Wnt antagonism inhibits hepatic stellate cell activation and liver fibrosis

Jason H. Cheng,1 Hongyun She,1,2 Yuan-Ping Han,3 Jiaohong Wang,1,2 Shigang Xiong,1 Kinji Asahina,1 and Hidekazu Tsukamoto1,2

Departments of 1Pathology and 3Surgery, Keck School of Medicine of the University of Southern California; 2Department of Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California

Submitted 11 June 2007; accepted in final form 12 November 2007

Cheng JH, She H, Han Y-P, Wang J, Xiong S, Asahina K, Tsukamoto H. Wnt antagonism inhibits hepatic stellate cell activation and liver fibrosis. Am J Physiol Gastrointest Liver Physiol 294: G39–G49, 2008. First published November 15, 2007; doi:10.1152/ajpgi.00263.2007.—Activation of hepatic stellate cells (HSC), a key event in liver fibrosis, is caused by diminished adipogenic transcription. This study investigated whether Wnt signaling contributes to "antiadipogenic" activation of HSC and liver fibrogenesis. Culture-activated HSC from normal rats and HSC from cholestatic rat livers were examined for expression of Wnt, Frizzled (Fz) receptors, and coreceptors by quantitative PCR. Wnt signaling was assessed by nuclear β-catenin and T cell factor (TCF) promoter activity. Dickkopf-1 (Dkk-1), a Wnt coreceptor antagonist, was transduced by an adenoviral vector to assess the effects of Wnt antagonism on culture activation of HSC and cholestatic liver fibrosis in mice. Messenger RNA for canonical (Wnt3a and 10b) and noncanonical (Wnt4 and 5a) Wnt genes, Fz-1 and 2, and coreceptors [low-density lipoprotein-receptor-related protein (LRP)6 and Ryk] are increased ~3–12-fold in culture-activated HSC compared with quiescent HSC. The nuclear β-catenin level and TCF DNA binding are markedly increased in activated HSC. TCF promoter activity is stimulated with Wnt1 but inhibited by Chibby, a protein that blocks β-catenin interaction with TCF, and by Dkk-1. Dkk-1 enhances peroxisome proliferator-activated receptor-γ (PPARγ)-driven PPAR response element (PPRE) promoter activity, a key adiogenic transcriptional parameter, abrogates agonist-stimulated contraction, and restores HSC quiescence in culture. High expression of Dkk-1 increases apoptosis of cultured HSC. Expression of Wnt and Fz genes is also induced in HSC isolated from experimental cholestatic liver fibrosis, and Dkk-1 expression ameliorates this form of liver fibrosis in mice. These results demonstrate antiadipogenic Wnt signaling in HSC activation and therapeutic potential of Wnt antagonism for liver fibrosis.

β-catenin; T cell factor; Dickkopf-1; PPARγ

HEPATIC STELLATE CELLS (HSC) constitute 5–8% of the total liver cell population and are desmin-positive pericytes that also function as a primary storage depot for vitamin A. Located in the subendothelial space of Disse of the liver, HSC serve to support a differentiated phenotype of hepatocytes via direct and indirect communications involving gap junctions, soluble factors, and matrix molecules. They also play a pivotal role in wound healing by remodeling and depositing extracellular matrix (ECM) proteins. Upon liver injury and subsequent paracrine stimulation by sinusoidal endothelial cells, Kupffer cells, platelets, and hepatocytes, as well as autocrine regulation, they undergo tightly programmed transdifferentiation known as activation, the transition into highly proliferative and contractile myofibroblastic cells. Phenotypic changes that accompany this cellular process include loss of retinyl esters, increased smooth muscle α-actin expression, proliferation, gelatinase expression, cytokine and chemokine production, leukocyte chemoattraction, synthesis and deposition of ECM proteins, and contractility (2, 5).

Liver fibrosis represents chronic wound repair and is causally associated with persistent HSC activation. Thus better understanding of molecular mechanisms that underlie HSC activation is an important prerequisite for development of new therapeutic modalities for cirrhosis, for which liver transplantation is presently only a clinically proven choice of treatment. To this end, we proposed several years ago and have supported by subsequent studies that HSC activation is characterized by the loss of adipogenic transcriptional regulation that is, in fact, required for the differentiated phenotype of HSC (7, 18, 28). The major transcriptional factors involved in adipocyte differentiation include proteins belonging to the CCAAT/enhanced binding protein family (C/EBP), peroxisome proliferator-activated receptor-γ (PPARγ), liver X receptor, and sterol regulatory element-binding protein 1 (SREBP-1) (21). Expression of these transcription factors and downstream genes are abundant in quiescent, differentiated HSC and are lost upon activation. A gain of function manipulation for these transcription factors by treatment with an adipocyte differentiation cocktail or ectopic transduction of PPARγ or SREBP-1c restores the quiescent phenotype of HSC (7, 28).

Wnts are a highly conserved family of secreted glycoproteins that regulate cellular differentiation and proliferation by binding to their Frizzled (Fz) receptor low-density lipoprotein-receptor-related protein (LRP)5/6 coreceptor complexes. In mice and humans, there are 20 Wnt genes (17), nine Fz, and two LRP coreceptors, LRP5 and LRP6, and the specificity of cellular effects are determined by the subsets of Wnts and signaling molecules expressed (6, 16). Three signal transduction pathways are activated by Wnt, the canonical Wnt pathway, the planar cell polarity pathway and the Wnt/Ca2+ pathway (6). Activation of the canonical pathway results in inhibition of glycogen synthase kinase 3 (GSK3) and allows stabilization of cytosolic β-catenin and its translocation to the nucleus, where it binds to the T cell factor/lymphocyte enhancer factor (TCF/LEF) family of transcription factors to stimulate the expression of multiple Wnt target genes, including c-myc, c-jun, and cyclin D1 (6, 15). Wnt signals are also regulated by secreted antagonists that bind directly to the ligand, such as Wnt inhibitory factor (WIF) and the secreted frizzled-related protein (sFRP) family, or prevent LRP co-receptor association with Fz. Dickkopf (Dkk), the best-charac-

http://www.ajpgi.org 0193-1857/08 $8.00 Copyright © 2008 the American Physiological Society

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
alyzed of the latter group of antagonists, binds to LRP6 and inhibits the Wnt-induced Fz-LRP5/6 complex formation that is essential to the canonical pathway. Canonical Wnt signaling through β-catenin is facilitated by the Wnt family members, such as Wnt1, Wnt3a, and Wnt10b, and Frizzled receptors such as Fz1 and Fz5 (15, 34).

Interestingly, both Wnt1 and Wnt10b potently suppress adipocyte differentiation via its inhibition of the adipogenic transcription factors C/EBPα and PPARγ (1, 24). This raises intriguing questions as to whether functional Wnt signaling exists in activated HSC and whether it is causally linked to the loss of adipogenic regulation and consequent activation of HSC. To this end, a recent study has shown that Wnt and Fz genes are expressed by a nonparenchymal liver cell fraction enriched with HSC and Kupffer cells (35). Activated rat HSC are also shown to express higher levels of Wnt4, Wnt5, and Fz2 in culture and experimental liver fibrosis model (11), and a most recent study suggests enhancement of activation and survival of human HSC via Wnt signaling (20). The present study systematically investigated the expression of Wnt, Fz, and coreceptors and the functionality of Wnt signaling by purified primary cultures of rat HSC, a rat HSC line designated as biliary fibrosis-derived stellate cells (BSC) (29), and HSC isolated from biliary liver fibrosis. We further tested the role of Wnt signaling in HSC activation by using the LRP coreceptor antagonist Dickkopf-1 (Dkk-1) in culture and an animal model. Indeed, our findings demonstrate the evidence of functional canonical Wnt signaling in activated HSC and restoration of adipogenic regulation and HSC quiescence by inhibition of Wnt signaling with Dkk-1. Furthermore, Dkk-1 is shown to prevent cholestatic liver fibrogenesis in an animal model.

MATERIALS AND METHODS

HSC and cell line. The use of animals for this study was approved by the Institutional Animal Care and Use Committee of the University of Southern California (protocols 9823 and 10655). HSC were isolated from normal male Wister rats as previously described (32) by the Non-Parenchymal Liver Cell Core of the Research Center for Alcoholic Liver and Pancreatic Diseases and Cirrhosis. HSC were also isolated from rats with cholestatic liver fibrosis 10 days following ligation and scission of the common bile duct. The purity of isolated HSC was examined by phase-contract microscopy, ultraviolet-excited fluorescence microscopy, and viability based on trypan blue exclusion (purity > 96%, viability > 94%). Freshly isolated normal rat HSC were cultured on 100-mm plastic dishes in low glucose DMEM supplemented with 10% FBS and antibiotics for 1, 3, or 7 days to examine quiescent, activating, or fully activated cells. HSC isolated from rats with bile duct ligation (BDL) and sham-operated rats were cultured overnight in low glucose DMEM with 3% FBS before extraction of RNA. A spontaneously immortalized HSC line (designated as BSC) was established from activated HSC isolated from experimental biliary liver fibrosis (29) and maintained in low glucose DMEM with 10% FBS.

Preparation of cellular and nuclear proteins and Western blot analysis. Total cellular and nuclear protein extracts were prepared as previously described (26). The nuclear proteins were resolved on a 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and incubated with primary antibodies (Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were visualized by a chemiluminescent method using the ECL detection system (Pierce).

Electrophoretic mobility shift assay. Nuclear extracts from rat HSC (15 μg protein each) were preincubated on ice for 15 min in a reaction mixture containing 20 mM HEPES (pH 7.6), 100 mM KCl, 0.2 mM EDTA, 2 mM DTT, 20% glycerol, and 200 μM/metyl-dl-ascorbate and were incubated for 30 min with 1–2 ng of an α2P-labeled double-stranded TCF consensus sequence (5′-GACCCCTTGTACCTTACCT and 5′-GAGGTTAGATCAAAGGT). As a control probe, we used an oligonucleotide corresponding to a footprint (FP) of the α(I) collagen promoter (FP4: –191/–173) with HSC nuclear extracts because the level of protein binding to this region is shown to be similar in quiescent and activated HSC (23). The sequences of this FP4 probe are 5′-GATCGCCGGGAGGGGGAGGCTGGT-3′ and 5′-GATCACCCAGCTCCCCCTCCCGCC-3′. For a supershift assay, an antibody against TCF-4 (Upstate Biotechnology, Lake Placid, NY) was added to the reaction mixture and incubated for an additional 20 min. The reaction mixture was then resolved on a 6% non-denaturing polyacrylamide gel followed by autoradiography.

Immunostaining. HSC cultured in 24-well plates were washed with PBS and fixed in cold methanol for 10 min. After three washes the cells were permeabilized with 0.15% Triton X-100 in PBS for 15 min. Following treatment with blocking solution consisting of 5% nonfat milk and subsequent PBS wash, dual immunofluorescence staining was performed by incubation with a 1:200 dilution of rabbit polyclonal antibody against β-catenin (Santa Cruz Biotechnology) and mouse monoclonal antibody against α-smooth muscle actin (α-SMA, Sigma) for 16 h at 4°C. C3-conjugated anti-rabbit IgG and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG were applied at a dilution of 1:200 for 4 h. Normal rabbit IgG (Thermo Fisher Scientific, Fremont, CA) was used as a negative control under the same condition of the β-catenin staining.

Apoptosis was assessed by TUNEL staining performed according to the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN), as well as immunostaining for active caspase 3 by using an antibody from R&D Systems (Minneapolis, MN). For immunostaining for Dkk-1 and green fluorescent protein (GFP) following infection with adenoviral vectors expressing these proteins, dual immunofluorescence staining was performed by incubation with a 1:1,000 dilution of rat monoclonal antibody against mouse Dkk-1 (R&D Systems) and a 1:500 dilution of rabbit polyclonal antibody against GFP (Invitrogen, Carlsbad, CA) for 1 h at room temperature. Alexa Fluor 568 goat anti-rat IgG and Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) were applied at a dilution of 1:400 for 30 min. Nuclei were counterstained by Prolong Gold Antifade Reagent with DAPI (Invitrogen). Fluorescence images were visualized under a microscope (Nikon Eclipse 90i; Nikon, Tokyo, Japan) and were captured using a digital camera (DS-Qi1; Nikon) and imaging software (NIS-Elements BR 2.30; Nikon).

Transient transfection and reporter gene assay. TCF promoter-luciferase constructs TOPFLASH and FOPFLASH (containing eight wild-type TCF/LEF binding sites and mutant consensus sequences, respectively) and pME18-TF, pLNCX-Wnt1, and MBP-Chibby expression vectors were gifts from Dr. Randall T. Moon (Univ. of Southern California, Los Angeles, CA). A PPAR response element (PPRE)-luciferase reporter construct and pCMX-PPARγ expression vector were gifts from Dr. Ronald Evans (Salk Institute, La Jolla, CA). For TCF or PPRE promoter activity assays, BSC in 24-well plates were transfected with TCF-luciferase (tk-TCF × 8-luciferase) or PPRE-luciferase construct (tk-PPRE × 3-luciferase) and an expression or empty vector. Transfection efficiency was assessed by using renilla phRL-TK vector (Promega, Madison, WI). A nonlipid cationic Targefect F2 reagent (Targeting Systems, San Diego, CA) was used at 1:1 molar ratio of total DNA (μg) and volume of F2 (μl). After 24 h of transfection, the cells were washed in PBS and solubilized for determination of both firefly (TCF/PPRE-luciferase) and renilla luciferase activities. Ratios of firefly and renilla luciferase activities were used to calculate relative changes in TCF and PPRE promoter activity.
Adenoviral Dkk-1 transduction. Adenoviral vector for Dkk-1 (Ad.Dkk-1) was a generous gift from Dr. Calvin Kuo (Stanford University). Primary cultures of rat HSC were infected with Ad.Dkk-1 or Ad.GFP at the multiplicities of infection (MOI) of 100, 300, or 500 on day 3 or 6 and cultured for 4 more days. Morphological effects were examined by phase contrast microscopy and lipid storage by oil red O staining. DNA synthesis was determined by the rate of [methyl-\(^{3}H\)] thymidine (New England Nuclear, Boston, MA) (0.5 \(\mu\)Ci/ml) incorporation into DNA. Expression of Dkk-1 and activation markers such as type I collagen were determined by immunoblot analysis. To determine the effects of Dkk-1 transduction in vivo, C57BL/6 mice were used to induce biliary liver fibrosis by BDL and scission. Ad.Dkk-1 or Ad.GFP (2 \(\times\) 10\(^{7}\) pfu) was injected into spleen at the time of BDL.

HSC contraction. Three days after culturing on plastic, HSC were infected with adenovirus-expressing GFP or Dkk-1. After additional culture for 4 days with 10% FBS, the cells were embedded in type I collagen at 5 \(\times\) 10\(^{5}\) cells per gel (0.6 ml). Then the cells were stimulated with endothelin-1 (ET-1) (100 nM), transforming growth factor \(\beta\) (TGF-\(\beta\)) (2 ng/ml), or 5% FBS for 30, 90, or 300 min for assessment of contraction.

Histology and hydroxyproline assay. Liver tissues were fixed in 3% paraformaldehyde and processed for hematoxylin and eosin and reticular staining by the Morphology Core of the Research Center for Alcoholic Liver and Pancreatic Diseases. The area of reticulin staining was quantified by a morphometric analysis. The hepatic content of hydroxyproline was determined with Ehrlich’s reagent after hydrolysis of the tissue as previously performed in our laboratory (10).

RNA Extraction and qRT-PCR. Total RNA was extracted from day 1 and day 7 HSC by using Trizol reagent by Invitrogen. Three micrograms of RNA were reverse transcribed at 37 °C for 1 h with murine Maloney leukemia virus reverse transcriptase and amplified by 40 cycles using the Stratagene SyberGreen kit for real-time PCR analysis. The sequences of the primers were designed using the Invitrogen OligoPerfect Designer: Wnt3a, 5'-CATCGCCAGTCAAGCTGAGCTCA; Wnt10b, 5'-CGTCTATGCCATGCGAGCTCA; LRP6, 5'-CCGTCCTATCTCAGCTATAAG and 5'-CTACGGTGCTCACGTA and 5'-CGAG and 5'-GACGTCTTGTTGCAAGTG; Wnt5a, 5'-GACGTCTTGTTGCAAGTG; Fz2, 5'-CATGGGCCAGCAGCACAG; Fz1, 5'-GAGTCACAGTGGTCCGAGTCTCAGTA and 5'-CCAGGTAGGGACAGGATTA; Fz2, 5'-CGTGTTCCATCTCAGCTATAGA and 5'-AAGATGAAACGGCAGGCACGAC; LRP6, 5'-TACGGTCTTCTACGGGACA and 5'-GGAACTGAGACTCGGACGAC; Ryk, 5'-AAGCGAGATTGCAAAAC and 5'-CAGGTCTGAGACTGGCTCA; Cts7, 5'-TGTGGCAGACGACTAAAGT and 5'-CGGAGCGCGAAAATGC; PPARy, 5'-CGTCTGAGCTCTAAGAAATACAAA and 5'-AGAATTCTGGTTTTTACAAATAAAGG; TGF-\(\beta\), 5'-TGCAGCCGCCCATTCCGCTCTAAGA and 5'-AGAATTCTGGTTTTTACAAATAAAGG; Dkk-1 (rat), 5'-GCTGCCCCTGGAGTTTTATGTGGTCCGACG; Dkk-1 (mouse), 5'-TGCAGCCGCCCATTCCGCTCTAAGA and 5'-AGAATTCTGGTTTTTACAAATAAAGG; G41Wnt, 5'-GACGTCTTGTTGCAAGTG; Fz1 and 2, and coreceptors (LRP6 and Ryk) in day 1 (quiescent) and day 7 (fully activated) HSC. The mRNA levels of the Wnt genes examined were all induced 3.5–12-fold in day 7 HSC compared with quiescent day 1 cells (Fig. 1A).

Increased nuclear \(\beta\)-catenin and TCF DNA binding in activated HSC. Having observed that both canonical Wnts and their receptors/coreceptors are induced in culture-activated HSC, autocrine induction of canonical Wnt signaling is suspected in these cells. Because canonical Wnt signaling induces a proliferative response and morphological alterations through stabilization and nuclear translocation of cytosolic \(\beta\)-catenin and activation of TCF/LEF-dependent transcription (34), we examined the nuclear levels of \(\beta\)-catenin in day 1 (quiescent) and day 7 (activated) HSC by Western blot analysis. A marked increase in the nuclear level of \(\beta\)-catenin is evident in day 7 HSC because \(\beta\)-catenin in the whole cell extract also increases (Fig. 1B). We then examined DNA binding of TCF, a transcriptional factor activated by \(\beta\)-catenin. Indeed, nuclear extracts from day 3 and 7 HSC show conspicuously increased TCF binding to DNA compared with that from quiescent HSC, as assessed by electrophoretic mobility shift assay (Fig. 1C, left). We also used a control probe for one of four footprints detected within the proximal \(\alpha\)-collagen promoter (FP4: -191/–173) (23). Analysis with this probe of nuclear extracts from day 1 vs. day 7 HSC shows an equal intensity of a shifted band (Fig. 1C, right) as previously reported (23), supporting the equal loading and quality of nuclear extracts of both samples. These results suggest that Wnt-stabilized \(\beta\)-catenin plays a role in HSC transdifferentiation by activating TCF-mediated transcription of target genes, including those that participate in proliferation such as cyclin D1 and antiadipogenic factors such as PPAR\(\beta\) (8, 33), a recently disclosed activation marker for HSC (9, 28). To confirm accumulation of nuclear \(\beta\)-catenin, we performed double immunostaining for \(\beta\)-catenin and an HSC activation marker, \(\alpha\)-SMA (Fig. 1D). In day 3 and 7 cells, \(\beta\)-catenin staining is intensified both along the plasma membrane and in nuclei as the staining for \(\alpha\)-SMA also increases. Morphometric analysis for \(\beta\)-catenin staining shows nuclear \(\beta\)-catenin positive cells increase from 8.9% on day 1 to 85 and 91% on day 3 and 7, respectively, and the cells with positive \(\beta\)-catenin staining along plasma membrane increase from less than 3% on day 1 to 75 and 93% on day 3 and 7 cells, respectively. These results not only confirm the nuclear localization of \(\beta\)-catenin in activated HSC but also suggest the potential role of this protein in cell adhesion and contractility.
Functional β-catenin in HSC. Next, we tested the functionality of canonical Wnt signaling by using a TCF promoter-luciferase reporter gene construct. For this analysis, we used the BSC cell line that was established by single cell cloning of spontaneously immortalized, activated HSC from cholestatic rat livers (29) since transfection of primary HSC is difficult. In the cells transiently cotransfected with a TCF expression vector and a TCF response element-luciferase reporter gene, TCF
expression expectedly stimulates a wild-type TCF promoter (TOPFLASH) in a dose-dependent manner, whereas no stimulation is evident with the mutant TCF promoter, FOPFLASH (Fig. 2A). To evaluate whether increased β-catenin in the nucleus of activated HSC serves as a functional coactivator for TCF promoter, we performed additional TCF reporter assays in HSC by using Chibby and TCF expression vectors. Chibby is a conserved nuclear protein that inhibits the binding of β-catenin to the TCF transcription factor by interacting directly with the COOH-terminal region of β-catenin while having no effect on β-catenin degradation (31). As such, overexpression of Chibby represses the transcriptional activation of the TCF reporter TOPFLASH in a dose-dependent fashion (Fig. 2B), confirming the functional role of nuclear β-catenin in this promoter activation.

Functional Wnt coreceptor and signaling in activated HSC. Having shown increased Wnt expression, elevated levels of nuclear β-catenin, increased TCF binding, and enhanced β-catenin-dependent TCF promoter activity in activated HSC or BSC, we examined next the existence of functional Wnt signaling at the receptor level. We used an expression vector for Dkk-1, which serves as a Wnt coreceptor (LRP6) antagonist by inhibiting ligand-induced oligomerization of Fz-LRP complex (27). The rationale for the use of Dkk-1 over other approaches such as gene silencing for Wnt and receptor genes is based on the following: 1) both LRP6 and Fz are induced in activated HSC (Fig. 1A) that are targets of Dkk-1; 2) induction of multiple Wnt and receptor genes in HSC (Fig. 1A) makes it difficult to block Wnt signaling by gene silencing, whereas Dkk-1 can block signaling induced by multiple Wnt gene

Fig. 2. Functional Wnt signaling in rat HSC line. A: effect of TCF expression on TCF response element reporter construct in a HSC line. Ectopic expression of TCF dose dependently induces the wild-type TCF promoter activity (TOPFLASH, pGL-OT) 17–44-fold compared with cotransfection with an empty expression vector. Mutant promoter–luciferase construct (FOPFLASH, pGL-OF) is unresponsive to TCF expression. *P < 0.05 compared with cotransfection with empty expression vector (pME18). B: expression of Chibby suppresses the β-catenin/TCF-driven TOPFLASH promoter activity as the evidence of functional β-catenin toward TCF promoter. Also note the ability of Chibby to suppress endogenous β-catenin-driven promoter activity in the absence of TCF expression vector. *P < 0.05 compared with TCF-driven pGL-OT. C: Dkk-1 transduction dose dependently suppresses TCF promoter activity. *P < 0.05 compared with TCF-driven pGL-OT. D: Wnt1 transduction increases TCF promoter activity. *P < 0.05 compared with cotransfection with empty expression vector.

Fig. 1. Evidence of induced Wnt signaling in activated rat HSC. A: expression of Wnt isoforms, Frizzled (Fz) receptors, and coreceptors are increased in culture-activated hepatic stellate cells (HSC). Total RNA extracted from quiescent HSC (day 1) and activated cells in culture (day 7) were analyzed for Wnt3a, Wnt10b, Wnt4, Wnt5a, Fz1, Fz2, low-density lipoprotein-receptor-related protein (LRP)6, and Ryk by quantitative real-time PCR (qRT-PCR). *P < 0.05 compared to day 1 HSC. B: nuclear and whole cell β-catenin levels are increased in activated (day 7) HSC as determined by immunoblot analysis. C: enhanced T cell factor (TCF) DNA binding is demonstrated by EMSA of nuclear extracts (15 μg protein) prepared from day 3 and day 7 HSC compared with that from quiescent day 1 HSC (left); specificity of TCF in the protein-DNA complex is confirmed with anti-TCF antibody. EMSA analysis with the control probe [a footprint-4 (FP4), the −191/−173 sequence of the proximal α(1) collagen promoter] demonstrates similar binding of proteins to this probe by nuclear extracts from day 1 and day 7 HSC (right). D: increased nuclear and plasma membrane localization of β-catenin in activated HSC. Immunofluorescent staining of β-catenin (red) and α-smooth muscle actin (α-SMA) (green) show that both day 3 (activating) and day 7 (fully activated) HSC exhibit intense β-catenin staining in both the plasma membrane and nuclei compared with quiescent day 1 cells (×200). The last photograph is for a negative control for β-catenin staining (stained with nonimmune IgG) but positive staining for α-SMA.
products; and 3) our qRT-PCR analysis demonstrates the undetectable level of Dkk-1 expression in both quiescent and activated HSC. Our results demonstrate that Dkk-1, indeed, suppresses TCF-driven promoter activity in a dose-dependent manner (Fig. 2C), supporting the presence of functional Wnt receptor complex for the canonical pathway in BSC. Next, we transduced Wnt1 to determine whether the expression of the ligand drives TCF promoter activity. Overexpression of Wnt1 increases the TCF-induced TOPFLASH promoter activity approximately two- to threefold (Fig. 2D). This rather modest extent of stimulation of the promoter activity is most likely due to sufficiently induced expression of endogenous Wnt ligands. In summary, these results provide another evidence for the existence of functional Wnt signaling.

Wnt antagonism with Dkk-1 restores adipogenic transcriptional regulation and quiescence in HSC. To test the role of Wnt signaling in HSC activation, we expressed Dkk-1 by an adenoviral vector in primary cultures of activated rat HSC and examined its effects on morphologic and biochemical parameters of HSC activation. As shown by an immunoblot in Fig. 3A, Dkk-1 adenovirus infection causes a decrease in nuclear β-catenin level as the evidence of canonical Wnt antagonism. The β-catenin level of whole cell extracts is also reduced in Dkk-1 transduced cells but to a lesser extent. Figure 3B shows representative images to demonstrate transduction efficiency of GFP and Dkk-1 in HSC infected with both Ad.GFP and Ad.Dkk-1 (100 MOI each) for 2 days. Almost all cells (more than 98%) are positive for both GFP (green) and Dkk-1 (red) immunostaining. The images also depict that Dkk-1-transduced cells are beginning to show morphologic changes with a retracted, star-shaped cell body and dendritic processes already at this 2 days postinfection time point. Four days after infection, the morphologic changes are more prevalent with increased intracellular lipid content (Fig. 3C), demonstrating a morphological reversal of HSC to the quiescent phenotype in a majority of the cells transduced with Dkk-1. DNA synthesis, as
assessed by \(^{3}\)H-thymidine incorporation, is inhibited by 30% and 65% by Dkk-1 at MOI of 100 and 300, respectively (Fig. 3D). Cyclin D1 and PCNA protein levels are also reduced by Dkk-1, further supporting antagonism of canonical Wnt signaling (Fig. 3E). Type I collagen expression is also inhibited by Dkk-1 transduction regardless of whether day 3 or day 3 HSC in culture are infected with the vector (Fig. 3E). Dkk-1 also reduces \(\alpha\)-SMA and TGF-\(\beta\)-1 mRNA expression, two bona fide fibrogenic parameters for activated HSC, while conspicuously increasing mRNA levels for C/EBP\(\alpha\) and PPAR\(\gamma\), key adipogenic transcription factors required for HSC quiescence (7, 28) (Fig. 3F). To further determine “proadipogenic” effects of Dkk-1 at the transcriptional level, we tested the effect of Dkk-1 on the activity of PPRE promoter induced by PPAR\(\gamma\). For this analysis, we cotransfected BSC with a PPRE-luciferase and Dkk-1 expression vectors. Expression of Dkk-1 increases basal PPRE promoter activity threefold and PPAR\(\gamma\)-induced activity twofold (the last bar graph, Fig. 3F). These results demonstrate that endogenous Wnt signaling suppresses PPAR\(\gamma\) expression and PPRE activity in activated HSC/BSC and suggests that this mechanism may serve to render antiadipogenic activation of HSC (28).

**Dkk-1 blocks HSC contraction.** Increased contractility is an important functional phenotype of activated HSC. We observed increased \(\beta\)-catenin staining along the plasma membrane in activated HSC (Fig. 1D) that is suggestive of its involvement in adherens junction and cell contractility. Thus we examined the effects of Wnt antagonism by Dkk-1 on HSC contractility induced by ET-1, TGF-\(\beta\), or FBS. For this analysis, day 3 HSC were infected with Ad.GFP or Ad.Dkk-1 and 4 days later cultured in 3D type I collagen gel. In this 3D culture system, the cells show intriguing morphological differences. Activated HSC infected with Ad.GFP display an extensive intercellular network with elongated dendritic processes (Fig. 4C, left), whereas Ad.Dkk-1-infected cells lack such network and appear quiescent in morphology (right). In addition, vitamin A storage, as assessed by UV-excited autofluorescence, is increased in Ad.Dkk-1-infected cells (Fig. 4B), suggesting a more quiescent phenotype and the viability of the cells. When these cells are stimulated with ET-1, TGF-\(\beta\) (2 ng/ml), or 5% FBS for 30, 90, or 300 min for assessment of contraction, the gel with Ad.GFP cells contracts even within 30 min with ET-1 and 90 min with TGF-\(\beta\) or 5% FBS, but such contraction is absent or weaker in the gel with Ad.Dkk-1-infected cells (Fig. 4A). These results demonstrate that Dkk-1 abrogates cell-to-cell interaction and agonist-induced contractility by activated HSC in 3D collagen.

**Dkk-1 at high MOI induces HSC apoptosis.** We tested next whether infection of culture-activated HSC with Ad.Dkk-1 at a high MOI for a prolonged period causes apoptosis. For this analysis, we infected day 3 HSC with a higher MOI of 500 and cultured them for 6 more days on plastic to detect apoptotic cells by TUNEL staining and immunostaining for active caspase 3. As shown in Fig. 5, the cells positive for TUNEL

---

**Fig. 6.** Wnt expression is increased in HSC from cholestatic liver fibrosis, and Wnt antagonism by Dkk-1 inhibits cholestatic liver fibrosis in mice. A: expression of Wnt, Fz, and coreceptor Ryk mRNA are induced in HSC isolated from cholestatic liver fibrosis in rats [bile duct ligation (BDL)] compared with the cells from sham-operated rats (Sham) as determined by qRT-PCR. \(*P < 0.05\): adenovirus-mediated transduction of Dkk-1 as demonstrated by immunoblot analysis. Note conspicuous expression of Dkk-1 in Ad.Dkk-1-infected mouse livers compared with Ad.GFP-infected mice. C: Dkk-1 transduction suppresses liver fibrosis induced by ligation and scission of the BDL in mice. Note marked attenuation of reticulin staining in a mouse given Ad.Dkk-1 compared with that given Ad.GFP. D: morphometric analysis of reticulin staining of mice subjected to sham-operation (Sham, \(n = 4\)) or BDL and scission (\(n = 6\) pairs) and Ad.GFP or Ad.Dkk-1 administration. Note a significant reduction of reticulin staining in Ad.Dkk-1 mouse livers. \(*P < 0.05\) compared with Sham given Ad.GFP. \(*P < 0.05\) compared with BDL given Ad.GFP. \(*P < 0.05\) compared with BDL mice given Ad.GFP. E: Dkk-1 inhibits liver mRNA levels of \(\alpha\)(I) procollagen and \(\alpha\)-SMA in BDL mice. \(*P < 0.05\) compared with Sham given Ad.GFP. **P < 0.05 compared with BDL mice given Ad.GFP.
AJP-Gastrointest Liver Physiol • VOL 294 • JANUARY 2008 • www.ajpgi.org

G47

Wnt AND STELLATE CELLS

A

Wnt 3a  Wnt 10b  Wnt4  Wnt5a

Relative mRNA Level

Sham  BDL  Sham  BDL  Sham  BDL  Sham  BDL

Fz1  Fz2  Ryk

Relative mRNA Level

Sham  BDL  Sham  BDL  Sham  BDL

B

BDL liver tissue

Adenovirus  GFP  Dkk-1

Dkk-1  (35 KDa)

β-Actin  (42 KDa)

C

x100  x200

Ad.GFP

Ad.Dkk-1

Sham  BDL

D

Reticulin Staining

Ad.GFP  Ad.Dkk-1

E

Hydroxyproline content (µg liver)

Ad.GFP  Ad.Dkk-1

F

α1(I) procollagen mRNA (Fold increase)

Ad.GFP  Ad.Dkk-1

α-SMA mRNA (Fold increase)

Ad.GFP  Ad.Dkk-1
and active caspase 3 are detected in Ad.Dkk-1-infected cells but less than in Ad.GFP cells. Counting the positive cells reveals that approximately three times higher percentage of apoptotic cells (31.2 + 9.4%) occur in the Ad.Dkk-1-infected cultures than the Ad.GFP cells (11.3 + 4.1%) under the condition we examined. We think the apoptosis rate observed even in Ad.GFP cells is caused by the high MOI (500) and the extended duration of culture after infection (6 days). Infection at 100 or 300 MOI for 4 days, as performed in the preceding experiments, causes much less apoptosis (<3%) in both groups, and there is no difference between the two.

**HSC isolated from cholestatic liver fibrosis show induced Wnt expression.** To extend our findings to the in vivo and pathological context, we have examined the expression of Wnt and Fz genes in HSC isolated from rat cholestatic liver fibrosis 10 days following ligation and scission of the common bile duct. As shown in Fig. 6A, qRT-PCR analysis reveals increased expression of Wnt3a, Wnt10b, Wnt4, Wnt5a, Fz1, Fz2, and Ryk in HSC from the BDL model compared with sham-operated animals (Sham), much like what is observed in culture-activated HSC. In addition, our qRT-PCR analysis for Dkk-1 demonstrates the Ct values for this parameter for both Sham and BDL HSC samples are close to or higher than the detection limit (Ct = 35) even with five times more sample cDNA applied for PCR, confirming the lack of Dkk-1 expression as seen in cultured HSC. In summary, these results suggest that induced Wnt expression and signaling in activated HSC are pathophysiologically relevant, particularly in cholestatic liver fibrosis.

**Dkk-1 suppresses liver fibrosis in an animal model.** Having established that HSC in cholestatic liver fibrosis have increased Wnt and Fz expression, we next tested the effects of Wnt antagonism with Dkk-1 on the development of cholestatic liver fibrosis. For this, adenovirus-expressing GFP or Dkk-1 was injected into spleen of mice at the time of BD and scission, and the livers were examined 10 days later. Immunoblot analysis of liver protein extracts confirms conspicuous expression of Dkk-1 in Ad.Dkk-1-infected BDL livers compared with Ad.GFP-infected BDL livers (Fig. 6B). In mice transduced with GFP by a control vector, their livers show morphologic evidence of periportal liver fibrosis characterized by deposition of collagen around proliferating bile ducts as detected by reticulin staining (Fig. 6C). In Dkk-1-transduced mouse livers, reticulin staining is significantly reduced (Fig. 6C for representative microphotographs and 6D for morphometric data). This finding is confirmed by the hepatic hydroxyproline content that is increased 3.5-fold in BDL mice compared with Sham, and this increase is attenuated by 70% in BDL mice with Dkk-1 transduction (Fig. 6D). Further, Dkk-1 expression reduces the mRNA levels of α1(I) procollagen by 49% and α-SMA by 74% (Fig. 6E). These results support our notion that Wnt signaling is critical in activation of HSC in vivo, and blocking Wnt signaling suppresses liver fibrosis.

**DISCUSSION**

Our previous studies demonstrate that the expression of adipogenic transcription factors is essential for the maintenance of HSC quiescence in vitro (7, 18, 28). Indeed, the treatment with PPARγ ligands is shown to inhibit liver fibrosis in animal models (4). In search for the mechanisms responsible for suppression of adipogenic transcriptional regulation in HSC during their activation, the present study examined whether Wnt signaling plays an antiadipogenic role in HSC as demonstrated in preadipocyte-adipocyte differentiation (24). In the latter cell types, Wnt signaling maintains preadipocytes in an undifferentiated state through inhibition of the adipogenic transcription factors C/EBPα and PPARγ (24), and this inhibition is likely mediated by the endogenous inhibitor of adipogenesis, Wnt10b, whose expression is induced in preadipocytes but decreased upon induction of differentiation. Regulation by this Wnt isoform through its respective receptors Fz1, Fz2, and/or Fz5, and coreceptors LRP5 and LRP6 most likely underlies this antidiadipogenic effect (1). In fact, ectopic expression of constitutively active Fz1 and Fz2 in preadipocytes inhibits adipogenesis via β-catenin-dependent and -independent pathways, respectively (12).

In support of our central hypothesis, the present study demonstrates the existence of functional canonical Wnt signaling in activated HSC. Expression of both canonical (Wnt3a and 10b) and noncanonical (Wnt4 and 5a) Wnt isoforms, Fz-1 and 2 receptors, and coreceptors (LRP6 and Ryk) is induced in activated HSC. Wnt-induced stabilization of β-catenin activates a TCF promoter-luciferase gene in the HSC line, whereas blockade of this signaling by using the coreceptor antagonist Dkk-1 results in both decreased TCF promoter activity and increased activity of a PPRE promoter. Furthermore, the expression of Dkk-1 by an adenoviral vector increases PPARγ expression and suppresses the bona fide parameters for activation of HSC including proliferation, type I collagen expression, and agonist-stimulated contraction. Additionally, a high level of Dkk-1 expression induces apoptosis of HSC. Finally, the implementation of this modality in an animal model of cholestatic liver fibrosis proves its antifibrotic efficacy that is most likely mediated via antagonism of antiadipogenic regulation by endogenous Wnt, the expression of which is shown to be increased in HSC in this form of liver fibrosis. These findings not only shed new insights into the molecular mechanisms of HSC activation and novel therapeutic framework for liver fibrosis but also highlight the biological importance of reciprocal antagonism between Wnt signaling and adipogenic transcriptional regulation in the context of HSC. Whereas Wnt inhibits PPARγ activity in adipocytes (24), PPARγ conversely suppresses Wnt/β-catenin signaling by proteasome-mediated degradation of β-catenin via stimulation of GSK3 (13, 19). Thus, PPARγ-induced inhibition of HSC activation or liver fibrosis that have previously been reported (4, 7, 18, 28) may be attributable to this inhibitory effect on Wnt signaling. Increased intracellular cAMP and consequent activation of CaMP response element-binding protein (CREB) are essential to adipocyte differentiation (22) and are also known to inhibit Wnt10b expression (1). PPARγ activity is posttranslationally inhibited in the HSC line by treatment with TNF-α (29) as shown in adipocytes (30). Since this cytokine induces Wnt10b (25), the inhibitory effect of TNF-α on PPARγ may also be mediated via Wnt signaling. Indeed, other factors known to inhibit adipogenesis and activate HSC, such as TGF-β, platelet-derived growth factor, and epidermal growth factor, may also stimulate Wnt10b expression, raising a possibility that Wnt signaling serves as the common downstream mediator of the fibrogenic effects rendered by these known mediators.
The present study did not test a therapeutic efficacy of Wnt antagonism using Dkk-1. This therapeutic approach will be best implemented by expressing Dkk-1 in a manner that specifically targets activated HSC in vivo. This is particularly important since global Dkk-1 expression may inhibit hepatocyte regeneration that may require canonical Wnt signaling. Future studies are required to test this selective mode of therapeutic modality.

HSC are known to express markers of different cell types including smooth muscle cells (desmin, α-SMA), neural and glial cells (GFAP, neurotropic factors and their receptors, nestin, synaptophysin), and adipocytes (leptin). Because Wnt signaling plays a pivotal role in mesodermal cell fate determination, the regulatory role of Wnt in this known plasticity of HSC is an intriguing question that deserves to be explored. To this end, the mechanisms of liver fibrosis may need to be approached from the shifted transdifferentiation of plastic HSC caused by regulatory molecules that determine mesenchymal cell fate.

ACKNOWLEDGMENTS

The authors acknowledge technical support rendered by Drs. Yoshihisa Nemoto and Qing-gao Deng. The authors are also grateful to Drs. Randall Moon (Univ. of Washington), Randall Widlitz (Univ. of Southern California), Ronald Evans, and Calvin Kuo (Stanford Univ.) for generous gifts of various reporter and expression plasmids and a viral vector as described in MATERIALS AND METHODS.

GRANTS

This work was supported by the National Institutes of Health grants, P50-AA11999 (Research Center for Alcoholic Liver and Pancreatic Diseases), R24-AA12885 (Non-Parenchymal Liver Cell Core), and Medical Research Service of Department of Veterans Affairs.

REFERENCES