GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Diet-Induced Obesity Enhances Progression of Hepatocellular Carcinoma through Tenascin-C/Toll-Like Receptor 4 Signaling

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Obesity is an independent risk factor for the development of liver fibrosis/cirrhosis and hepatocellular carcinoma (HCC). Tenascin-C (TnC), an extracellular matrix protein, is transiently expressed during tissue injury and plays a role in fibrogenesis and tumorigenesis. However, the mechanistic role of TnC signaling in the development of HCC remains unknown. We developed a diet-induced obesity HCC mouse model and examined TnC expression and liver injury. To determine the cellular mechanism of TnC signaling in promoting inflammation and hepatocyte epithelial-mesenchymal transition and migration, we used primary hepatocytes and hepatoma and macrophage cell lines. Further, to determine whether elevated TnC expression correlated with obesity-associated HCC, we measured plasma TnC in obese patients with various levels of liver injury. Increased tissue inflammation accompanied with elevated hepatic stellate cell-derived TnC and Toll-like receptor 4 expression was observed in the diet-induced obesity HCC animal model. *In vitro* studies found enhanced Toll-like receptor 4 signaling activated by TnC, promoting an increased inflammatory response, hepatocyte transformation, and migration. Further, obese patients with cirrhosis alone and in combination with HCC showed significant increases in plasma TnC compared with healthy volunteers and patients with less severe liver injury. Overall, these studies suggest TnC/Toll-like receptor 4 signaling as an important regulator in HCC; inhibiting this signaling axis may be a viable therapeutic target for impeding HCC. (*Am J Pathol* 2016, 186: 145–158; <http://dx.doi.org/10.1016/j.ajpath.2015.09.015>)

Nonalcoholic fatty liver disease (NAFLD) is estimated to affect nearly 30% of the US population.¹ A subset of patients with NAFLD develop nonalcoholic steatohepatitis (NASH), characterized pathologically by hepatic steatosis, lobular inflammation, and ballooning. Although genetic variation, such as with patatin-like phospholipid domain containing protein 3,² may explain some individual susceptibility to NASH, fibrosis progression, and, ultimately, hepatocellular carcinoma (HCC), the exact links in this pathway are largely unresolved. HCC is the fifth most common cancer in the world and the second leading cause of cancer-related mortality.³ Although incidence of HCC arising in the context of NASH is currently lower than that of chronic viral hepatitis,^{4,5} the increasing prevalence of NAFLD and NASH will lead to a greater number of patients

at risk of developing HCC, particularly because obesity in and of itself is an independent risk factor for liver cancer.⁶ Thus, the mechanisms underlying the development of HCC, both in obesity and NASH, are an attractive and clinically relevant area of investigation.

One of the key events in the promotion of HCC development is activation of hepatic stellate cells (HSCs).^{7–10} On liver injury, HSCs transdifferentiate from an adipogenic-like cell to that of a highly contractile, proliferative, myofibroblast-like cell.¹¹ In addition to change in structural characteristics, activated HSCs also increase secretion of several extracellular

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matrix proteins, including tenascin-C (TnC).^{12,13} TnC, a hexameric extracellular matrix glycoprotein, is minimally detected in normal/healthy tissue, but it is transiently expressed during tissue injury, mediating both an inflammatory and fibrogenic response, promoting effective tissue repair.¹⁴ In liver disease, TnC is up-regulated in fibrotic areas and perisinusoidal spaces,^{15,16} with HSCs being the primary cellular source.¹⁷ Conversely, TnC deficiency attenuates development of fibrosis.^{13,18} Moreover, TnC concentrations are increased in visceral adipose tissue of obese individuals with NAFLD, contributing to the sustained inflammatory response and matrix remodeling.¹⁹ Elevation of TnC expression is associated with an invasive phenotype of primary cultured HCC cells²⁰ and is shown to promote epithelial-mesenchymal transition (EMT) in colorectal cancer cells.²¹ Further, EMT was found to play a pivotal role in migration of malignant hepatocytes during HCC progression.²²

TnC interacts with several extracellular matrix molecules and cell receptors, including Toll-like receptor 4 (TLR4), increasing proinflammatory cytokine expression [eg, tumor necrosis factor (TNF)- α , IL-6, IL-8].²³ Liver cells (eg, HSCs, Kupffer cells, endothelial cells, and hepatocytes), benign tumors, and malignant cancer cells express TLR4.^{24,25} In normal immune cells, TLR4 was shown to enhance antitumor immunity; however, with chronic inflammation, TLR4 signaling augments cancer development.²⁵ TLR4 expression is elevated in murine livers after diet-induced obesity.²⁶ Further, TLR4 and TLR2 expression increases in livers from patients with hepatitis, cirrhosis, or HCC.²⁷ Conversely, mice with impaired TLR4 signaling are less susceptible to liver fibrosis.²⁸ In a metastatic model, silencing TLR4 resulted in a lowering of tumor burden²⁴; conversely, TLR4 activation by lipopolysaccharide (LPS) contributed to inflammation-driven tumor promotion in HCC.²⁹

Herein, we present data showing that elevated expression of TnC by HSCs promotes enhanced TLR4 signaling, leading to inflammation, hepatocyte transformation, and migration, which is correlated with development of obesity-associated HCC in a mouse model. Furthermore, TnC concentrations are also increased in plasma from patients with NASH cirrhosis, and this concentration is further increased in patients with HCC. Interfering with and/or blocking TnC/TLR4 signaling may prove to be a therapeutic target for preventing HCC.

Materials and Methods

Cell Lines

Human hepatoma cell line Huh 7.0 and mouse macrophage cell line Raw 264.7 were cultured in Dulbecco's modified Eagle's medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 2.0 mmol/L glutamine, and 10% fetal bovine serum. HepG2 and Hep3B human hepatoma cell lines were cultured in minimal essential medium with the same supplements as described for Huh 7.0 cells.

Primary Cell Isolation

Adult male Sprague-Dawley rats (200 to 250 g for hepatocytes and >500 g for HSCs; Charles River Laboratories, Durham, NC) were used for these studies. All experiments were reviewed and approved by the Carolinas Medical Center (Charlotte, NC) Institutional Animal Care and Use Committee (protocols 09-12-04A and 09-13-01A). Primary rat hepatocytes were isolated and plated with a modified two-step collagenase perfusion as previously reported.³⁰ Primary rat HSCs were isolated by pronase/collagenase perfusion digestion, followed by subsequent density-gradient centrifugation, as previously reported.³¹

Cell Treatments

Huh 7.0 cells, primary rat hepatocytes, and Raw 264.7 cells were treated with 1 $\times$ phosphate-buffered saline (control), 1 μ g/mL LPS (diluted in 1 $\times$ phosphate-buffered saline), or 50 nmol/L TnC $\pm$ 1 μ g/mL LPS for 6, 15, or 24 hours. To confirm the involvement of the TLR4 signaling pathway, Huh 7.0 cells and Raw 264.7 cells were preincubated with either 25 μ mol/L TLR4-specific antagonist (VIPER; Imgenex, San Diego, CA) or control peptide (CP7; Imgenex) 90 minutes before treatment with TnC $\pm$ LPS. Primary rat HSCs were treated on day 7 for 72 hours with dimethyl sulfoxide control or 10 μ mol/L SR9009 (EMD Millipore, Billerica, MA).

Mouse Model

Male C57BL/6 mice (aged 21 to 25 days; Charles River Laboratories) were randomized, weighed, and injected intraperitoneally one time with diethylnitrosamine (DEN; Sigma-Aldrich, St. Louis, MO) or olive oil vehicle before mice were randomly assigned to a 10% kcal% fat, control diet (CD; Research Diets, Inc., New Brunswick, NJ; D12450B) or a 60% kcal% fat (40% unsaturated: 60% saturated fat lard), high-fat diet (HFD; Research Diets; D12492) at 5 weeks as previously reported.³² At 42 weeks, mice were euthanized, and the livers were resected and stored (-80°C). All work with mice performed at Carolinas Medical Center was reviewed and approved by the Institutional Animal Care and Use Committee at this institution (protocol no. 12-07-01A). Plasma, snap-frozen liver, and formalin-fixed, paraffin-embedded liver from male C57BL/6 mice fed the CD or HFD for 10 and 20 weeks were obtained from The Jackson Laboratories (Bar Harbor, ME).

Patient Samples

Whole blood was collected and submitted to the Liver, Biliary and Pancreatic Repository according to an institutional review board-approved protocol for Carolinas HealthCare System. Obese patients [body mass index (BMI; calculated as kg/m^2) > 30] undergoing bariatric surgery, hepatobiliary surgery, or with chronic liver disease were

eligible for enrollment. Obese patients were negative for hepatitis B and hepatitis C viruses. Plasma samples were obtained from healthy volunteers (BMI < 30) as a control group. Concentrations of TnC in plasma were determined with the use of the human TnC (FN III-C) Assay enzyme-linked immunosorbent assay (ELISA) kit (IBL, Minneapolis, MN). For statistical analysis, TnC results were stratified by fibrosis stage and NAFLD activity score,³³ determined by liver biopsy at the time of bariatric surgery. Patients with cirrhosis and HCC were diagnosed by cross-sectional imaging³⁴ and compatible clinical symptoms/signs of cirrhosis.³⁵ Patient characteristics are described in [Table 1](#).

Histologic Analysis

Immunohistochemistry

Sections (4 μ m) of formalin-fixed, paraffin-embedded liver tissue were blocked with 2.5% normal horse serum and incubated with either anti-TLR4 antibody (dilution 1:75; Abcam, Cambridge, MA;) or anti-CD68 antibody (dilution 1:250; Abcam) for macrophage detection. Detection of bound antibody was visualized with an anti-rabbit IgG ImmPRESS polymerized reporter enzyme staining system (Vector Labs, Burlingame, CA) with diaminobenzidine (Vector Labs). The nuclei were counterstained with Mayer's hematoxylin. Representative liver sections were examined microscopically ($\times 20$) on an Olympus BX40 microscope (Olympus America, Inc., Center Valley, PA) with a DP72 camera and CellSense standard 2.0 software (Olympus America, Inc.) and scored by three blinded independent investigators with the use of the following scoring scale: 0, no staining to <1%; 1, faint staining, 1% up to 10%; 2, moderate staining, 10% up to 40%; 3, heavy staining, >40%. Individual scores were averaged to medians $\pm$ SEM, providing a numerical score for each liver section and then used for statistical analysis.

Naphthol AS-D-Chloroacetate Esterase (Leder stain)

Sections (4 μ m) of formalin-fixed liver tissue were incubated in Naphthol AS-D-Chloroacetate Esterase working solution and then counterstained with Gill's hematoxylin, followed by permanent coverslipping with Permount. Representative liver sections were examined microscopically ($\times 40$) on an Olympus BX40 microscope (Olympus America, Inc.) with a DP72 camera and CellSense standard 2.0 software (Olympus America, Inc.), and neutrophils were counted and averaged from four randomly selected areas per slide by two independent investigators.

Immunofluorescence

Four representative sections (4 μ m) of formalin-fixed, paraffin-embedded liver tissue were incubated with the following primary antibodies: rabbit anti-TnC (dilution 1:100; Abcam) and goat anti- α -smooth muscle actin (α -SMA; dilution 1:100; Abcam) overnight then detected with

the following secondary antibodies: anti-rabbit cyanine 3 (dilution 1:250; Jackson ImmunoResearch, West Grove, PA) and Alexa Fluor donkey anti-goat IgG 488 (dilution 1:2500; Life Technologies, Carlsbad, CA). The nuclei were stained with DAPI (Life Technologies). To suppress nonspecific background staining, slides were incubated with Sudan Black B (Sigma-Aldrich) before being coverslipped with DABCO (Electron Microscopy Sciences, Hatfield, PA). In addition to merging green and red fluorescence to detect HSCs that express TnC, areas of close proximity of TnC and α -SMA were also denoted with arrows, which is likely more accurate because TnC is a secreted protein and may not exactly colocalize with α -SMA (ie, visualized by yellow fluorescence). Further, side-by-side comparison of two images was shown to be the most reliable method for visually comparing relative distribution of two probes.³⁶ Slides were examined with the use of a Zeiss 710 Confocal Microscope with Z-Stack (Carl Zeiss, Oberkochen, Germany) and $\times 40$ magnification.

Quantitative Real-Time PCR

Total RNA was extracted from primary rat hepatocytes, using TRIzol Reagent according to the manufacturer's (Invitrogen, Carlsbad, CA) protocol. Total RNA was extracted from mouse liver tissue, Huh 7.0 cells, and Raw 264.7 cells with the use of the Zymo Quick-RNA Mini-Prep with TRIzol and DNase treatment (Zymo Research, Irvine, CA). cDNA was synthesized by iScript cDNA synthesis kit according to the manufacturer's protocol (Bio-Rad, Hercules, CA) with the use of 1 μ g total RNA. The reverse transcription was performed as previously reported.³¹ Mouse-, rat-, and human-specific primers are listed in [Table 2](#).

Immunoblotting Analysis

Protein lysates from mouse liver tissue were prepared with ProPrep protein extraction solution according to manufacturer's protocol (Boca Scientific, Boca Raton, FL). Protein concentration was determined by Pierce bicinchoninic acid (BCA) protein assay kit. Equal amounts of protein were loaded on a 4% to 12% Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Thermo Scientific, Waltham, MA). The following antibodies were used: anti-TLR4 (Abcam and Santa Cruz Biotechnology, Inc., Dallas, TX), anti-TnC (Abcam and R and D Biosciences, Littleton, CO), and anti-glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Inc.). Human coded plasma samples were loaded equally on a 4% to 12% Bis-Tris gel and transferred to a polyvinylidene difluoride membrane and probed for anti-TnC (Millipore, Billerica, MA). Protein expression was quantified by scanning densitometry by Quantity One Software version 4.6.3 (Bio-Rad Laboratories, Hercules, CA) and normalized to glyceraldehyde-3-phosphate dehydrogenase loading control.

Table 1 Demographic and Clinical Characteristics of Study Subjects

Variable	Metavir score					F4 + HCC nonobese (n = 15)
	F0 (n = 36)	F1 (n = 31)	F2–F3 (n = 22)	F4 (n = 26)	F4 + HCC (n = 13)	
Sex, n (%)						
Male	5 (13.9)	5 (16.1)	4 (18.2)	12 (46.2)	10 (76.9)	13 (86.7)
Female	31 (86.1)	26 (83.9)	18 (81.8)	14 (53.9)	3 (23.1)	2 (13.3)
Age, year	43.4 ± 1.5	47.1 ± 1.4	49.0 ± 2.2	55.4 ± 1.8	67.5 ± 2.3	63.4 ± 2.4
BMI, kg/m ²	43.6 ± 1.1	44.2 ± 1.0	41.5 ± 1.8	37.5 ± 1.5	31.9 ± 1.7	26.8 ± 0.8
AST (IU/L)	21.6 ± 1.4	32.6 ± 4.1	41.2 ± 6.1	51.9 ± 3.6	87.9 ± 18.3	74.6 ± 11.0
ALT (IU/L)	21.9 ± 2.0	41.7 ± 7.0	47.2 ± 11.3	36.9 ± 3.0	59.2 ± 13.4	57.7 ± 1.2
AFP (IU/L)						
Median	0 (0)	0 (0)	1.15 (2/22)*	3.65 (20/26)*	7.6 (12/13)*	17.0 (13/15)*
Range	0 (0)	0 (0)	1.1–1.2	1.4–12.7	0.9–1,032,400	2.8–2399
Tumor size, n (%)						
≤3 cm	NA	NA	NA	NA	8 (61.5)	9 (60.0)
>3 cm	NA	NA	NA	NA	5 (38.5)	6 (40.0)
Steatosis, n (%)						
0	21 (58.3)	2 (6.5)	0/21 (0)*	0/9 (0)*	1/5 (20.0)*	0/3 (0)*
1	11 (30.6)	12 (38.7)	14/21 (66.7)*	5/9 (55.6)*	3/5 (60.0)*	3/3 (100)*
2	4 (11.1)	14 (45.2)	4/21 (19.0)*	3/9 (33.3)*	1/5 (20.0)*	0/3 (0)*
3	0 (0)	3 (9.7)	3/21 (14.3)*	1/9 (11.1)*	0/5 (0)*	0/3 (0)*
Lobular inflammation, n (%)						
0	28 (77.8)	8 (25.8)	2/20 (10.0)*	2/9 (22.2)*	1/5 (20.0)*	0/3 (0)*
1	8 (22.2)	18 (58.1)	7/20 (35.0)*	4/9 (44.4)*	4/5 (80.0)*	2/3 (66.7)*
2	0 (0)	5 (16.1)	9/20 (45.0)*	3/9 (33.3)*	0/5 (0)*	1/3 (33.3)*
3	0 (0)	0 (0)	2/20 (10.0)*	0/9 (0)*	0/5 (0)*	0/3 (0)*
Ballooning, n (%)						
0	32 (88.9)	17 (54.8)	4/20 (20.0)*	3/9 (33.3)*	1/5 (20.0)*	1/3 (33.3)*
1	4 (11.1)	12 (38.7)	12/20 (60.0)*	5/9 (55.6)*	4/5 (80.0)*	2/3 (66.7)*
2	0 (0)	2 (6.5)	4/20 (20.0)*	1/9 (11.1)*	0/5 (0)*	0/3 (0)*
NAFLD Activity Score, n (%)						
0	19 (52.8)	1 (3.2)	0/20 (0)*	0/9 (0)*	0/5 (0)*	0/3 (0)*
1	9 (25.0)	5 (16.1)	1/20 (5.0)*	1/9 (11.1)*	1/5 (20.0)*	0/3 (0)*
2	3 (8.3)	2 (6.5)	3/20 (15.0)*	1/9 (11.1)*	1/5 (20.0)*	1/3 (33.3)*
3	4 (11.1)	12 (38.7)	4/20 (20.0)*	3/9 (33.3)*	2/5 (40.0)*	2/3 (66.7)*
4	1 (2.8)	8 (25.8)	5/20 (25.0)*	2/9 (22.2)*	1/5 (20.0)*	0/3 (0)*
5	0 (0)	3 (9.7)	4/20 (20.0)*	2/9 (22.2)*	0/5 (0)*	0/3 (0)*
6	0 (0)	0 (0)	1/20 (5.0)*	0/9 (0)*	0/5 (0)*	0/3 (0)*
7	0 (0)	0 (0)	0/20 (0)*	0/9 (0)*	0/5 (0)*	0/3 (0)*
8	0 (0)	0 (0)	2/20 (10.0)*	0/0 (0)*	0/5 (0)*	0/3 (0)*

*Liver biopsies/AFP not available for all patients.

AFP, α -fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HCC, hepatocellular carcinoma; NA, not applicable; NAFLD, nonalcoholic fatty liver disease.

NF- κ B Nuclear Expression

Huh 7.0 and Raw 264.7 cells were seeded on 4-well Nunc LabTek II chamber slides (Nunc International, Rochester, NY). Cells were preincubated with either 25 μ mol/L VIPER or CP7 for 90 minutes before being treated with 50 nmol/L TnC $\pm$ 1 μ g/mL LPS for 1 hour (Raw 264.7 cells) or 3 hours (Huh 7.0 cells). Cells were then fixed and incubated with the following primary antibodies: rabbit anti-NF- κ B (dilution 1:100; Huh 7.0 cells; Invitrogen; or dilution 1:100; Raw 264.7 cells; eBioscience, San Diego, CA) overnight at 4°C and were detected with secondary donkey anti-rabbit 488 (dilution 1:1500; Invitrogen). The nuclei were stained

with DAPI (Life Technologies). Slides were examined with a Zeiss 710 Confocal Microscope with Z-Stack and $\times 40$ magnification.

ELISA

TNF- α concentrations were quantified from mouse liver tissue lysates, spent media from cultured rat primary hepatocytes, and spent media from Raw 264.7 cells with the use of a rat TNF- α ELISA antibody pair set, standard (Sigma-Aldrich) and ELISA support pack (eBiosciences). IL-6 concentrations from mouse liver tissue, rat primary hepatocyte media, and Raw 264.7 cell media were quantified with the use of a mouse

Table 2 Real-Time PCR Primers

Primer name	Sequence
Human primers	
hTNF α (+)	5'-CCCGACTATCTCGACTTTGC-3'
hTNF α (-)	5'-GGTTGAGGGTGTCTGAAGGA-3'
hIL-6 (+)	5'-AAATGCCAGCCTGCTGACGAAG-3'
hIL-6 (-)	5'-AACAAACAATCTGAGGTGCCCATGCTA-3'
hCdh1 (+)	5'-CTGGTTCAGATCAAATCCAAC-3'
hCdh1 (-)	5'-GCGTGAGAGAAGAGAGTGTATG-3'
hVimentin (+)	5'-ATCCAAGTTTGCTGACCTCTC-3'
hVimentin (-)	5'-GCCATTTCTCCTTCATATTC-3'
hCOL4A1 (+)	5'-CTGGAAATCCCGGACCAC-3'
hCOL4A1 (-)	5'-GGGAAATCCTCTTTACCTTTC-3'
hTLR4 (+)	5'-CTGCCCATGTGTCAGGCCTTAT-3'
hTLR4 (-)	5'-AATGCCACCTGGAAGACTCT-3'
hGAPDH (+)	5'-GAGTCAACGGATTTGGTCGT-3'
hGAPDH (-)	5'-TTGATTTTGGAGGGATCTCG-3'
Mouse primers	
mTNF α (+)	5'-ACTGAACCTCGGGGTGATCGGTCC-3'
mTNF α (-)	5'-GTGGGTGAGGAGCAGTAGTCG-3'
mIL-6 (+)	5'-GACAAAGCCAGAGTCCTTCAGAGAG-3'
mIL-6 (-)	5'-CTAGGTTTGCCGAGTAGATCTC-3'
mVimentin (+)	5'-CATTCAGACAGGATGTTG-3'
mVimentin (-)	5'-TCTTCATCGTGCAGTTTC-3'
mTNC (+)	5'-ACCATGCTGAGATAGATGTTCCAA-3'
mTNC (-)	5'-CTTGACAGCAGAAACACCAATCC-3'
m α -SMA (+)	5'-ATCGTCCACCGCAAATGC-3'
m α -SMA (-)	5'-AAGGAAGTGGAGGCGCTG-3'
mGAPDH (+)	5'-CATGGCCTTCCGTGTTCTCTA-3'
mGAPDH (-)	5'-CCTGCTTCACCACCTTCTTGA-3'
Rat primers	
rTNF α (+)	5'-AGACCCTCACACTCAGATCATCTTCT-3'
rTNF α (-)	5'-CACGCTGGCTCAGCCACT-3'
rIL-6 (+)	5'-GTGGCTAAGGACCAAGACCA-3'
rIL-6 (-)	5'-GGTTTGCCGAGTAGACCTCA-3'
rTLR4 (+)	5'-ATCATCCAGGAAGGCTTCCA-3'
rTLR4 (-)	5'-GCTGCCTCAGCAAGGACTTCT-3'
rTnC (+)	5'-TGTTGTGCTTCGAAGGCTATG-3'
rTnC (-)	5'-GCTCATTCGAGTCTTCACCA-3'
rCdh1 (+)	5'-GGCATCACCACAGAGACC-3'
rCdh1 (-)	5'-CCAGTCTCGTTTCTGTCTTCTC-3'
rVimentin (+)	5'-GGAGGAGAGCAGGATTTCTC-3'
rVimentin (-)	5'-GGTGTCGAACCAGAGGAAGTG-3'
rCOL4A1 (+)	5'-TTCACACCTGAGCACACACA-3'
rCOL4A1 (-)	5'-CGCCTTCAAGCATAGTGA-3'
rGAPDH (+)	5'-ATCCCCTAACATCAAATGG-3'
rGAPDH (-)	5'-ACTGTGGTCATGAGCCCTTC-3'

IL-6 mouse ELISA kit (Abcam). IL-6 concentrations were quantified from Huh 7.0 cells with the use of a human IL-6 ELISA antibody pair set (BD Biosciences, San Jose, CA), standard (eBiosciences), and ELISA support pack (eBiosciences).

Migration Assay

Migration assays were performed with Platypus Technologies Oris Cell Migration Assays (Madison, WI) in a 96-well

plate with preinserted stoppers before treatment (detection zone). Huh 7.0 cells were fluorescently labeled with Cell-Tracker Green CMFDA fluorescent probe (Molecular Probes, Inc., Eugene, OR). Stoppers were removed, and cells were preincubated with either 25 μ mol/L VIPER or CP7 for 90 minutes before being treated with 50 nmol/L TnC $\pm$ 1 μ g/mL LPS for 24 hours. Images were captured with an Olympus IX51 microscope (Olympus America, Inc.) at $\times 2$ and $\times 4$ magnification. Distance migrated was assessed by measuring the area of the detection zone at 0 and 24 hours for each well with the use of the Olympus CellSense standard 2.0 software (Olympus America, Inc.).

Statistical Analysis

Unless otherwise stated, all data are expressed as means $\pm$ SEM. Two-way analysis of variance with all pairwise multiple comparisons by Bonferroni *t*-test was performed for all *in vivo* experiments. One-way analysis of variance with Tukey-Kramer or Bonferroni, where appropriate, was performed for all *in vitro* experiments. For CD68 macrophage and Naphthol AS-D-Chloroacetate Esterase neutrophil analysis, data are expressed as medians $\pm$ SEM on a whisker plot. Nonparametric one-way analysis of variance with all pairwise comparisons was performed. All statistical analyses were performed with SigmaPlot 11.0 statistical software (Systat Software, Inc., San Jose, CA). *P* < 0.05 was considered significant.

Results

HFD Elevates Expression of TnC from Activated HSCs

To fully elucidate the deleterious effects of obesity-associated HCC, we developed a DIO HCC mouse model.³² C57BL/6 male mice (21 days) were injected intraperitoneally with 5 mg/kg DEN to promote tumor development, or olive oil as a control, and assigned to either CD (10% kcal %fat) or HFD (60% kcal %fat). Necropsies were performed after 37 weeks on the diet. As reported in an earlier publication, HFD alone led to a 30% (3 of 10) tumor incidence compared with 10% (1 of 10) incidence in CD, and HFD potentiated tumor incidence in mice injected with DEN 89% (8 of 9) compared with 60% (6 of 10) in CD + DEN.³² HFD (with or without DEN) enhanced mRNA expression of both α -SMA, a marker of activated HSCs, and TnC, a marker of liver injury (Figure 1A) compared with CD. However, only expression of TnC mRNA was augmented in the HFD + DEN mice compared with the CD + DEN mice (Figure 1A). Similarly, confocal microscopy showed elevated α -SMA and TnC protein expression in the HFD $\pm$ DEN mice compared with the CD mice, and TnC expression correlated with HSCs (ie, α -SMA⁺ cells) (Figure 1, B and C). Although yellow/orange fluorescence signal intensity was moderate, we have also denoted areas of close proximity of TnC and

Figure 1 HFD elevates expression of TnC from activated HSCs. **A:** Enhanced mRNA levels of α -SMA and TnC (RT-qPCR) from mouse liver tissue expressed as a fold-change relative to the CD mice. **B and C:** Formalin-fixed, paraffin-embedded whole liver sections were stained for α -SMA (green) and TnC (red), and localization was visualized by confocal microscopy. α -SMA expression was used as a positive control for HSCs. **White arrowheads** indicate dual staining and/or close proximity of α -SMA and TnC expression which can be visualized in merged images. **B:** TnC and α -SMA expression was calculated by quantifying pixel intensity with the use of Fiji Image J. Data are expressed as means $\pm$ SEM. $n = 7$ to 8 mice per treatment group (**A**); $n = 3$ to 4 mice per treatment group (**B**). * $P < 0.05$ versus CD; † $P < 0.05$ versus CD + DEN. CD, control diet; DEN, diethylnitrosamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HFD, high-fat diet; HSC, hepatic stellate cell; RT-qPCR, quantitative real-time RT-PCR; TnC, tenascin-C; α -SMA, α -smooth muscle actin.

α -SMA expression, because TnC is a secreted protein and may not exactly colocalize with α -SMA. Further, the most reliable method for visually comparing relative distribution of two probes is side-by-side comparison of two images.³⁶ α -SMA and TnC protein expression were also elevated in the CD + DEN mice compared with the CD mice. Furthermore, similar results were observed at earlier stages of liver injury, using a separate group of mice from a different DIO mouse model (10 weeks and 20 weeks HFD alone without DEN injections), whereby TnC expression was concomitantly increased with HSC activation (Supplemental Figure S1, A and B), demonstrating HFD alone can lead to HSC activation and TnC expression. To corroborate our confocal assessment and what is presented in the literature,¹⁷ showing HSCs as the primary source of hepatic TnC, primary HSCs and hepatocytes were isolated, and TnC expression was determined. Human HSC lines (TWNT-4 and LX2) and culture-activated primary HSCs, which mimic cellular events similar to those observed during hepatic injury, showed significantly higher amounts of TnC mRNA than freshly isolated primary hepatocytes and human hepatoma cell lines (Hep3B, HepG2, and

Huh7) (Supplemental Figure S2, A and B). Overall, these results identify HSCs as the main cellular source of TnC, although hepatoma cells can express TnC, but at significantly lower values.

HFD Increases Liver Inflammation

Previous studies found increased tumor burden in HFD mice compared with CD and increased markers of metabolic syndrome, such as serum high-density lipoprotein, low-density lipoprotein, and leptin.³² The livers of the HFD mice showed increased inflammation as assessed by TNF- α and IL-6 mRNA levels (Figure 2A). However, the HFD + DEN mice did not show increased inflammatory gene expression compared with the CD + DEN mice. Similarly, HFD elicited EMT as observed by increased expression of vimentin (Figure 2A). To further confirm an enhanced inflammatory response in our DIO HCC model, we examined infiltration of inflammatory cells (eg, macrophage, neutrophil) and observed increased immune cell count in livers of mice fed the HFD compared with the CD and in the HFD + DEN mice compared with the CD + DEN mice (Figure 2, B–D).

Figure 2 HFD increases liver inflammation. **A:** Cytokine (TNF- α and IL-6) and epithelial-mesenchymal transition gene (*Vim*) mRNA expression from C57BL/6 mice injected with DEN or olive oil (control) and randomly assigned to CD or HFD. Total RNA was extracted from mouse liver, and mRNA levels of each gene were analyzed by RT-qPCR and are expressed as a fold change relative to CD mice. **B:** Formalin-fixed, paraffin-embedded whole liver sections were stained with chromagen for CD68/macrophage and visualized by microscopy. Chromagen scoring (**C**) and neutrophil count (**D**) are from Leder-stained whole liver sections. Data are expressed as means $\pm$ SEM (**A**); each data point represents an individual field from mouse liver sections. $n = 7$ to 8 mice per treatment group (**A**); $n = 4$ per treatment group (**C** and **D**). * $P < 0.05$ versus CD; $^{\dagger}P < 0.05$ versus HFD. CD, control diet; DEN, diethylnitrosamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HFD, high-fat diet; RT-qPCR, quantitative real-time RT-PCR; TNF, tumor necrosis factor; Vim, vimentin.

Inhibition of TLR4 Signaling Prevents TnC-induced Elevations in Inflammatory Gene Expression

To more closely delineate the cellular signaling mechanism of TnC-induced inflammation in our DIO HCC mouse model, murine macrophages were treated with TnC $\pm$ LPS, a known activator of TLR4 signaling. TnC treatment resulted in significant elevations in TNF- α and IL-6 mRNA (Figure 3A). These levels were augmented by LPS (Figure 3A). Elevations in inflammatory cytokine mRNA expression correlated with secreted TNF- α and IL-6 in cell culture supernatant fluid

examined by ELISAs (Figure 3B). In addition, these responses were completely abrogated by pretreatment with VIPER³⁷ as gauged by inflammatory cytokine expression returning to near control levels (Figure 3, A and B). The scrambled peptide (CP7) served as a control for VIPER treatments with no detectable effects to cells (data not shown). Because hepatocytes are also a source of TNF- α and IL-6, TnC treatment also increased expression of these cytokines in both primary rat hepatocytes (Supplemental Figure S3, A and B) and Huh 7.0

Figure 3 Inhibition of TLR4 signaling prevents TnC-induced elevations in inflammatory gene expression in macrophages. Raw 264.7 cells were preincubated with either 25 μ M VIPER or 25 μ M CP7 90 minutes before treatment with 0.05 μ M TnC $\pm$ 1.0 μ g/mL LPS. **A:** Total RNA was extracted, and mRNA expression for inflammatory cytokines (TNF- α , IL-6) was analyzed by RT-qPCR at 6 hours. **B:** TNF- α (6 hours) and IL-6 (24 hours) protein amounts analyzed by ELISA were normalized to total protein. **C:** NF- κ B translocation was assessed by immunofluorescence. Cells were grown on cover slides and treated as described in *Materials and Methods*, followed by fixation and immunostaining with anti-p65 NF- κ B antibody and visualized by confocal microscopy from three independent experiments. Data are expressed as means $\pm$ SEM. $n \geq 3$. * $P < 0.05$ versus CP7 control; $^{\dagger}P < 0.05$ versus CP7 + TnC. CP7, control peptide; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; ND, not detectable; TLR, Toll-like receptor; TnC, tenascin-C; RT-qPCR, quantitative real-time RT-PCR; TNF, tumor necrosis factor; VIPER, TLR4-specific antagonist.

Figure 4 TLR4 expression is modulated by HFD and TnC. **A:** Formalin-fixed, paraffin-embedded whole liver sections were stained with chromagen for TLR4. TLR4 localization was visualized by microscopy, and three independent blind scores were performed from each experimental group. **B:** TLR4 scoring data. **C:** Huh 7.0 cells were treated with 0.05 $\mu\text{mol/L}$ TnC for 6 hours. Total RNA was extracted, and mRNA levels for TLR4 were analyzed by RT-qPCR and are expressed as a fold change relative to control. Data are expressed as means $\pm$ SEM. $n = 3$ to 4 mouse liver tissue sections per treatment group (**B**); $n \geq 3$ (**C**). * $P < 0.05$ versus CD; $^{\dagger}P < 0.05$ versus all groups. CD, control diet; DEN, diethylnitrosamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HFD, high-fat diet; RT-qPCR, quantitative real-time RT-PCR; TLR, Toll-like receptor; TnC, tenascin-C.

cells (Supplemental Figure S3C), and VIPER diminished this induction ($P = 0.062$) (Supplemental Figure S3C).

NF- κ B is a known downstream target of TLR4 signaling, which on activation, translocates from the cytoplasm to the nucleus to initiate inflammatory gene transcription.³⁸ To further establish TnC-mediated inflammatory response via intact TLR4 signaling, NF- κ B translocation was assessed in macrophages by confocal microscopy. Cells treated with TnC resulted in a shift in NF- κ B fluorescence to the nucleus compared with control cells (Figure 3C). In addition, LPS treatment further enhanced NF- κ B nuclear fluorescence compared with TnC alone, which was abrogated by VIPER. Collectively, these data support that intact TLR4 signaling is necessary for TnC-induced inflammatory response.

TLR4 Expression Is Modulated by HFD and TnC

TLR4 protein expression was elevated in livers of mice from all treatment groups compared with CD, as found by immunohistochemistry (Figure 4, A and B). Interestingly, DEN alone showed increased expression of TLR4. We also observed a significant increase *in vitro* in TLR4 mRNA expression in Huh 7.0 cells by TnC (Figure 4C), suggesting TnC regulates TLR4 expression.

TnC-Induced EMT Is Mediated through TLR4 in Hepatocytes

This study, and others, showed that TnC can elicit a response through TLR4²³; therefore, we examined if the TLR4/TnC signaling axis played a role in hepatocyte EMT.

TnC treatment in Huh 7.0 cells (Figure 5A) decreased mRNA expression of epithelial markers, Cdh1, and COL4A1 and increased expression of the mesenchymal marker, vimentin. These responses were abrogated by VIPER pretreatment, as gauged by EMT mRNA levels returning to near control levels (Figure 5A). Similar results were observed in primary rat hepatocytes (Cdh1 $P = 0.064$ and vimentin $P = 0.058$) (Supplemental Figure S3D). Elevation of TLR4 mRNA in TnC-treated Huh 7.0 cells was not inhibited by VIPER pretreatment (Supplemental Figure S4), indicating that the inhibitory effects of VIPER on inflammatory cytokines and EMT markers observed are not a result of decreased expression of TLR4 but are related to inhibition of intact TLR4 signaling cascade and downstream targets.

To further establish TnC-mediated EMT response via TLR4 signaling in hepatocytes, NF- κ B translocation was assessed by confocal microscopy. Cells treated with TnC or LPS showed increased nuclear NF- κ B fluorescence compared with control cells (Figure 5B), and VIPER abrogated this effect. These data further support that TnC-induced EMT in hepatocytes is mediated through intact TLR4 signaling.

TnC-Mediated Enhancement of Hepatoma Migration Is Regulated by TLR4

Because increased TnC expression is associated with the invasive phenotype of HCC cells,^{20,39} we examined the effects of TnC on cell migration in Huh 7.0 cells. TnC significantly increased cell migration in comparison with

Figure 5 Inhibition of TLR4 signaling prevents TnC-induced elevations in epithelial-mesenchymal transition gene expression. **A:** Huh 7.0 cells were preincubated with either 25 $\mu\text{mol/L}$ VIPER or 25 $\mu\text{mol/L}$ CP7 90 minutes before treatment with 0.05 $\mu\text{mol/L}$ TnC. Total RNA was extracted, and mRNA levels for epithelial-mesenchymal transition genes [*Cdh1*, *COL4A1*, and *Vim*] at 15 hours were analyzed by RT-qPCR. **B:** NF- κ B translocation in Huh 7.0 cells was assessed by immunofluorescence. Cells were grown on cover slides and treated as described in *Materials and Methods*, followed by fixation and immunostaining with anti-p65 NF- κ B antibody and visualized by confocal microscopy. Data are expressed as means $\pm$ SEM of fold change relative to control. $n \geq 3$ (**A**); $n = 4$ independent experiments (**B**). * $P < 0.05$ versus CP7 control. CP7, control peptide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide; RT-qPCR, quantitative real-time RT-PCR; TLR, Toll-like receptor; TnC, tenascin-C; VIPER, TLR4-specific antagonist.

control cells (Figure 6), and VIPER pretreatment abrogated this response, indicating that the effects of TnC on hepatoma cell migration are mediated through the TLR4 signaling.

Plasma TnC Is Elevated in Patients with Cirrhosis and HCC

Because secreted TnC was elevated from culture-activated HSCs (Supplemental Figure S5) and was increased in our DIO HCC model, we assessed plasma TnC concentrations from obese patients with and without liver disease. Plasma TnC concentrations, by ELISA, were significantly elevated in patients with NASH cirrhosis (Metavir score = 4) compared with healthy volunteers and with bariatric surgery patients with various stages of fibrosis (Metavir score = 0, 1, 2, and 3) (Table 1 and Figure 7A). No statistical difference was found in TnC concentrations when stratified by NAFLD activity score (≤ 2 versus ≥ 3 ; data not shown). Interestingly, plasma from patients with NASH cirrhosis and HCC (4 + HCC) had significantly higher plasma TnC than patients without HCC. Western blot analyses showed two TnC variants, and both variants increased significantly in NASH cirrhotic patients, with or without HCC (Figure 7B). Further, NASH cirrhotic patients with HCC had 1.5 times higher TnC expression than those without HCC (Figure 7C).

Figure 6 Inhibition of TLR4 signaling prevents TnC-mediated enhancement of cell migration. Huh 7.0 cells were fluorescently labeled and seeded on Platypus migration assay 96-well plates with stoppers and allowed to attach for a minimum of 3 hours. Stoppers were removed, and cells were preincubated with either 25 $\mu\text{mol/L}$ VIPER or 25 $\mu\text{mol/L}$ CP7 90 minutes before treatment with 0.05 $\mu\text{mol/L}$ TnC for 24 hours. Distance migrated was assessed by measuring the area of the detection zone at 0 and 24 hours for each well. Representative images after migration, 24 hours after TnC was visualized by microscopy. Data are expressed as means $\pm$ SEM. $n = 4$. * $P < 0.05$ versus CP7 control; † $P < 0.05$ versus CP7 + TnC. CP7, control peptide; TLR, Toll-like receptor; TnC, tenascin-C; VIPER, TLR4-specific antagonist.

Discussion

HSCs Are the Primary Source of Elevated TnC

HFD enhanced an inflammatory response, EMT, and development of HCC in a DEN-initiated HCC murine model, and these pathologies were associated with increased HSC-derived TnC and TLR4 expression. Further *in vitro* mechanistic studies showed TnC-mediated inflammation, hepatocyte EMT, and migration through intact TLR4 signaling (Figure 8). Our study supports activated HSCs as the main cellular source of TnC, but they were not exclusively nor consistently the only origin, because primary hepatocytes and hepatoma cells in culture also expressed TnC but at a substantially lower value. In addition, TnC expression in hepatoma cells could be stimulated with TNF- α .³⁹ Further, results similar to ours were observed in canine hepatitis in which an important relation between TnC and α -SMA was obtained when correlation was assessed without considering location. However, when correlation was analyzed per location, the overlap was not significant.⁴⁰ Isolation of nonparenchymal and hepatocytes from livers of the DIO HCC mice will help clarify cell-specific TnC expression, and it is likely that TnC from different cells contributes a unique pathologic function affecting cell transformation, migration, and metastasis.

TnC/TLR4 Signaling Promotes Inflammatory Response

In this study, plasma TnC concentrations were elevated in NASH cirrhotic patients with further increases in patients with HCC. Extracellular matrix proteins can have dynamic signaling properties and are now appreciated to play a crucial role in the pathophysiology of inflammatory diseases and cancer.^{41,42} TnC was identified as an endogenous activator of TLR4-mediated immunity in arthritic joint disease.²³ It can promote hepatic fibrosis by augmenting inflammatory responses, such as through increased expression of transforming growth factor- β , enhancing HSC recruitment. Contrarily, inhibition of TnC attenuates development of hepatic fibrosis.¹³ Further, TLR4 promotes the development of HCC,²⁹ and, interestingly, TLR4 signaling is already heightened in fatty liver²⁶; thus establishing TnC/TLR4 signaling axis as an important contributor in NAFLD-associated HCC would provide new therapeutic strategies. Because TnC can be detected in plasma, it could also serve as a noninvasive biomarker for cirrhosis and HCC.

The pathways linking NAFLD to HCC are undefined. HCC is associated with an aberrant immune response. Several proinflammatory cytokines (eg, TNF- α and IL-6) are elevated in HCC tumors and detected in serum and plasma,⁴³ and these cytokines have proliferative and anti-apoptotic effects.⁴⁴ In addition, these cytokines promote obesity-enhanced HCC.⁴⁴ In our mouse model, elevated concentrations of TNF- α and IL-6 mRNA were observed in livers of the HFD mice compared with the CD mice.

Figure 7 TnC expression in human plasma is elevated in obesity-associated cirrhosis and progression to HCC. **A:** TnC concentrations were measured by ELISA in plasma samples from healthy volunteers (BMI < 30) and patients with a primary diagnosis of obesity (BMI > 30) and Metavir scores of 0 to 4. Patients with Metavir score of 4 (NASH cirrhosis) were compared with patients with HCC (obese and nonobese etiologies). **B:** Representative immunoblot of TnC protein expression from human plasma samples. **C:** TnC concentrations were analyzed from immunoblots of TnC protein from NASH cirrhotic patients (Metavir score of 4, BMI > 30) with HCC compared with those without HCC. Data are expressed as means $\pm$ SEM. $n = 4$ (C). * $P < 0.05$ versus healthy volunteers and Metavir scores 0 to 3 (A); $^{\dagger}P < 0.05$ versus Metavir score 4 (A); * $P < 0.05$ versus Metavir score 4 (C). BMI, body mass index (calculated as kg/m²); ELISA, enzyme-linked immunosorbent assay; HCC, hepatocellular carcinoma; NASH, nonalcoholic steatohepatitis; TnC, tenascin-C.

Interestingly, the HFD + DEN mice did not show increased tissue cytokine expression, although inflammation is clearly elevated as seen by increased inflammatory cell infiltration by immunohistochemistry. It will be important to determine differences in inflammatory response between tumor and nontumor tissue to more precisely understand the role of TnC in disease progression. Previous studies suggested that enhanced TnC expression in the inflammatory HCC tumor

Figure 8 Proposed mechanism for TnC-mediated TLR4 signaling in the promotion of obesity-associated HCC. On liver injury, from diet-induced obesity, activated HSCs increase secretion of TnC, which signals through TLR4 to promote inflammation and hepatocyte transformation, migration, and eventual development of HCC. EMT, epithelial-mesenchymal transition; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; LPS, lipopolysaccharide; NAFLD, nonalcoholic fatty liver disease; TnC, tenascin-C; TLR, Toll-like receptor.

microenvironment may play an important role in HCC recurrence and metastasis.³⁹ Further, TnC increased expression of proinflammatory cytokines in a murine macrophage cell line and in primary rat hepatocytes, showing that TnC can promote an inflammatory response from both immune cells and hepatocytes. TnC was shown to play a key role in regulating inflammation mediated through miR-155 during pathogenic activation of TLR4⁴⁵; thus, it will be interesting to see if this miRNA or others contribute to HFD-induced inflammation. Preliminary data from our group indicates that exosomal miR-155 is elevated in plasma of NAFLD + HCC and alcoholic liver disease ALD + HCC patients ($n = 4$) compared with patients with fibrosis only. However, patients with hepatitis C virus + HCC show a different miRNA profile, suggesting that the cause is important for miRNA expression and regulation of disease development and progression.

TnC/TLR4 Signaling Promotes EMT

HCC initiation and progression are two important points of therapeutic intervention. In primary rat hepatocytes and hepatoma cells, TnC, mediated through TLR4, increased EMT and/or migration, indicating enhancement of metastatic potential. Previous studies found that silencing TLR4 inhibits invasion, survival, and tumorigenicity of prostate cancer cells, whereas LPS enhanced the invasive potential and EMT of HCC cells with elevated TLR4 expression.⁴⁶ EMT enhances cell migration and invasion, and increased vimentin expression may play an important role in heightened cell migration.⁴⁷ Other signaling mechanisms for promoting EMT were identified, such as transforming growth factor- β , platelet-derived growth factor, and mitogen-activated protein kinase^{22,48–50}; therefore, the crosstalk with these pathways and TnC/TLR4 needs further investigation. Activated HSCs, the primary cellular source of TnC, not only elicited an inflammatory response as

shown in this study but also were shown to stimulate growth, invasion, and migration of hepatoma cells.⁵¹ Further, senescent HSCs, characterized by a secretory profile, were shown to promote obesity-induced hepatic carcinogenesis.⁵² Moreover, elevations in macrophage-derived IL-6, as seen here, were also shown to favor EMT in HCC.⁵³ In addition, NF- κ B translocation was suggested as a useful marker of EMT and invasiveness in other cancers.⁵⁴ Thus, HSC-derived TnC signaling through intact TLR4 signaling on hepatocytes may provide potential therapeutic targets for preventing the initiation (ie, hepatocyte transformation) and progression (ie, growth and metastasis) of HCC.

Targeting TnC/TLR4 Signaling as a Therapy

Although results presented herein were performed in a DIO HCC mouse model, supported by mechanistic studies, the TnC/TLR4 signaling axis will be relevant to HCC development regardless of cause, because TLR4 signaling was shown to play a key role in tumor invasion, migration, and poor prognosis of HCC.⁵⁵ Recent studies from our group identified transcriptional repressor Rev-erb as a regulator of TnC in HSCs, and a specific Rev-erb agonist, SR9009, inhibited the development of fibrosis.³¹ *In vitro* studies showed SR9009 decreased expression of secreted TnC from activated HSCs (Supplemental Figure S6A). Therefore, it will be interesting to test if this agonist can also block the development of obesity/NAFLD-associated HCC by inhibiting TnC-mediated liver injury. Further, hepatocytes exposed to conditioned media from HSCs pretreated with SR9009 (media diminished of TnC) showed a significant reduction in proinflammatory cytokine expression compared with hepatocytes exposed to control HSC media (cells treated only with dimethyl sulfoxide) (Supplemental Figure S6B), suggesting TnC from activated HSCs promotes an inflammatory response. Moreover, to definitively determine the contribution of TnC/TLR4 signaling in the

development of obesity/NAFLD-associated HCC, we have proposed the use of this agonist in our DIO HCC animal model with wild-type and TLR4 knockout mice. Although the use of TnC knockout mice was shown to play a role in the development of fibrosis, the TnC knockout mice have several defects, including behavioral abnormalities, defects in healing, and abnormally low concentrations of fibronectin.⁵⁶ It is also difficult to assess if these knockout animals would even survive our DIO HCC animal model study. Moreover, because TnC has several key functions that numerous disease disorders, the best approach would be to knockdown/inhibit TnC specifically in the HSC. Therefore, studies are under way to develop an animal model in which TnC expression can be modulated specifically in the HSC. In addition, because the study presented relied on data collected at one terminal end point, further investigation into the effects of HFD and DEN at earlier time points during disease progression may provide greater insight into the mechanism of obesity-associated HCC.

TnC as a Diagnostic Indicator for Progression of Cirrhosis to HCC

HCC is one of the most common and aggressive malignancies worldwide.⁵⁷ The prognosis for HCC is poor because of its late detection, and patients with HCC usually survive for less than a year. However, if HCC is detected at early stages, curative treatments (ie, ablation, resection, transplantation) are attainable.⁵⁸ Thus, defining an early marker for HCC is needed and would improve current effectiveness of screening and hence, prognosis of this insidious disease. NASH cirrhosis is predicted to be the leading indication for liver transplantation in the next decade.⁵⁹ However, screening modalities, such as ultrasound scanning, are less sensitive in obese patients, and serial surveillance imaging by computed tomography or magnetic resonance imaging is not cost effective. Moreover, α -fetoprotein is an insensitive biomarker and is not recommended for screening by the American Association for the Study of Liver Diseases.⁶⁰ Presently, it is not possible to predict NAFLD outcome by routine blood and tissue biomarkers. Although most NAFLD-associated HCC develops in the background of cirrhosis, there are increasing cases of HCC arising in NAFLD patients without cirrhosis,⁶¹ thereby compounding the difficulty in finding a useful biomarker because of various disease presentations (ie, with or without inflammation, fibrosis, or cirrhosis). The yearly cumulative incidence of HCC in NASH cirrhotic patients was reported to be 2.6% per year.⁶² Further, risk factors for developing HCC in NASH cirrhotic patients were i) older age at time of cirrhosis diagnosis, ii) higher BMI, and iii) alcohol consumption. A population of HSCs was characterized by an inflammatory but not a fibrogenic phenotype and may contribute to NASH-associated HCC in noncirrhotic livers.⁶³ Although NAFLD cirrhotic patients with HCC had lower BMIs than their noncirrhotic counterparts, it is not

surprising, because cirrhosis is typified by weight loss and profound catabolism due to the effects of advanced liver disease. Plasma TnC concentrations were substantially higher in NASH cirrhotic patients with HCC and nonobese cirrhotic patients with HCC (nonobese causes include hepatitis C virus, hepatitis B virus, alcoholic liver disease) than those without HCC. It will be important to systematically correlate tumor number and size with plasma concentrations of TnC to determine whether TnC can serve as an early, more sensitive biomarker of HCC than α -fetoprotein, which was shown to have limited use for diagnosis, particularly at the early stages.⁶⁴ Further, α -fetoprotein is also associated with chronic liver disease and is not specific for HCC.^{65,66} Our studies showed that TnC concentrations were significantly elevated regardless of tumor size and number (Table 1), and TnC expression is further elevated in patients with metastatic versus nonmetastatic HCC.³⁹ Further, it will be important to determine TnC expression directly from tumor versus surrounding nontumor tissue to assess its contribution to initiation and progression of HCC. Not surprisingly, this study also found HCC patients, regardless of cause, were considerably older than non-HCC patients, because age is a known risk factor for HCC and many other cancers.⁶² Overall, the data presented herein indicate that HSC-derived TnC is elevated in obesity-associated HCC and that TnC can initiate an inflammatory response and EMT through intact TLR4 signaling, suggesting TnC as a therapeutic target for HCC, and further, as a good diagnostic indicator for progression of cirrhosis to HCC.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.09.015>.

References

1. Bohinc BN, Diehl AM: Mechanisms of disease progression in NASH: new paradigms. *Clin Liver Dis* 2012, 16:549–565
2. Singal AG, Manjunath H, Yopp AC, Beg MS, Marrero JA, Gopal P, Waljee AK: The effect of PNPLA3 on fibrosis progression and development of hepatocellular carcinoma: a meta-analysis. *Am J Gastroenterol* 2014, 109:325–334
3. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D: Global cancer statistics. *CA Cancer J Clin* 2011, 61:69–90
4. El-Serag HB: Epidemiology of viral hepatitis and hepatocellular carcinoma. *Gastroenterology* 2012, 142:1264–1273.e1
5. White DL, Kanwal F, El-Serag HB: Association between nonalcoholic fatty liver disease and risk for hepatocellular cancer, based on systematic review. *Clin Gastroenterol Hepatol* 2012, 10: 1342–1359.e2

6. Calle EE, Rodriguez C, Walker-Thurmond K, Thun MJ: Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003, 348:1625–1638
7. Amann T, Bataille F, Spruss T, Muhlbauer M, Gabele E, Scholmerich J, Kiefer P, Bosserhoff AK, Hellerbrand C: Activated hepatic stellate cells promote tumorigenicity of hepatocellular carcinoma. *Cancer Sci* 2009, 100:646–653
8. Geng ZM, Li QH, Li WZ, Zheng JB, Shah V: Activated human hepatic stellate cells promote growth of human hepatocellular carcinoma in a subcutaneous xenograft nude mouse model. *Cell Biochem Biophys* 2014, 70:337–347
9. Zhao W, Zhang L, Xu Y, Zhang Z, Ren G, Tang K, Kuang P, Zhao B, Yin Z, Wang X: Hepatic stellate cells promote tumor progression by enhancement of immunosuppressive cells in an orthotopic liver tumor mouse model. *Lab Invest* 2014, 94:182–191
10. Zhao W, Zhang L, Yin Z, Su W, Ren G, Zhou C, You J, Fan J, Wang X: Activated hepatic stellate cells promote hepatocellular carcinoma development in immunocompetent mice. *Int J Cancer* 2011, 129:2651–2661
11. Friedman SL: Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiol Rev* 2008, 88:125–172
12. Chiang DJ, Pritchard MT, Nagy LE: Obesity, diabetes mellitus, and liver fibrosis. *Am J Physiol Gastrointest Liver Physiol* 2011, 300:G697–G702
13. El-Karef A, Yoshida T, Gabazza EC, Nishioka T, Inada H, Sakakura T, Imanaka-Yoshida K: Deficiency of tenascin-C attenuates liver fibrosis in immune-mediated chronic hepatitis in mice. *J Pathol* 2007, 211:86–94
14. Midwood KS, Hussenet T, Langlois B, Orend G: Advances in tenascin-C biology. *Cell Mol Life Sci* 2011, 68:3175–3199
15. Ramadori G, Schwogler S, Veit T, Rieder H, Chiquet-Ehrismann R, Mackie EJ, Meyer zum Buschenfelde KH: Tenascin gene expression in rat liver and in rat liver cells. In vivo and in vitro studies. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1991, 60:145–153
16. Yamada S, Ichida T, Matsuda Y, Miyazaki Y, Hatano T, Hata K, Asakura H, Hirota N, Geerts A, Wisse E: Tenascin expression in human chronic liver disease and in hepatocellular carcinoma. *Liver* 1992, 12:10–16
17. Van Eyken P, Geerts A, De Bleser P, Lazou JM, Vrijssen R, Sciort R, Wisse E, Desmet VJ: Localization and cellular source of the extracellular matrix protein tenascin in normal and fibrotic rat liver. *Hepatology* 1992, 15:909–916
18. Carey WA, Taylor GD, Dean WB, Bristow JD: Tenascin-C deficiency attenuates TGF-beta-mediated fibrosis following murine lung injury. *Am J Physiol Lung Cell Mol Physiol* 2010, 299: L785–L793
19. Catalan V, Gomez-Ambrosi J, Rodriguez A, Ramirez B, Rotellar F, Valenti V, Silva C, Gil MJ, Salvador J, Fruhbeck G: Increased tenascin C and Toll-like receptor 4 levels in visceral adipose tissue as a link between inflammation and extracellular matrix remodeling in obesity. *J Clin Endocrinol Metab* 2012, 97:E1880–E1889
20. Lin ZY, Chuang WL: Genes responsible for the characteristics of primary cultured invasive phenotype hepatocellular carcinoma cells. *Biomed Pharmacother* 2012, 66:454–458
21. Takahashi Y, Sawada G, Kurashige J, Matsumura T, Uchi R, Ueo H, Ishibashi M, Takano Y, Akiyoshi S, Iwaya T, Eguchi H, Sudo T, Sugimachi K, Yamamoto H, Doki Y, Mori M, Mimori K: Tumor-derived tenascin-C promotes the epithelial-mesenchymal transition in colorectal cancer cells. *Anticancer Res* 2013, 33:1927–1934
22. van Zijl F, Zulehner G, Petz M, Schneller D, Kornauth C, Hau M, Machat G, Grubinger M, Huber H, Mikulits W: Epithelial-mesenchymal transition in hepatocellular carcinoma. *Future Oncol* 2009, 5: 1169–1179
23. Midwood K, Sacre S, Piccinini AM, Inglis J, Trebaul A, Chan E, Drexler S, Sofat N, Kashiwagi M, Orend G, Brennan F, Foxwell B: Tenascin-C is an endogenous activator of Toll-like receptor 4 that is essential for maintaining inflammation in arthritic joint disease. *Nat Med* 2009, 15:774–780
24. Yu LX, Yan HX, Liu Q, Yang W, Wu HP, Dong W, Tang L, Lin Y, He YQ, Zou SS, Wang C, Zhang HL, Cao GW, Wu MC, Wang HY: Endotoxin accumulation prevents carcinogen-induced apoptosis and promotes liver tumorigenesis in rodents. *Hepatology* 2010, 52: 1322–1333
25. Mai CW, Kang YB, Pichika MR: Should a Toll-like receptor 4 (TLR-4) agonist or antagonist be designed to treat cancer? TLR-4: its expression and effects in the ten most common cancers. *Oncotargets* 2013, 6:1573–1587
26. Gabele E, Dostert K, Dorn C, Patsenker E, Stickel F, Hellerbrand C: A new model of interactive effects of alcohol and high-fat diet on hepatic fibrosis. *Alcohol Clin Exp Res* 2011, 35:1361–1367
27. Soares JB, Pimentel-Nunes P, Afonso L, Rolanda C, Lopes P, Roncon-Albuquerque R Jr, Goncalves N, Boal-Carvalho I, Pardal F, Lopes S, Macedo G, Lara-Santos L, Henrique R, Moreira-Dias L, Goncalves R, Dinis-Ribeiro M, Leite-Moreira AF: Increased hepatic expression of TLR2 and TLR4 in the hepatic inflammation-fibrosis-carcinoma sequence. *Innate Immun* 2012, 18:700–708
28. Cong M, Iwaisako K, Jiang C, Kisseleva T: Cell signals influencing hepatic fibrosis. *Int J Hepatol* 2012, 2012:158547
29. Dapito DH, Mencin A, Gwak GY, Pradere JP, Jang MK, Mederacke I, Caviglia JM, Khiaabani H, Adeyemi A, Battaller R, Lefkowitz JH, Bower M, Friedman R, Sartor RB, Rabadan R, Schwabe RF: Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. *Cancer Cell* 2012, 21:504–516
30. Samson CM, Schrum LW, Bird MA, Lange PA, Brenner DA, Rippe RA, Behrns KE: Transforming growth factor-beta1 induces hepatocyte apoptosis by a c-Jun independent mechanism. *Surgery* 2002, 132:441–449
31. Li T, Eheim AL, Klein S, Uschner FE, Smith AC, Brandon-Warner E, Ghosh S, Bonkovsky HL, Trebicka J, Schrum LW: Novel role of nuclear receptor rev-erbalpha in hepatic stellate cell activation: potential therapeutic target for liver injury. *Hepatology* 2014, 59: 2383–2396
32. Thompson KJ, Swan RZ, Walling TL, Iannitti DA, McKillop IH, Sindram D: Obesity, but not ethanol, promotes tumor incidence and progression in a mouse model of hepatocellular carcinoma in vivo. *Surg Endosc* 2013, 27:2782–2791
33. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ: Nonalcoholic Steatohepatitis Clinical Research Network: Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005, 41:1313–1321
34. Wald C, Russo MW, Heimbach JK, Hussain HK, Pomfret EA, Bruix J: New OPTN/UNOS policy for liver transplant allocation: standardization of liver imaging, diagnosis, classification, and reporting of hepatocellular carcinoma. *Radiology* 2013, 266:376–382
35. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanyal AJ; American Gastroenterological Association, American Association for the Study of Liver Diseases, American College of Gastroenterology: The diagnosis and management of non-alcoholic fatty liver disease: practice guideline by the American Gastroenterological Association, American Association for the Study of Liver Diseases, and American College of Gastroenterology. *Gastroenterology* 2012, 142:1592–1609
36. Dunn KW, Kamocka MM, McDonald JH: A practical guide to evaluating colocalization in biological microscopy. *Am J Physiol Cell Physiol* 2011, 300:C723–C742
37. Lysakova-Devine T, Keogh B, Harrington B, Nagpal K, Halle A, Golenbock DT, Monie T, Bowie AG: Viral inhibitory peptide of TLR4, a peptide derived from vaccinia protein A46, specifically inhibits TLR4 by directly targeting MyD88 adaptor-like and TRIF-related adaptor molecule. *J Immunol* 2010, 185:4261–4271
38. Dallot E, Mehats C, Oger S, Leroy MJ, Breuille-Fouche M: A role for PKCzeta in the LPS-induced translocation NF-kappaB p65 subunit in cultured myometrial cells. *Biochimie* 2005, 87:513–521

39. Nong Y, Wu D, Lin Y, Zhang Y, Bai L, Tang H: Tenascin-C expression is associated with poor prognosis in hepatocellular carcinoma (HCC) patients and the inflammatory cytokine TNF- α -induced TNC expression promotes migration in HCC cells. *Am J Cancer Res* 2015, 5:782–791
40. Mekonnen GA, Ijzer J, Nederbragt H: Tenascin-C in chronic canine hepatitis: immunohistochemical localization and correlation with necro-inflammatory activity, fibrotic stage, and expression of alpha-smooth muscle actin, cytokeratin 7, and CD3+ cells. *Vet Pathol* 2007, 44:803–813
41. Lu P, Weaver VM, Werb Z: The extracellular matrix: a dynamic niche in cancer progression. *J Cell Biol* 2012, 196:395–406
42. Sorokin L: The impact of the extracellular matrix on inflammation. *Nat Rev Immunol* 2010, 10:712–723
43. Budhu A, Wang XW: The role of cytokines in hepatocellular carcinoma. *J Leukoc Biol* 2006, 80:1197–1213
44. Park EJ, Lee JH, Yu GY, He G, Ali SR, Holzer RG, Osterreicher CH, Takahashi H, Karin M: Dietary and genetic obesity promote liver inflammation and tumorigenesis by enhancing IL-6 and TNF expression. *Cell* 2010, 140:197–208
45. Piccinini AM, Midwood KS: Endogenous control of immunity against infection: tenascin-C regulates TLR4-mediated inflammation via microRNA-155. *Cell Rep* 2012, 2:914–926
46. Jing YY, Han ZP, Sun K, Zhang SS, Hou J, Liu Y, Li R, Gao L, Zhao X, Zhao QD, Wu MC, Wei LX: Toll-like receptor 4 signaling promotes epithelial-mesenchymal transition in human hepatocellular carcinoma induced by lipopolysaccharide. *BMC Med* 2012, 10:98
47. Bonnomet A, Brysse A, Tachsidis A, Waltham M, Thompson EW, Polette M, Gilles C: Epithelial-to-mesenchymal transitions and circulating tumor cells. *J Mammary Gland Biol Neoplasia* 2010, 15:261–273
48. Dooley S, Hamzavi J, Ciucian L, Godoy P, Ilkavets I, Ehnert S, Ueberham E, Gebhardt R, Kanzler S, Geier A, Breitkopf K, Weng H, Mertens PR: Hepatocyte-specific Smad7 expression attenuates TGF- β -mediated fibrogenesis and protects against liver damage. *Gastroenterology* 2008, 135:642–659
49. Gotzmann J, Fischer AN, Zojer M, Mikula M, Proell V, Huber H, Jechlinger M, Waerner T, Weith A, Beug H, Mikulits W: A crucial function of PDGF in TGF- β -mediated cancer progression of hepatocytes. *Oncogene* 2006, 25:3170–3185
50. Reichl P, Haider C, Grubinger M, Mikulits W: TGF- β in epithelial to mesenchymal transition and metastasis of liver carcinoma. *Curr Pharm Des* 2012, 18:4135–4147
51. Sun B, Zhang X, Cheng X, Zhang Y, Chen L, Shi L, Liu Z, Qian H, Wu M, Yin Z: Intratumoral hepatic stellate cells as a poor prognostic marker and a new treatment target for hepatocellular carcinoma. *PLoS One* 2013, 8:e80212
52. Yoshimoto S, Loo TM, Atarashi K, Kanda H, Sato S, Oyadomari S, Iwakura Y, Oshima K, Morita H, Hattori M, Honda K, Ishikawa Y, Hara E, Ohtani N: Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* 2013, 499:97–101
53. Capece D, Fischietti M, Verzella D, Gaggiano A, Ciccirelli G, Tessitore A, Zazzeroni F, Alesse E: The inflammatory microenvironment in hepatocellular carcinoma: a pivotal role for tumor-associated macrophages. *Biomed Res Int* 2013, 2013:187204
54. Arora R, Yates C, Gary BD, McClellan S, Tan M, Xi Y, Reed E, Piazza GA, Owen LB, Dean-Colomb W: Panepoxydione targets NF- κ B and FOXM1 to inhibit proliferation, induce apoptosis and reverse epithelial to mesenchymal transition in breast cancer. *PLoS One* 2014, 9:e98370
55. Liu WT, Jing YY, Yu GF, Han ZP, Yu DD, Fan QM, Ye F, Li R, Gao L, Zhao QD, Wu MC, Wei LX: Toll like receptor 4 facilitates invasion and migration as a cancer stem cell marker in hepatocellular carcinoma. *Cancer Lett* 2015, 358:136–143
56. Mackie EJ, Tucker RP: The tenascin-C knockout revisited. *J Cell Sci* 1999, 112(Pt 22):3847–3853
57. Pisani P, Parkin DM, Bray F, Ferlay J: Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer* 1999, 83:18–29
58. Bruix J, Gores GJ, Mazzaferro V: Hepatocellular carcinoma: clinical frontiers and perspectives. *Gut* 2014, 63:844–855
59. Charlton MR, Burns JM, Pedersen RA, Watt KD, Heimbach JK, Dierkhising RA: Frequency and outcomes of liver transplantation for nonalcoholic steatohepatitis in the United States. *Gastroenterology* 2011, 141:1249–1253
60. Bruix J, Sherman M: American Association for the Study of Liver Diseases: Management of hepatocellular carcinoma: an update. *Hepatology* 2011, 53:1020–1022
61. Baffy G, Brunt EM, Caldwell SH: Hepatocellular carcinoma in non-alcoholic fatty liver disease: an emerging menace. *J Hepatol* 2012, 56:1384–1391
62. Ascha MS, Hanounieh IA, Lopez R, Tamimi TA, Feldstein AF, Zein NN: The incidence and risk factors of hepatocellular carcinoma in patients with nonalcoholic steatohepatitis. *Hepatology* 2010, 51:1972–1978
63. Schnabl B, Purbeck CA, Choi YH, Hagedorn CH, Brenner D: Replicative senescence of activated human hepatic stellate cells is accompanied by a pronounced inflammatory but less fibrogenic phenotype. *Hepatology* 2003, 37:653–664
64. Gupta S, Bent S, Kohlwes J: Test characteristics of alpha-fetoprotein for detecting hepatocellular carcinoma in patients with hepatitis C. A systematic review and critical analysis. *Ann Intern Med* 2003, 139:46–50
65. Lok AS, Lai CL: alpha-Fetoprotein monitoring in Chinese patients with chronic hepatitis B virus infection: role in the early detection of hepatocellular carcinoma. *Hepatology* 1989, 9:110–115
66. Yuen MF, Lai CL: Serological markers of liver cancer. *Best Pract Res Clin Gastroenterol* 2005, 19:91–99