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Suppression of fibrogenic signaling in hepatic stellate cells by Twist1-dependent microRNA-214 expression: Role of exosomes in horizontal transfer of Twist1

Li Chen,[1] Ruju Chen,[1] Sherri Kemper,[1] Alyssa Charrier,[1,2] and David R. Brigstock[1,2,3]

[1]*The Research Institute at Nationwide Children's Hospital, Columbus, Ohio;* [2]*Molecular, Cellular, and Developmental Biology Program, The Ohio State University, Columbus, Ohio;* [3]*Department of Surgery, Wexner Medical Center, The Ohio State University, Columbus, Ohio*

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Chen L, Chen R, Kemper S, Charrier A, Brigstock DR. Suppression of fibrogenic signaling in hepatic stellate cells by Twist1-dependent microRNA-214 expression: Role of exosomes in horizontal transfer of Twist1. *Am J Physiol Gastrointest Liver Physiol* 309: G491–G499, 2015. First published July 30, 2015; doi:10.1152/ajpgi.00140.2015.—A hallmark of liver fibrosis is the activation of hepatic stellate cells (HSC), which results in their production of fibrotic molecules, a process that is largely regulated by connective tissue growth factor (CCN2). CCN2 is increasingly expressed during HSC activation because of diminished expression of microRNA-214 (miR-214), a product of dynamin 3 opposite strand (DNM3os) that directly suppresses CCN2 mRNA. We show that an E-box in the miR-214 promoter binds the basic helix-loop-helix transcription factor, Twist1, which drives miR-214 expression and results in CCN2 suppression. Twist1 expression was suppressed in HSC of fibrotic livers or in cultured HSC undergoing activation in vitro or after treatment with ethanol. Furthermore, Twist1 decreasingly interacted with DNM3os as HSC underwent activation in vitro. Nanovesicular exosomes secreted by quiescent but not activated HSC contained high levels of Twist1, thus reflecting the suppression of cellular Twist1 during HSC activation. Exosomal Twist1 was intercellularly shuttled between HSC and stimulated expression of miR-214 in the recipient cells, causing expression of CCN2 and its downstream effectors to be suppressed. Additionally, the miR-214 E-box in HSC was also regulated by hepatocyte-derived exosomes, showing that functional transfer of exosomal Twist1 occurs between different cell types. Finally, the levels of Twist1, miR-214, or CCN2 in circulating exosomes from fibrotic mice reflected fibrosis-induced changes in the liver itself, highlighting the potential utility of these and other constituents in serum exosomes as novel circulating biomarkers for liver fibrosis. These findings reveal a unique function for cellular or exosomal Twist1 in CCN2-dependent fibrogenesis.

connective tissue growth factor; CCN2; exosome; fibrosis; E-box

LIVER INJURY IS CHARACTERIZED by a phenotypic and functional transformation of normally quiescent hepatic stellate cells (HSC) into α-smooth muscle actin (α-SMA)-expressing myofibroblastic cells, which promote wound closure and produce a collagen matrix that supports hepatocyte repopulation (9, 11). Whereas this activated HSC phenotype is relatively short lived in acute injury, it persists during chronic injury and results in unrelenting deposition of large amounts of collagen that is a hallmark of hepatic fibrosis, a serious pathology that compromises normal hepatic structure and function and is a harbinger of cirrhosis, hepatocarcinoma, and end-stage liver disease (10). Transforming growth factor-β (TGF-β) plays a central role in stimulating pathways of fibrogenesis in activated HSC, but it is a challenging therapeutic target because it also regulates critical immune responses and has important tumor-suppressive actions. On the other hand, the fibrogenic properties of TGF-β are mediated via connective tissue growth factor (CCN2, also known as CTGF), a complex matricellular molecule that is produced downstream of TGF-β and directly regulates many of the differentiated functions of activated HSC, including mitogenesis, chemotaxis, adhesion, matrigenesis, and fibrogenesis (15). CCN2 is produced at high levels in activated HSC, whereas its expression in quiescent HSC is substantially suppressed. We recently identified microRNA-214 (miR-214) as a direct negative regulator of CCN2 in primary mouse HSC or the human LX-2 HSC line (4). Via its direct binding of the CCN2 3′ untranslated region (UTR) in quiescent HSC, miR-214 inhibits CCN2 expression in HSC, whereas, in activated HSC, miR-214 expression is suppressed, thereby allowing CCN2 to be expressed. CCN2 and miR-214 are thus dynamically and reciprocally expressed as a function of HSC activation (4).

MiR-214 is located within the intron of the dynamin 3 (DNM3) gene and is encoded with miR-199a, producing a 7.9-kb noncoding DNM3 opposite strand transcript, termed DNM3os (27, 41). Because factors that drive DNM3os transcription will enhance miR-214-dependent suppression of CCN2 expression and thus have potential therapeutic utility, we sought to identify the element(s) in the miR-214 promoter and their associated transcription factor(s) that account for the high levels of miR-214 expression that occur in quiescent HSC. Here we show that an E-box in the miR-214 promoter is a binding site for the basic helix-loop-helix (bHLH) transcription factor, Twist1, which drives miR-214 promoter activity and miR-214 expression, resulting in CCN2 suppression. Functional assays show that Twist1 decreasingly interacts with DNM3os as HSC undergo culture-induced activation, consistent with the finding that HSC demonstrate an activation-dependent suppression of Twist1 expression. Moreover, nanovesicular exosomes secreted by quiescent HSC or hepatocytes contain Twist1, which is intercellularly shuttled to recipient HSC in which the E-box is targeted, resulting in regulation of the miR-214-CCN2 axis. Finally, serum exosomes contain Twist1, miR-214, or CCN2 at levels that reflect their fibrosis-induced changes in the liver, suggesting that the molecular payload in circulating exosomes offers new possibilities in the search for noninvasive biomarkers of liver fibrosis.

Address for reprint requests and other correspondence: D. Brigstock, Rm. WA2011, Research Bldg. 2, Nationwide Children's Hospital, 700 Children's Dr., Columbus, OH 43205 (e-mail: David.Brigstock@NationwideChildrens.Org).

pGL 4.11[Luc2P]-DNM3os promoter-transfected cells was compared with that in mock-transfected cells.

EMSA. 5×10^7 primary mouse HSC (D1) were lysed in EMSA lysis buffer according to the manufacturer's recommendations (Thermo Scientific, Rockford, IL). After centrifugation (10,000 revolution/min, 1 min) of the cell lysate, the pellet (nuclei) was collected, resuspended in extraction buffer, and centrifuged (14,000 revolution/min, 5 min, 4°C), and the supernatant was used for EMSA.

Wild-type or mutant DNM3os promoters were labeled at their 3′ end using biotin (0.5 μM; Thermo Scientific) and terminal deoxynucleotidyltransferase (0.2 U/μl; Thermo Scientific), and purified using chloroform:isoamyl alcohol (1:1). Nuclear protein (1 μg) was incubated for 25 min at room temperature with labeled oligonucleotides (20 fmol/reaction assay) in binding buffer [50% glycerol, 1% NP-40, 100 mM $MgCl_2$, 200 mM EDTA, 1× binding buffer, 1 M KCl, and 1 μg/μl Poly(dI:dC)]. For some groups, *1*) 0.5 μg/μl anti-Twist1 antibody (Abcam) was added for super-shift evaluation, *2*) 4 pmol unlabeled oligonucleotide was used to competitively inhibit formation of shifted complexes, or *3*) nuclear protein was omitted to verify its requirement for complex formation. Samples were mixed with 5× loading buffer (Thermo Scientific) and electrophoresed on a 5% DNA retardation gel in 0.5× TBE buffer. Complexes were transferred and cross linked to a nylon membrane before incubation with streptavidin-horseradish peroxidase conjugate (1:300; Thermo Scientific) at 37°C for 30 min and analysis by chemiluminescence (Thermo Scientific).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed with an EpiTect ChIP kit (Qiagen). Briefly, 2×10^6 HSC were cross linked with 1% formaldehyde for 10 min at room temperature, after which the reaction was terminated with 0.125 M glycine. The cells were then isolated and sonicated on ice to generate DNA shear fragments of ~200-1,000 bp. The lysates were pelleted, precleared, precipitated with 10 μg/ml Twist1 antibody (Millipore, Temecula, CA) or control IgG, and allowed to rotate overnight at 4°C with magnetic protein A beads (Qiagen). The immune complexes were collected and eluted using ChIP-grade proteinase K. The cross links were destroyed by heating the samples at 45°C for 30 min, and the DNA recovered underwent ChIP-PCR using DNM3os primers (see above) according to the manufacturers' instructions.

Analysis of exosomal Twist1. Exosomes were isolated as described from HSC-conditioned medium on *days 3* or *20*. Cryogenic transmission electron microscopy of purified exosomes was performed as described (4). Exosomal Twist1 protein or mRNA were determined by, respectively, Western blot using anti-Twist1 (Abcam) or qRT-PCR, the latter of which was normalized to let-7a, which we determined to be an optimal exosomal housekeeping miR for these studies (data not shown). Exosome uptake in P6 HSC was shown by confocal microscopy of the cells after incubation for 12 h with exosomes isolated from *day 3* HSC and subsequently stained with PKH26.

Exosomes were purified from primary HSC after transfection of *day 6* cells for 48 h with or without 100 nM Twist1 siRNA. Suppressed exosomal Twist1 levels in the exosomes from siRNA-treated cells was confirmed by RT-PCR using exosomal let-7a as a reference control. Exosomal proteins were evaluated by Western blot as described above. Control or Twist1-deficient exosomes were added (3 μg/ml) for 48 h to P4 primary HSC, after which Twist1 levels in the cells were evaluated by RT-PCR or Western blot (see above). Cells were further analyzed by RT-PCR for miR-214, CCN2, α-SMA, or collagen α1(I).

Exosomal regulation of DNM3os was shown by assessment of dual luciferase activity in *day 6* HSC transfected with pGL 4.11[Luc2P]-DNM3os wild-type or mutant plasmids, or vector alone, when cocultured for 24 h with *day 1* HSC or AML12 hepatocytes that had been treated for 24 h with or without 100 μM GW4869, an inhibitor of nSMase2 that is required for biosynthesis of ceramide on which exosome production depends (3, 4).

Circulating exosomes were harvested using PureExo Exosome Isolation Kits (101Bio, Palo Alto, CA) from serum of mice treated for up to 5 wk with CCl_4 as described above. Total RNA from exosomes in 200 μl of serum was prepared using miRNeasy mini kits (Qiagen) as described above. Each reaction was run in triplicate, and all samples were normalized to let-7a.

Statistical analysis. All experiments were performed at least three times with triplicate measurements. For controls, error bars were derived by setting the mean value as 1 and defining variance of replicates from 1. Treatment groups were then expressed as fold of means ± SE. The data from qRT-PCR or luciferase activity assays were analyzed by Student's *t*-test using Sigma plot 12.0 software (SPSS, Chicago, IL), and P values <0.05 were considered statistically significant.

RESULTS

Suppression of Twist1 expression during fibrosing liver injury or during HSC activation in vivo or in vitro. Analysis of total hepatic RNA showed that hepatic Twist1 expression was high in livers recovered from control oil-treated mice but was significantly decreased in livers from CCl_4-treated mice (Fig. 1*A*). This response was associated with suppressed expression of hepatic miR-214 and stimulated expression of CCN2, α-SMA, or collagen α1(I) (Fig. 1*A*). Isolated activated HSC from this 5-wk injury model showed an overall similar expression pattern in that Twist1 or miR-214 were inhibited and CCN2, α-SMA, or collagen α1(I) were enhanced (Fig. 1*A*). Consistent with these findings, Western blot analysis showed that Twist1 protein levels were suppressed in fibrotic livers or in activated HSC recovered from fibrotic livers and that CCN2 protein levels increased under the same conditions (Fig. 1*A*). Immunostaining for Twist1 in liver sections showed that it was present in desmin-positive nonparenchymal cells (presumptive quiescent HSC) in control animals, but, after CCl_4 injury, Twist1 staining was absent from activated HSC, which stained positively for α-SMA as well as desmin (Fig. 1*B*, *top*, arrows). Some parenchymal cells also strongly stained positively for Twist1, but this was only weakly reduced after CCl_4 treatment (Fig. 1*B*, *top*). Nonetheless, because background hepatocyte staining might potentially confound our interpretation of Twist1 staining in HSC, we alternatively isolated HSC from the livers of control or fibrotic animals to verify their Twist1 status in vivo. As assessed by immunostaining, quiescent HSC isolated from control animals were positive for desmin or Twist1 but not for CCN2, α-SMA, or collagen α1(I). In contrast, activated HSC isolated from animals treated with CCl_4 for 5 wk were positive for desmin, CCN2, α-SMA, or collagen α1(I) but not for Twist1 (Fig. 1*B*, *bottom*). Thus, because HSC activation in vivo was associated with the loss of Twist1 mRNA expression or protein production (Fig. 1, *A* and *B*), this phenomenon was the focus of the studies described herein.

In a TAA liver fibrosis model exhibiting enhanced staining in HSC for CCN2, α-SMA, or collagen α1(I), decreased expression of hepatic Twist1 mRNA or miR-214 and increased expression of hepatic CCN2 mRNA were also documented (Fig. 1*C*). Analysis of HSC isolated from normal livers and maintained in vitro showed that there was a large decrease in Twist1 expression between *days 2* and *4* of culture and then a more gradual decline in its expression up to *day 14* of culture as the cells became progressively activated and expressed decreasing levels of miR-214 and