay (Biocolor Ltd., Carrickfergus, UK).

Statistical analysis

Each experiment was performed between 3 and 8 times. Data were typically obtained from 3-5 animals in each experimental group using duplicate or triplicate determinations for each animal. For comparison of individual data points, students *t*-test was applied and statistical significance set at *p* < 0.05.

Results

Production of native CCN2 by mouse hepatocytes *in vivo*

We first verified that mouse hepatocytes naturally produce CCN2 *in vivo* in response to hepatic insult. Ethanol administration caused a robust increase in CCN2 mRNA expression in the liver parenchyma as assessed by *in situ* hybridization and an increase in the frequency of CCN2-positive hepatocytes from 13% to 85% as assessed by FACS (Figure 1B), thus supporting studies of CCN2 expression in mouse hepatocytes using a transgenic approach.

Cloning and expression of pALB/CCN2 in mouse hepatocytes

The functionality of pALB/hCCN2 was verified by transient transfection of mouse FL83B hepatocytes *in vitro*. Using species-specific CCN2 primers, mock-transfected cells were shown by RT-PCR to contain only the 734 bp mCCN2 transcript whereas this transcript plus the 632bp hCCN2 transcript was present in pALB/hCCN2- transfected cells (Figure 1C,

panels a,b). As assessed by immunoprecipitation, transfected cells also contained greater quantities of the 38kDa CCN2 protein when compared to the mock-transfected cells (Figure 1C, panel d). β -actin mRNA expression and protein production were comparable in mock- and CCN2-transfected cells (Figure 1C, panels c,e). It was thus concluded that the pALB/hCCN2 plasmid was functional in hepatocytes.

Production and phenotypic characterization of hCCN2 transgenic mice

Limited PCR performed on tail DNA showed that the intensity of the hCCN2 PCR product was greater in *hccn2*^{+/+} (homozygous) than in *hccn2*^{+/-} (heterozygous) mice, and was absent from wild-type (*hccn2*^{-/-}) mice (Figure 2A, upper panel). These data were supported by Southern blotting using a hCCN2 probe (Figure 2A, lower panel) and were subsequently verified by Q-PCR (Figure 2B). The hCCN2 transgene copy number was 2.89 ± 0.15 for *hccn2*^{+/-} (n=35) and 4.77 ± 0.16 for *hccn2*^{+/+} (n=11).

Hepatic hCCN2 mRNA was verified by RT-PCR which further demonstrated that hCCN2 expression was greater in *hccn2*^{+/+} than *hccn2*^{+/-} mice, and that no hCCN2 expression was evident in *hccn2*^{-/-} littermates (Figure 2C). Hepatic expression of endogenous mCCN2 mRNA was identical between all three strains of mice (Figure 2C). Immunohistochemistry revealed stronger CCN2 staining in the parenchyma of *hccn2*^{+/+} livers as compared to *hccn2*^{-/-} livers (Figure 2C). Additionally increased amounts of immunoreactive CCN2 in *hccn2*^{+/+} mice compared to *hccn2*^{-/-} were demonstrated by Western blot of liver lysates (Figure 2E, inset) and FACS analysis of isolated hepatocytes (Figure 2E).

CCN2 transgenic mice were alert, healthy, active, and exhibited normal growth curves, weights, appetite, fertility, pregnancy rate, gestational period, litter size and birth weight (data not shown). Individual mice in the ZY2 transgenic were maintained up to 29 months of age with no clinical symptoms in either males or females. Neither *hccn2*^{+/+} nor *hccn2*^{+/-} mice exhibited any histological abnormalities. H and E staining of liver sections was unremarkable and showed no differences compared to wild-type littermates (Figure 3A). The staining pattern of α -SMA or collagen in transgenic mice was also unremarkable and was identical to that of wild-type mice, with α -SMA present in the smooth muscle cells of the blood vessels and collagen fibers localized to the vasculature as expected (Figure 3A). Serum liver function tests (AST, ALT) were not significantly altered in transgenic mice (Figure 3B).

Enhanced susceptibility of CCN2 transgenic mice to liver fibrosis

When animals were chronically treated with a dose of CCl₄ that was selected to induce sub-maximal stimulation of hepatic fibrosis in wild-type mice, the fibrotic response to this dose was noticeably enhanced in transgenic mice (Figures 4,5). This difference was evident when liver sections were stained either for α -SMA (Figure 4A) or collagen (Figure 5A) and was more pronounced in homozygous mice than heterozygous mice. As assessed by image analysis, the amount of staining for α -SMA or collagen was positively correlated with the *hccn2* genotype and a statistically significant increase was apparent in *hccn2*^{+/+} mice as compared to *hccn2*^{-/-} mice ($p < 0.05$) (Figures 4B, 5B). A statistically significant exacerbated fibrotic response to CCl₄ in *hccn2*^{+/+} mice was also evident when hepatic extracts were assessed for α -SMA levels by Western blotting (Figure 4C) or for acid-soluble collagen content by Sircol™ assay (Figure 5C) ($p < 0.05$). As assessed by Q-RT-PCR, treatment of either *hccn2*^{-/-} mice or *hccn2*^{+/+} mice with CCl₄ caused an increase in the mRNA expression of α -SMA, collagen $\alpha 1$ (1), MMP-2, TIMP-1 (Figure 6A). However, CCl₄-induced expression of all transcripts was greater in *hccn2*^{+/+} mice compared and was statistically higher ($p < 0.05$) for α -SMA, collagen $\alpha 1$ (1), and TIMP-1. Expression of TGF- $\beta 1$ mRNA in wild type or transgenic mice was moderately enhanced after chronic CCl₄

treatment but this response was not exacerbated by the presence of the CCN2 transgene (Figure 6A). Finally, in CCl₄-treated animals, circulating HA levels were significantly greater in *hccn2* *+/+* mice as compared to *hccn2* *-/-* mice ($p < 0.05$) (Figure 6B).

To verify that the pro-fibrotic effect of the CCN2 transgene after liver injury was not restricted to the effect of hepatotoxins such as CCl₄, mice alternatively underwent BDL and their livers were examined for collagen deposition two weeks later. As detected by Sirius Red staining, there was a marked increase in BDL-induced collagen deposition in *hccn2* *+/+* mice as compared to *hccn2* *-/-* mice ($p < 0.05$; Figure 7) whereas no fibrotic response was present in sham-operated mice (data not shown). These data thus showed that the effects of distinct fibrotic stimuli were exacerbated by expression of the hepatic hCCN2 transgene.

Discussion

Research over the last 15-20 years has firmly established that CCN2 is involved in fibrosis. While many studies have focused on aspects of CCN2 gene regulation at the molecular level, the precise role of CCN2 in fibrogenic cascades has been difficult to ascertain because of the lack of availability of high quality purified CCN2 with which to perform critical studies and because its actions as a matricellular protein are difficult, if not impossible, to fully model using *in vitro* approaches. Moreover, only a limited number of transgenic systems have been reported for CCN2 (24-26) and none of these have addressed either its action in the liver or, somewhat surprisingly, its fibrotic properties. To address these deficiencies, we created transgenic mice that overproduced hepatic CCN2 under the control of the albumin enhancer/promoter in hepatocytes, a legitimate cell type given its ability produce CCN2 in response to various insults, often downstream of TGF- β (Figure 1; (10-15))

Our major finding was that hepatocyte overexpression of CCN2 *per se* was non-fibrotic but that it resulted in a potentiation of CCl₄- or BDL-stimulated hepatic fibrosis. It is important to note that, under normal circumstances, there was no histopathology or evidence of HSC activation in the livers of CCN2 transgenic mice. *In vitro* studies have previously shown that CCN2 does not interact with quiescent HSC but that it potentiates fibrogenic and survival pathways in activated HSC which express up-regulated levels of CCN2 receptors such as integrin $\alpha v \beta 3$ (27). This fact, coupled with the absence of other injury cascades in transgenic livers that might otherwise cause HSC activation, likely explains the inability of the CCN2 transgene alone to drive fibrosis.

Fibrosing stimuli ordinarily cause the elaboration of multiple signaling pathways as opposed to changes in CCN2 expression alone. Thus, the pathophysiological role of CCN2 is best evaluated in the context of the fibrogenic cascades of which it is a normal component. We therefore conducted additional studies in transgenic mice subjected to CCl₄ treatment or BDL and found that the presence of the hepatocyte CCN2 transgene constitutes a 'first hit' that sensitized the liver to a different 'second hit', resulting in more advanced fibrosis.

The increase in fibrosis-susceptibility in CCN2 transgenic mice may be related to the additive effects of the hCCN2 protein on intrinsic CCN2 target pathways that were activated by CCl₄ or BDL, or their co-operative binding interactions with other molecules in the fibrogenic cascade such as fibronectin or TGF- β 1. For example, CCN2 binds to fibronectin (28) and increases integrin-dependent adhesion to fibronectin by fibroblasts (29), chondrocytes (30), hepatic oval cells (31) or activated mouse HSC (D.R. Brigstock and G. Huang, unpublished observations). Also, TGF- β 1 binds directly to CCN2, which increases TGF- β bioactivity due to its enhanced affinity for the Type II TGF- β receptor (32), a phenomenon that may account for the reported synergism between CCN2 and TGF- β in

stimulating dermal fibrosis *in vivo* (33). A synergistic action of this type seems plausible in our model since many fibrotic readouts downstream of TGF- β were amplified in CCl₄-treated transgenic mice versus wild type mice, even though expression of CCl₄-induced TGF- β itself was comparable between the two groups of animals. While there are no other transgenic CCN2 fibrosis models with which to draw comparisons, expression of a CCN2 transgene in mouse podocytes *in vivo* was insufficient to cause glomerular damage, but when challenged in a setting of a streptozotocin-induced type 1 diabetes the transgenic mice exhibited increased glomerular pathology as compared to wild-type mice (25). This exacerbated response included enhanced matrix expansion which was proposed to reflect the combined matrigenic actions of CCN2 and other factors such as TGF- β (25). Lastly, co-operative interactions between CCN2 and TGF- β *in vivo* were shown in Balb/c mice which, though genetically resistant to Smad-3-mediated bleomycin-induced lung fibrosis, became susceptible to fibrosis when treated concomitantly with bleomycin and a CCN2-expressing adenovirus (34). With respect to genetic-resistance, the FVB strain used in our study is relatively resistant to hepatic fibrosis (35) raising the question of whether this phenomenon might account for the lack of hepatic phenotype in the FVB hCCN2 transgenic mice. However, we have back-crossed the hCCN2 transgene on to the more susceptible Balb/c strain (35) and have found that liver histology remains normal over all generations examined (up to and including F11; data not shown). Thus the various *in vivo* studies to date show that CCN2 overexpression alone does not cause tissue damage and/or fibrosis and that additional changes in the micro-environment are required for the deleterious functions of CCN2 to be manifested, a mode of action that is entirely consistent with its function as a matricellular protein.

In conclusion, our studies demonstrate that expression of an albumin promoter-driven CCN2 transgene in mouse hepatocytes *in vivo* does not cause hepatic injury or fibrosis but renders the livers more susceptible to the injurious actions of another fibrotic stimulus. These studies provide further evidence in support for a central role of CCN2 in hepatic fibrosis and emphasize the role of the micro-environment in regulating the pro-fibrotic action of CCN2.

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Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
α-SMA	alpha-smooth muscle actin
BDL	bile duct ligation
CCN2	connective tissue growth factor
CCl₄	carbon tetrachloride
FACS	fluorescence-activated cell sorting
HSC	hepatic stellate cells

hCCN2	human CCN2
mCCN2	mouse CCN2
Q-RTPCR	quantitative real time PCR
PCR	polymerase chain reaction
RT-PCR	reverse-transcriptase PCR
TGF-β	transforming growth factor beta

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Figure 1. Cloning and testing of human CCN2 transgene construct and production of native CCN2 by hepatocytes *in vivo*

(A) A 1.9kb fragment containing the human CCN2 gene was placed downstream of the albumin-enhancer to produce pALB/hCCN2 as described in Materials and Methods. Primers P1 and P2 were used for genotyping. (B) Wild-type FVB/n mice were administered (a) 6g/kg ethanol or (b) saline by i.p. injections at 0, 8 and 16 hours. Mice were sacrificed 8 hours later and either (a,b,) fixed tissue was processed for *in situ* hybridization to detect CCN2 mRNA or (c) hepatocytes were isolated from fresh tissue and tested for the presence of CCN2 protein by FACS analysis using a CCN2 polyclonal antibody. (C) FL83B hepatocytes were either mock transfected (lane 1) or transiently transfected with pALB/hCCN2 (lane 2) for 48 hours, after which RNA or protein were subsequently extracted from parallel cultures of cells. The figure shows the results of reverse-transcriptase PCR using primers designed to detect (a) mCCN2 mRNA, (b) hCCN2 mRNA or (c) mouse β-actin. Immunoprecipitation using anti-CCN2 (d) showed that higher levels of CCN2 protein were present in lysates from hCCN2-transfected cells as compared to the mock-transfected counterparts, while levels of β-actin determined by Western blot (e) were comparable.

Figure 2. Genotypic characterization and CCN2 production in livers of hCCN2 transgenic mice (A) Semi-quantitative PCR (upper panel) and Southern blot (lower panel) of DNA isolated from mouse tails. (B) Q-PCR in which each DNA sample was analyzed in 3 separate reactions (mean $\pm$ s.d). Representative data are shown for three individual mice, each of which has a distinct *hccn2* genotype. (C) RT-PCR of hepatic RNA from wild-type or transgenic mice using mCCN2-specific or hCCN2-specific primers confirming the presence of hCCN2 transcripts in transgenic but not wild-type mice. (D) CCN2 immunohistochemistry of liver sections from (a) wild-type (*hccn2*^{-/-}) or (b) transgenic (*hccn2*^{+/+}) mice. (E) FACS analysis of CCN2-positive hepatocytes isolated from the livers of wild-type or transgenic (*hccn2*^{+/+}) mice. The inset shows a CCN2 Western blot of whole liver lysate normalized for total protein loading from wild type (lane 1) or transgenic (*hccn2*^{+/+}) (lane 2) mice.

Figure 3. CCN2 transgenic mice exhibit normal hepatic histology and LFTs

(A) Histological features of livers from wild-type or transgenic mice. Liver sections of mice were stained with H&E (top row), anti-α-SMA (middle row), or Sirius Red (bottom row) (20×; insets show detail at 40×). Data are representative of 24 mice observed from 8 individual experiments (n=3 per group) (B) Serum ALT and AST levels in individual wild-type or transgenic mice over the first 28 weeks of life.

Figure 4. Enhanced CCl₄-induced α-SMA production in CCN2 transgenic mice as compared to wild type mice

(A) Mice were treated i.m. once per day on 4 successive days each week for 5 weeks with either 30 μl of vegetable oil or a mixture of 0.5 μl CCl₄ in 29.5 μl of vegetable oil. Livers were resected, fixed, and processed for α-SMA immunohistochemistry. Staining is representative of 24 mice observed from 8 individual experiments (n=3 per group). (B) α-SMA staining on tissue sections was quantified by image analysis of ten 20× fields (mean ± s.d). *p<0.05 versus “*hccn2* ^{-/-}”. (C) Presence of α-SMA in hepatic tissue extracts relative to that of β-actin as assessed by densitometric scanning of Western blots from 4 animals per group (mean ± s.d). *p<0.05 versus “*hccn2* ^{-/-}”. The inset shows a representative Western blot.

Figure 5. Enhanced CCl₄-induced collagen deposition in CCN2 transgenic mice as compared to wild type mice

(A) Mice were treated as described in Figure 4 and liver sections were stained with Sirius Red to detect collagen. Staining is shown at 20× and is representative of 24 mice observed from 8 individual experiments (n=3 per group). (B) Sirius Red staining on tissue sections was quantified by image analysis of ten 20× fields (mean ± s.d). *p<0.05 versus "*hccn2* ^{-/-}". (C) SircolTM assay of acid-soluble collagen in hepatic tissue extracts (mean ± s.d.; n=5 per group). *p<0.05 versus "*hccn2* ^{-/-}".

Figure 6. Enhanced CCl_4 -induced production of fibrosis-related gene products in CCN2 transgenic mice as compared to wild type mice

(A). Q-RT-PCR of mRNA from $hccn2^{-/-}$ or $hccn2^{+/+}$ livers after chronic exposure to CCl_4 for 5 weeks. Data are triplicate determinations (mean $\pm$ s.d.) of relative mRNA expression from 3 mice in each group and are representative of three experiments. * $p < 0.05$ v " $hccn2^{-/-}$, Oil"; ** $p < 0.05$ v " $hccn2^{+/+}$, Oil"; # $p < 0.05$ v " $hccn2^{-/-}$, CCl_4 ". (B) Serum HA levels in $hccn2^{-/-}$ or $hccn2^{+/+}$ mice after chronic CCl_4 treatment for 5 weeks (mean $\pm$ s.d.; $n=5$ per group). * $p < 0.05$ v " $hccn2^{-/-}$ ".

Figure 7. Enhanced BDL-induced collagen deposition CCN2 transgenic mice as compared to wild type mice

(A) Mice were subjected to BDL and, two weeks later, livers were removed, sectioned and stained with Sirius Red (20×). (B) Quantification of Sirius Red staining of tissue sections by image analysis of ten 20× fields from 4 mice in each group (mean ± s.d). *p<0.05 versus “*hccn2* ^{-/-}”