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TLR4 Signaling via NANOG Cooperates With STAT3 to Activate *Twist1* and Promote Formation of Tumor-Initiating Stem-Like Cells in Livers of Mice

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BACKGROUND & AIMS: Obesity and alcohol consumption contribute to steatohepatitis, which increases the risk for hepatitis C virus (HCV)-associated hepatocellular carcinomas (HCCs). Mouse hepatocytes that express HCV-NS5A in liver up-regulate the expression of Toll-like receptor 4 (TLR4), and develop liver tumors containing tumor-initiating stem-like cells (TICs) that express NANOG. We investigated whether the TLR4 signals to NANOG to promote the development of TICs and tumorigenesis in mice placed on a Western diet high in cholesterol and saturated fat (HCFD). **METHODS:** We expressed HCV-NS5A from a transgene (NS5A Tg) in *Tlr4*^{-/-} (C57Bl6/10ScN), and wild-type control mice. Mice were fed a HCFD for 12 months. TICs were identified and isolated based on being CD133+, CD49f+, and CD45-. We obtained 142 paraffin-embedded sections of different stage HCCs and adjacent nontumor areas from the same patients, and performed gene expression, immunofluorescence, and immunohistochemical analyses. **RESULTS:** A higher proportion of NS5A Tg mice developed liver tumors (39%) than mice that did not express HCV NS5A after the HCFD (6%); only 9% of *Tlr4*^{-/-} NS5A Tg mice fed HCFD developed liver tumors. Livers from NS5A Tg mice fed the HCFD had increased levels of TLR4, NANOG, phosphorylated signal transducer and activator of transcription (pSTAT3), and TWIST1 proteins, and increases in *Tlr4*, *Nanog*, *Stat3*, and *Twist1* messenger RNAs. In TICs from NS5A Tg mice, NANOG and pSTAT3 directly interact to activate expression of *Twist1*. Levels of TLR4, NANOG, pSTAT3, and TWIST were increased in HCC compared with nontumor tissues from patients. **CONCLUSIONS:** HCFD and HCV-NS5A together stimulated TLR4-NANOG and the OB-R-pSTAT3 signaling pathways, resulting in liver tumorigenesis through an exaggerated mesenchymal phenotype with prominent *Twist1*-expressing TICs.

Keywords: HCC; HCV; Obesity; NASH.

Obesity and infection by hepatitis C virus (HCV) are connected pathophysiologically to hepatocarcinogenesis.^{1–5} The risk for hepatocellular carcinoma (HCC) increases from 8.6-fold to 47.8-fold as a result of concomitant obesity in HCV-infected patients.⁴ Obesity induced by a high-cholesterol high-fat diet (HCFD) is

associated with increased levels of serum bacterial endotoxin derived from the hepatic portal and/or the systemic gut; these increased levels stimulate the expression of proinflammatory cytokines in the liver and adipose tissues, subsequently leading to liver injury.^{5–7} Such HCFD-mediated changes superimposed on HCV infection lead to an increased incidence of overt diabetes,⁸ potentially establishing a self-reinforcing oncogenic cycle.

HCC, the fifth most common cancer in the world and the third leading cause of cancer mortality, has a low 5-year survival rate because of a lack of effective therapeutic options.^{9,10} An understanding of the molecular mechanisms of hepatocarcinogenesis will be required for the development of improved therapeutic models for this disease. The HCV-NS5A protein, a major target of therapeutic efforts, suppresses activity of interferon-induced, double-stranded, RNA-activated protein kinase PKR,¹¹ accounting for the resistance of most HCV strains to interferon treatment. Furthermore, NS5A transactivates many gene promoters.¹² We recently showed that HCV infection and the associated expression of the NS5A protein lead to excessive tumor necrosis factor α production, fulminant hepatitis, and a 6-fold increase in mortality in response to gram-negative bacterial-derived lipopolysaccharide (LPS) ligand.¹³ These effects are mediated through increased expression of the innate immune receptor Toll-like receptor 4 (TLR4), a transmembrane receptor that activates nuclear factor- κ B and induces a proinflammatory and tumorigenic gene

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Abbreviations used in this paper: AFP, _____; ChIP, chromatin immunoprecipitation; EMT, epithelial mesenchymal transition; HCC, hepatocellular carcinoma; HCFD, high-cholesterol fat diet; HCV, hepatitis C virus; LFD, low-fat diet; LPS, lipopolysaccharide; mRNA, messenger RNA; OB-R, _____; PCR, polymerase chain reaction; pSTAT3, phosphorylated signal transducer and activator of transcription 3; shRNA, short hairpin RNA; Tg, transgene; TICs, tumor-initiating stem-like cells; TLR4, Toll-like receptor 4; TSS, transcription initiation/start site; USC, _____; WT, wild-type.

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expression program in HCV-infected livers. Likewise, increased TLR4 signaling in NS5A-positive hepatocytes after chronic and excessive alcohol consumption promotes the expansion of highly malignant, CD133⁺/CD49f⁺/Nanog⁺

liver tumor-initiating stem-like cells (TICs) in alcohol-associated hepatocarcinogenesis.¹⁴ Nevertheless, the significance of TLR4 in hepatocarcinogenesis associated with obesity and HCV infection and the role of proteins involved

in the metastatic properties of TICs has not been addressed directly.

Long-term consumption of a HCFD increases levels of gut-derived bacterial endotoxin in the plasma.¹⁵ We previously showed increased expression of TLR4 (a receptor for endotoxin) in hepatocytes of NS5A-transgene (Tg) mice.¹⁴ Based on these findings, we postulated that synergism between HCV and obesity in liver disease progression involved TLR4-dependent signaling. We also reasoned that the TLR4-NANOG pathway might play a major role in mediating the synergism between obesity and HCV in the pathogenesis of HCC via generation of CD133⁺/Nanog⁺ TICs. Our RNA microarray analysis on TICs derived from HCFD fed mice showed a significant increase in *Twist1*. We previously showed that leptin and its receptor (OB-R) augmented phosphorylated signal transducer and activator of transcription 3 (pSTAT3) in TICs,¹⁶ these results led us to hypothesize that adipose tissue-derived leptin-pSTAT3 and TLR4-NANOG signals are needed for activation of *Twist1* in TICs. Here, we provide evidence that TLR4 drives oncogenesis in part through the transcriptional induction of *Twist1*, a master regulator of epithelial mesenchymal transition (EMT),^{17–19} to generate cells with stem-like properties and a predisposition to the EMT. This signaling module therefore represents a new candidate target in the treatment of obesity- and HCV-associated HCC.

Materials and Methods

Additional details are described in the [Supplementary Materials and Methods](#) section and in [Supplementary Tables 3–6](#).

Mouse Studies

All experiments on mice were approved by the USC Institutional Animal Care and Use Committee. Transgenic mice expressing the HCV-NS5A gene under control of the *ApoE* promoter^{20,21} were obtained from Professor Ratna Ray (Saint Louis University, St. Louis, MO). TLR4-deficient mice (C57BL/6/10ScN), control mice (C57BL/6/10ScN), and C57BL/6 mice were purchased from Jackson Laboratories. To generate wild-type

(WT), NS5A, *Tlr4*^{-/-}, and *Tlr4*^{-/-}-NS5A mice on a more congenic genetic background, NS5A Tg (FVB strain) and *Tlr4*^{-/-} mice were cross-bred on a C57BL/6 background (Jackson Laboratories) for more than 8 generations at USC. Littermates on mixed C57BL/6-NS5A transgenic and *Tlr4*^{-/-} mice (Jackson Laboratories) were intercrossed for at least 8 generations to produce WT, NS5A, *Tlr4*^{-/-}, and *Tlr4*^{-/-}-NS5A mice on a more congenic genetic background. Both sexes of mice were used for experiments. The HCFD diet was modified from TD.03350 (Harkan Teklad, Inc) as previously described.^{22,23} Where indicated, mice were fed ad libitum with an ethanol-containing Lieber-DeCarli diet containing 3.5% ethanol or isocaloric dextrin (Bioserv, Frenchtown, NJ) HCFD beginning at 8 weeks of age for a period of 12 months. Other mice were fed modified high-fat AIN-93G purified ethanol liquid diet with anhydrous milk fat, lard, corn oil, and 1% cholesterol (DYET 710362; Dyets, Inc) or Lieber-DeCarli Regular Control Diet (DYET 710027).

Human Subjects

Paraffin-embedded tissue sections were obtained in accordance with the approved Institutional Review Board. There were 3 institutions (University of Southern California, University of California at Los Angeles, and University of Minnesota) that granted Institutional Review Board approval for the supplied specimens. Specimens were obtained from the Liver Tissue Cell Distribution System at the University of Minnesota according to the following criteria: surgically excised HCC tissues from 8 patients $\pm$ HCV infection, $\pm$ history of alcoholism, $\pm$ obesity/diabetes/body mass index greater than 30. Eighteen specimens also were obtained from the Hepatobiliary and Liver Transplantation Service at the USC Keck School of Medicine. A total of 116 cases of HCC were identified from 2002 to 2011 by searching the University of California at Los Angeles Department of Pathology database using the following search terms: liver, hepatocellular carcinoma, resection, and transplant. All patient identifiers were removed to protect confidentiality. Samples were obtained from both sexes between the ages of 42 and 80. Histologically, all samples showed varying degrees of microvesicular and macrovesicular steatosis and inflammation in addition to different stages of HCC. These paired 116 specimens were the livers that had been dissected with the tumor

Figure 1. NS5A Tg mice fed HCFD with or without LPS frequently developed tumors. (A) Summary of WT and *Tlr4*^{-/-} HCV-NS5A Tg mice fed control diet or HCFD with or without LPS from 8 weeks of age for 12 months. N, number of experimental mice. WT-HCFD; **P* < .05 ***P* < .01 ****P* < .005, green scripts and symbols, statistical analysis in comparison with LFD; purple scripts and symbols, statistical analysis in comparison with HCFD. (B) Plasma endotoxin and leptin levels in mice fed LFD or HCFD. (C) Gross images of nonpathologic liver from control diet (1, 2) and liver tumor with multiple nodules from HCFD (3–6) and HCFD + LPS (7). *Lower panel:* Histology of respective groups. The HCFD tumor shown (arrow) is a dysplastic nodule. (D) Frequencies of liver dysplastic nodules and HCCs in LFD- or HCFD-WT or NS5A Tg mice fed LFD or HCFD for 12 months. Representative H&E staining of tumor sections from WT or NS5A Tg mice fed HCFD or LPS + HCFD. The histopathology of the tumors (arrows) shown are dysplastic nodules or HCCs based on their hypercellularity. Nodular lesions differ from the surrounding liver parenchyma with cytologic or structural atypia. (E) Normal liver/liver tumor lysates from WT and NS5A Tg mice fed control chow or HCFD were analyzed for LPS-induced TLR4 signaling. *Upper panel:* Tumor necrosis factor-receptor-associated factor 6 (TRAF6) interaction with transforming growth factor α -activated kinase 1 (TAK1), was enhanced in NS5A Tg mice fed HCFD. The interaction between TAK1 and TRAF6 was examined by immunoblots after immunoprecipitation (IP) with TAK1 antibody. As a positive control (shown in last 3 lanes), mice were challenged with LPS; LPS was injected (2 mg/kg) 30 minutes, 1 hour, or 2 hours, respectively, before liver tissues were collected for analysis. The relative densitometry units and details are available in [Supplementary Figure 1A](#). *Bottom panel:* LPS-induced phosphorylation of IKK- β in the liver was increased in NS5A Tg mice fed HCFD. Positive controls (last 3 lanes), as explained previously. (F) Data summary of body weight changes over a 12-month feeding period and statistics are available in panel A. Scale bar: 50 μ m.

and adjacent noncancerous areas from the same patients. Clinicopathologic information is described in [Supplementary Figure 10](#) and summarized in [Supplementary Table 1](#).

Results

HCFD Promotes Liver Oncogenesis in NS5A Tg Mice in a TLR4-Dependent Manner

We used an in vivo loss-of-function strategy to test the role of TLR4 in this interplay between NS5A and obesity. Hepatocyte-specific NS5A Tg^{20,21} and WT mice with or without TLR4 deficiency (*Tlr4*^{-/-})¹⁴ were maintained on a low-fat diet (LFD) or an HCFD with or without supplemental LPS for 12 months ([Figure 1A](#)). HCFD consumption resulted in an obese population (WT and NS5A Tg mice); however, this outcome remarkably was prevented by TLR4 deficiency in either genotype ([Figure 1A and F](#)). In HCFD mice, we observed a liver tumor incidence of 39% in NS5A Tg mice compared with 6% in WT mice. By contrast, we observed a significant decrease of tumor incidence to 9% in *Tlr4*^{-/-} NS5A Tg mice ([Figure 1A and C](#)). Conversely, LPS supplementation in the HCFD (100 mg/kg) further increased the incidence to 47% in NS5A Tg mice ([Figure 1A](#)). This observation indicated a significant contribution of the LPS-TLR4 pathway in hepatocarcinogenesis. In addition, the presence of NS5A in HCFD-fed mice significantly increased the liver to body ratio, which coincided with severe liver hepatomegaly and inflammation ([Figure 1A and C](#) and [Supplementary Table 2](#)).

As predicted, HCFD, and HCFD + LPS feeding markedly increased plasma endotoxin and leptin levels in all tested cohorts ([Figure 1B](#)). Several liver malignancies were observed in NS5A Tg mice, but not in the control animals. Additional observed pathologies included nonalcoholic steatohepatitis-like bloating ([Figure 1C](#)), dysplastic nodules (nonmalignant), and HCCs ([Figure 1D](#)). Activation of TLR4 signaling was assessed by co-immunoprecipitation of transforming growth factor α -activated kinase 1-tumor necrosis factor receptor-associated factor 6, and immunoblotting for p-IKK- β .¹⁴ Concomitant TLR4 activation through tumor necrosis factor receptor-associated factor 6-transforming growth factor α -activated kinase 1-p-IKK- β was evident in HCFD-fed NS5A Tg ([Figure 1E](#), and [Supplementary Figure 1](#)), but not in LFD-fed cohorts. As a positive control for TLR4 activation parameters, a single intraperitoneal dose of LPS (2 mg/kg) was given to chow-fed WT mice before sample collection (last 3 lanes of [Figure 1E](#), top). Collectively, these results showed that HCV-NS5A and HCFD acted synergistically to induce liver tumors in a manner dependent on TLR4.

Twist1 Identified as one of the Most Conspicuously Up-Regulated Genes in TLR4-Dependent NS5A- and HCFD-Driven Hepatocarcinogenesis

To understand the molecular basis of enhanced liver oncogenesis in HCFD-NS5A mice, we performed RNA microarray analysis. This identified 131 differentially up-regulated and 43 down-regulated transcripts in HCFD-fed

NS5A Tg mice ([Figure 2A](#) and [Supplementary Figure 2](#)). Some of the more highly up-regulated transcripts of different functional categories are listed in [Figure 2A](#). These include the stemness marker *Nanog*, oncogene *Igf2bp3*, and EMT and tumor metastasis regulator *Twist1*.^{19,24,25} *Nanog* and *Igf2bp3* have been found to be critical in self-renewal and tumorigenic activity of TICs isolated from liver tumors of alcohol-fed NS5A mice.¹⁴ To confirm that TLR4 activation in the liver is from TICs, we performed immunofluorescence staining on control, HCFD, and HCFD + LPS livers ([Supplementary Figure 3](#)). This analysis confirmed that the source of TLR4 in the HCFD and HCFD + LFD livers is from TICs (TLR4 co-staining with NANOG) and not from the resident macrophages (Kupffer cells). For this study, we further examined the molecular mechanisms through which *Twist1* promoted EMT and tumor metastasis in HCFD-fed NS5A-derived TICs. To substantiate the microarray data we performed quantitative real-time polymerase chain reaction (PCR) analysis to measure *Twist1* gene expression. As expected, *Twist1* messenger RNA (mRNA) was induced significantly in HCFD-fed NS5A Tg mice compared with HCFD-fed WT mice or LFD-fed NS5A Tg mice ([Figure 2B](#)). These analyses also showed that *Twist1* transcription was reduced in the HCFD-fed *Tlr4*^{-/-} NS5A Tg cohort ([Figure 2B](#)), suggesting that the presence of TLR4 was permissive or required for *Twist1* induction.

TLR4 Signaling Transactivates Twist1

To further establish whether TLR4 regulates *TWIST1*, human HCC cell line Huh7 cells were transfected with the NS5A gene expression vector. We then transduced lentivirus expressing *TLR4* or scrambled short hairpin RNA (shRNA) in these NS5A/vector-expressing cells and further stimulated these cells with or without LPS. As shown in [Figure 2C](#), LPS treatment up-regulated *TWIST1* mRNA levels in NS5A-transfected Huh7 cells transduced with scrambled shRNA, but not in any other groups with shRNA knockdown of TLR4. *TWIST1* induction was abrogated significantly by TLR4 blockade. When a dominant-negative variant of TLR4 lacking the cytoplasmic domain (mutant TLR4; TLR4 Δ Cyt) was transduced into these cells, a similar and more conspicuous reduction of *TWIST1* expression was observed. We then tested whether TLR4 signaling can transcriptionally activate *TWIST1*. Huh7 cells were transfected with *TWIST1* promoter (nt -700/-1) luciferase plasmid constructs²⁶ and assayed for activity upon LPS treatment. A potent *TWIST1* promoter activity was observed that was responsive to the LPS-TLR4 signaling axis ([Figure 2D](#)), indicating that TLR4 does indeed transactivate *TWIST1*.

Twist1 Blockade Reduces TIC Self-Renewal, Migration, and Tumorigenesis

To show that TLR4 is responsible for *Twist1* induction in TICs, we isolated CD133+/CD49f+/CD45- cells for examination of gene expression to show that these cells indeed express higher levels of stemness genes and *Twist1* ([Figure 3A](#)). The functionality of *Twist1* in TICs was analyzed by silencing expression using lentivirus expressing *Twist1* shRNA. *Twist1* silencing did not affect TLR4 or

Figure 2. TLR4-mediated TWIST1 induction. (A) Chief summary of RNA microarray analysis. *Twist1*, a key regulator of EMT signaling, was significantly higher in NS5A + HCDF compared with WT + HCDF. (B) Quantitative analysis of *Twist1* from liver/liver tumor tissues of all cohorts, as listed in Figure 1A. Heightened *Twist1* expression (NS5A Tg mice fed HCDF) was abrogated by TLR4 deficiency. Data normalized to glyceraldehyde-3-phosphate dehydrogenase expression are listed as the fold-change ($P < .05$). (C) LPS induced TWIST1 in Huh7 cells transduced with an NS5A expression vector ($P < .05$ compared with cells transduced with an empty vector). This was suppressed by lentiviral expression of shRNA for *TLR4* and also in cells transduced with the dominant-negative TLR4 vector (*TLR4*ΔCyt). (D) LPS induced TWIST1 promoter activity. Huh7 cells transfected with TWIST1 promoter-luciferase construct were stimulated with LPS (10 μ g/mL) in culture. Other experimental procedures in this figure are the same as described earlier. TLR4 knockdown or mutation abrogated TWIST1 promoter activity, but adding TLR4 rescued it. Relative light unit (RLU) values were normalized by the *Renilla* luciferase activity driven by the SV40 promoter, which were used as a transfection control ($P < .05$).

NANOG (downstream of the LPS-TLR4 axis¹⁴) protein expression (Figure 3B), but up-regulated epithelial cell markers *albumin* and *E-cadherin* expression while down-regulating expression of a mesenchymal cell marker, *N-cadherin* (Figure 3C); thus indicating that *Twist1* silencing changes the mesenchymal phenotype to the epithelial phenotype. These data indicated that *Twist1* acts downstream of the TLR4 signaling cascade and contributes significantly to the maintenance of mesenchymal phenotype based on its effect on *albumin*, *E-cadherin*, and *N-cadherin*. To further investigate this phenomenon, we assessed the phenotypic changes in TICs after *Twist1* blockade. TIC morphology was altered from a spindle (mesenchymal) shape to a tadpole-like (epithelial) shape (Figure 3D, inset); there also was increased cell size (Supplementary Figure 4A). Moreover, *Twist1* blockade significantly reduced cell proliferation (Supplementary Figure 4B), self-renewal ability as assayed by colony formation in soft agar (Figure 3D), spheroid formation (Supplementary Figure 4C), and cell migration by scratch assay (Figure 3E). We then tested implanted cells for tumorigenic potential in NOG mice. Subcutaneously transplanted *Twist1* or scrambled shRNA TICs were monitored for tumor size over a period of 35 days. Gross and optical image analysis of

live tumor-bearing mice showed reduced tumor size in *Twist1* knockdown groups (Figure 3F, panels 3 and 4). As expected, tumor volume and weight were reduced significantly (Figure 3F, panels 1 and 2). Histologic examination of xenografted TICs showed that the resulting tumor showed HCC morphology (Figure 3F, panel 5). These results showed that *Twist1*, regulated through the LPS-TLR4 axis, plays a significant role in maintaining the mesenchymal and tumorigenic properties of TICs.

NANOG and pSTAT3 Regulate Twist1

We next investigated the molecular mechanisms responsible for TLR4-dependent activation of *Twist1*. We performed *Twist1* promoter-reporter assays, using promoter constructs²⁶ containing either WT (nt -700 to -1) or mutated regions upstream of the transcription initiation/start site (TSS). The activation of these reporter constructs was analyzed in cells transduced with either scrambled or *Tlr4* shRNA. From this analysis we established that the region between -209 to -51 is essential for the basal and *Tlr4*-dependent induction of *Twist1* in TICs (Figure 4A and Supplementary Figure 5, Huh7 cells). In particular, a deletion between nts -102 and -74 markedly reduced *Twist1* promoter activity, indicating that this region contained

Figure 3. *Twist1* is required for mesenchymal morphology of TICs, down-regulation of *Twist1* reduces TIC cancer-initiating property. (A) CD133+/CD49f+/CD45- cells were isolated from tumors of 2 different HCDF-fed NS5A Tg mice and examined for stemness gene expression by quantitative real-time PCR. (B) To silence *Twist1* expression, lentivirus shRNA *Twist1* was transduced in TICs. Immunoblot analysis confirmed decreased TWIST1 expression in TICs and showed unchanged expression of NANOG and TLR4 (n = 3). (C) mRNA levels were validated by quantitative real-time PCR. Expression profile of EMT-regulated genes, including mesenchymal markers (*Twist1* and *N-cad*) and epithelial markers (*albumin* and *E-cadherin*) were analyzed (n = 3; *P < .05). (D) Light-field microscopy showed an altered morphology of TICs after *Twist1* knockdown. Scrambled TIC (1) parenchymal cell phenotype drastically changed to a tadpole shape (2) after *Twist1* knockdown (40×; n = 10; insets are enlarged images). In vitro oncogenicity was tested via soft agar colony formation assay. Silencing *Twist1* in TICs (4) significantly reduced colony-forming ability in contrast to control cells (3). The number of colonies formed was normalized and summarized (n = 3; *P < .05). (E) shRNA knock down of *Twist1* diminished the ability of TICs to effectively migrate in contrast to the scrambled shRNA control, as shown by in vitro cell migration assay. The images were captured at 0 hours and 24 hours after scratching the cell layer with a 100 μL pipet tip (n = 3; *P < .05). (F) Analyses at day 35 after TIC transplantation (subcutaneously injected into NOG mice). *Twist1* silencing reduced the overall tumor volume (1) and weight (2). (3) Gross image of subcutaneous tumors. (4) Noninvasive bioluminescence imaging shows the decrease in overall tumor growth (n = 4 NOG mice/cohort; *P < .05). (5) H&E staining of xenografted tumor in NOG mice shows HCC histology. Scale bar: 50 μm. cad, cadherin; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

essential cis-elements. Long-term treatment of mice with HCFD activated *Tlr4*-*Nanog* signaling and increased leptin and endotoxin levels in the plasma. Furthermore, we previously showed that leptin and its receptor (OB-R) augmented pSTAT3 in TICs.¹⁶ In addition, NANOG is known to cooperate with STAT3 for maintenance of pluripotency in mouse embryonic stem cells.²⁷ Thus, we reasoned for activation of *Twist1* in TICs, the adipose tissue-derived leptin-pSTAT3 signal and the TLR4-NANOG signal are needed. In silico analysis using Transcription Element Search System and Transfac identified consensus NANOG and STAT3 binding sites on the *Twist1* promoter region. To evaluate the functions of these transcription factors, we mutated (Figure 4B) the respective NANOG and STAT3 binding sites in the corresponding luciferase reporter construct and discovered that the STAT3-1 (STAT3 site distal to TSS) and NANOG-1 (NANOG site proximal to TSS) sites were critical for *Twist1* promoter activity. As shown in Figure 4B, mutations on these specific binding sites markedly attenuated reporter responsiveness to both LPS and leptin induction. In addition, when key upstream cellular signals (*Tlr4*, *Nanog*, and *Stat3*) were blocked, *Twist1* promoter activity was abrogated significantly (Figure 4C). This result was substantiated further after chromatin immunoprecipitation (ChIP)-quantitative PCR analysis with antibodies specific for NANOG and pSTAT3 (Figure 4D). Single antibody immunoprecipitation of either NANOG or pSTAT3 enriched the NANOG-1 and STAT3-1 binding sites in quantitative PCR, signifying that these 2 transcription factors might cooperatively transactivate *Twist1* in response to LPS and leptin. As further validation of this model, sequential ChIP analysis was performed. As shown in Figure 4E, NANOG and pSTAT3 mutually bound each other in the process of transactivating *Twist1*.

Mouse and Human HCC Have Accentuated Expression of TLR4, p-STAT3, and TWIST1

The involvement of both LPS-TLR4-NANOG and Lepin-OB-R-pSTAT3 signaling pathways for *Twist1* induction was examined by immunoblotting analysis of lysates from liver tumors isolated from HCFD-fed NS5A Tg mice and normal livers of chow-fed mice. As expected, TLR4, STAT3, pSTAT3,

and TWIST1 all were up-regulated (Supplementary Figure 6A). The mRNA levels of TLR4, STAT3, and TWIST1 also were increased in quantitative real-time PCR analysis (Supplementary Figure 6B-D). Furthermore, immunostaining showed co-localization of TWIST1 with pSTAT3 and NANOG, as well as co-localization of pSTAT3 with NANOG in tumor-bearing HCFD and HCFD + LPS NS5A Tg liver specimens (Figure 5 and Supplementary Figure 7), but less co-localization or fewer numbers of CD133+/CD49F+ or AFP+ cells in LFD-fed NS5A Tg or HCFD-fed *Tlr4*-/- NS5A Tg mice (Supplementary Figure 7). The major source of TLR4 in the liver of wild-type mice is from nonparenchymal cells, including Kupffer cells and stellate cells. The TICs derived from mouse models have significant induction of TLR4. As shown in Supplementary Figure 7, the LFD cohort with immunofluorescence staining shows TLR4-positive cells, which are presumably Kupffer cells or stellate cells. However, in HCFD and HCFD + LPS the TLR4-positive cells have NANOG co-expression, indicating that the TLR4 origin is not only from Kupffer cells or stellate cells, but also from the TICs or hepatocytes. This is corroborated further in Supplementary Figure 7 in which co-staining of TWIST1-NANOG and CD133-CD49F is present in HCFD but not in LFD. Nonparenchymal areas of mice fed both HCFD and LFD have TLR4 staining whereas co-staining of TLR4-NANOG or TLR4-AFP are present mainly in the HCFD group but not in the LFD group and in groups of *Tlr4*-/-NS5A Tg mice. In liver of NS5A Tg mice, both parenchymal (albumin+) and nonparenchymal staining of TLR4 are positive (Supplementary Figure 8), whereas the nonparenchymal area of WT mice fed LFD mainly have positive staining of TLR4 (Supplementary Figure 7), indicating that hepatocytes and TICs of NS5A Tg mice have increased levels of TLR4, which are associated with strong staining patterns of AFP and TWIST1.

We next assessed the clinical relevance of our findings by analyzing the expression of these proteins in patient-derived HCC samples. Immunofluorescence staining detected co-localization of TWIST1 with TLR4, pSTAT3, and NANOG (Figure 6A and Supplementary Figure 10). Moreover, paired immunohistochemical analyses of 142 patient samples (116 as a tissue microarray analysis) (Supplementary Figure 9 and Supplementary Table 1) were

Figure 4. NANOG and STAT3 influence *Twist1* promoter activation in NS5A TICs. (A) LPS induces *Twist1* promoter activity in TICs. *Twist1* promoter analysis with various deletion constructs showed the importance of the TSS proximal segment (nt -209/-1). Relative light unit (RLU) values were normalized by *Renilla* luciferase activity driven by a constitutively active SV40 promoter (p*Twist1* nts -1 to -700; **P* < .05; color matched; p*Twist1* nts -1 to -209; **P* < .05; n = 3). (B) NANOG and STAT3 activate the *Twist1* promoter. NANOG and STAT3 binding elements in the *Twist1* promoter region (nts -209 to -51) were mutated by in vitro mutagenesis (p*Twist1* 1-209WT; **P* < .05; color matched; n = 3). (C) Silencing *Tlr4* and *Nanog* using lentivirus expressing shRNA or *Stat3* and *Stat3D* (retrovirus expressing dominant-negative Stat3). **P* < .05; color matched; n = 3). (D) Upper panel: Schematic representation of the *Twist1* promoter region showing the locations probed for the consensus binding sequences for NANOG (yellow script), STAT3 (green lettering), and the specificity control (SC) regions analyzed by ChIP (white script). Immediately below the schematic representation are NANOG ChIP-quantitative PCR (black bar graphs) and STAT3 ChIP-quantitative PCR (blue bar graphs) analyses, which showed the enrichment of NANOG and STAT3 in TICs after LPS (10 µg/mL) and leptin (5 ng/mL) treatment. The fold-enrichment values are relative expression values normalized to the IgG controls (SC3; **P* < .05; SC2; **P* < .05; SC1; **P* < .05; biological replicates 4; n = 2). (E) Protein-protein-DNA interaction shown by sequential ChIP-quantitative PCR indicated that NANOG and STAT3 bind each other on the *Twist1* promoter region in TICs after LPS (10 µg/mL) and leptin (5 µg/mL) treatment. The fold-enrichment values are relative expression values normalized to the IgG controls (SC3, SC2, SC1; **P* < .05; color matched; **P* < .05; biological replicates 4; n = 2). qPCR, qualitative PCR.

Figure 5. Induction of NANOG, pSTAT3, and TWIST1 in HCCD and HCCD + LPS NS5A Tg cohorts. Confocal immunofluorescence microscopy showed co-localization of TWIST1 with (A) NANOG and (B) pSTAT3 (C) Co-localization of pSTAT3 with NANOG in tumors obtained from HCCD and HCCD + LPS NS5A Tg liver specimens. This immunoreactivity is completely absent in low-fat diet liver tissues (magnification, 40× oil; n = 15 samples/cohort; n = 3). Quantifications of the immunofluorescence data were performed using Metamorph software. Scale bar: 50 μm.

performed to validate the significance of TWIST1 and NANOG in human tissue sections from 3 different cohorts (Figure 6B and C and Supplementary Figure 9A). To corroborate our findings and to gain insights on the correlation of *Twist1* with grade, survival, and relapse in HCC patients, we performed an in silico analysis using the OncoPrint Gene browser. Two independent libraries from the repository were analyzed: The Cancer Genome Atlas liver (probing 97 HCC and 59 paired normal liver tissue) and Guichard liver²⁸ (probing 99 HCC and 86 normal liver).

Both showed the significant impact of *Twist1* on HCC (Figure 6D and Supplementary Figure 9B).

Twist1 Overexpression Promotes Tumor Formation

Our results indicated that *Twist1* silencing reduces TIC-derived tumorigenesis (Figure 3F) and that *Twist1* is downstream of TLR4 (Figure 4). We then investigated whether overexpression of *Twist1* beyond the basal level in TICs can enhance its role in malignant tumor development

Figure 6. Accentuated TWIST1 co-localization with TLR4, P-STAT3, and NANOG in human patient samples. (A) Confocal immunofluorescence imaging studies showed TLR4, P-STAT3, and NANOG often co-localized with TWIST1 in HCC patient liver specimens (tumor), but absent in noncancerous liver tissue (adjacent) (magnification, 40 $\times$ oil; n = 8 samples/cohort; n = 3; red boxes show cropped images). (B) Paired IHC staining performed at USC corroborated with immunofluorescence, which showed the significant increase in NANOG and TWIST1 expression in HCC tumor samples (100 $\times$ magnification; n = 18 samples, paired; n = 3). (C) Tissue microarray analysis confirmed the correlation of TWIST1 and NANOG in a large number of patient HCC tumor samples (100 $\times$ magnification; n = 116 samples, paired). Adjacent, parent noncancerous liver; tumor, human HCC. The liver is removed to transplant new liver. (D) In silico analysis using the OncoPrint Gene browser, probing for *Twist1* correlation with grade, survival, and relapse in HCC patients via Guichard libraries. Scale bar: 50 μ m.

and metastasis. In addition, we asked how *Tlr4* silencing can influence this outcome. To test this hypothesis, we transplanted TICs expressing scrambled or *Tlr4* shRNA (Supplementary Figure 11), TICs containing empty vector, or TICs constitutively expressing *Twist1* into NOG recipient mice (Supplementary Figure 11A). Overexpression of *Twist1* indeed promoted tumor growth and significantly increased

final tumor volume and weight (Figure 7A and B). Concomitant *Tlr4* silencing (Supplementary Figure 11B) reduced the overall tumor volume and weight, indicating that TLR4 acts upstream of *Twist1*. Constitutive overexpression of *Twist1* resulted in increased metastasis to the lung and the liver, suggesting that it has an important role in metastatic progression (Figure 7C).

Figure 7. *Twist1* overexpression drives tumor growth independently of *Tlr4*. TICs were transduced with lentivirus expressing shRNA for *Tlr4* or scrambled shRNA followed by a second transduction with retrovirus expressing *Twist1* overexpression (OE) plasmid vector or empty vector (Emp). These cells were injected subcutaneously into the rear flanks of NOG mice (1 million cells/injection). (A) Tumor volume measured at days 15, 25, and 30 (also whenever an unexpected death occurred) showed an increasing trend in the tumor volume with intact *Tlr4* and *Twist1* overexpression when compared with their respective controls (sh-Tlr4 + OE vs sh-Tlr4 + Emp; *** $P < .001$; $n = 4$ NOG mice/cohort; $n = 2$; statistics performed using 2-way analysis of variance). (B) Significant increase in the overall tumor weight (*** $P < .001$, **** $P < .0001$, $n = 4$ NOG mice/cohort; $n = 2$). (C) Overexpression of *Twist1* promotes liver and lung metastasis irrespective of the endogenous *Tlr4* expression in TICs. (D) A schematic representation of the proposed link between oncogenic TLR4/NANOG signaling, OB-R/pSTAT3, and an effective TWIST1 pathway in generating TICs.

Discussion

TICs comprise a small percentage of cells with stem-like properties resident in tumors and have been documented in a wide variety of cancerous tissues.²⁹ EMT remodels cells and thus plays a key role in the acquisition of malignant traits.^{30,31} In this report, we show that TLR4 is required for liver oncogenesis and the expansion of liver TICs in HCFD-fed HCV-NS5A Tg mice. Analysis of gene expression in TICs showed that *Twist1*, a master regulator of EMT,¹⁷⁻¹⁹ was increased 11-fold, which was not observed in TICs derived from alcohol diet-fed NS5A Tg mice.¹⁴ The findings described an unexpected convergence of the NANOG and STAT3 signaling pathways. We have identified an important functional link between the NANOG pathway, by activation

of upstream LPS-TLR4 signaling, and the STAT3 pathway, driven by leptin-OB-R signaling. These 2 pathways cooperate to activate *Twist1* and augment TIC motility (Figure 7D).

These studies implicate that lifestyle diseases, including obesity and alcoholism, promote genesis, mesenchymal phenotype, and metastatic characteristics of TICs through synergistic interactions between the LPS-TLR4-NANOG pathway and leptin-Ob-R-STAT3 (Figure 7D). Therefore, investigation of the effects of inhibitor combinations to prevent this synergistic interaction, including TLR4 antagonist or inhibitors targeting STAT3, NANOG, and/or TWIST1, is warranted for further investigation in preclinical mouse models.

In conclusion, stemness markers NANOG and STAT3 are activated downstream of the LPS-TLR4 and leptin-OB-R pathways, respectively. NANOG and STAT3 cooperate to drive increased *Twist1* levels, promoting the mesenchymal phenotype and metastasis in TICs (Figure 7D) and contributing to HCC development.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2015.11.002>.

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Conflicts of interest

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