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Downregulation of the Wnt antagonist Dkk2 links the loss of Sept4 and myofibroblastic transformation of hepatic stellate cells

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ABSTRACT

Background/Aims: Sept4, a subunit of the septin cytoskeleton specifically expressed in quiescent hepatic stellate cells (HSCs), is downregulated through transdifferentiation to fibrogenic and contractile myofibroblastic cells. Since *Sept4*$^{-/-}$mice are prone to liver fibrosis, we aimed to identify the unknown molecular network underlying liver fibrosis by probing the association between loss of *Sept4* and accelerated transdifferentiation of HSCs. *Methods:* We compared the transcriptomes of *Sept4*$^{+/+}$ and *Sept4*$^{-/-}$ HSCs undergoing transdifferentiation by DNA microarray and quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. Because *Dickkopf2* (*Dkk2*) gene expression is reduced in *Sept4*$^{-/-}$ HSCs, we tested whether supplementing Dkk2 could suppress myofibroblastic transformation of *Sept4*$^{-/-}$ HSCs. We tested the involvement of the canonical Wnt pathway in this process by using a lymphoid enhancer-binding factor/transcription factor-luciferase reporter assay. *Results:* We observed consistent upregulation of *Dkk2* in primary cultured HSCs and in a carbon tetrachloride liver fibrosis in mice, which was decreased in the absence of *Sept4*. Supplementation with Dkk2 suppressed the induction of pro-fibrotic genes (*α-smooth muscle actin* and 2 collagen genes) and induced an anti-fibrotic gene (*Smad7*) in *Sept4*$^{-/-}$ HSCs. In human liver specimens with inflammation and fibrosis, Dkk2 immunoreactivity appeared to be positively correlated with the degree of fibrotic changes. *Conclusions:* Pro-fibrotic transformation of HSCs through the loss of *Sept4* is, in part, due to reduced expression of *Dkk2* and its homologues, and the resulting disinhibition of the canonical Wnt pathway.

1. Introduction

Chronic liver inflammation caused by viral infections, alcohol and other toxic chemicals, and metabolic disorders is accompanied by tissue remodeling and fibrosis. These interrelated pathological processes are major issues in hepatology because they are implicated in major clinical disorders, namely liver cirrhosis and carcinoma. Previous studies have highlighted hepatic stellate cells (HSCs) as pivotal players contributing to liver fibrosis [1,2]. Upon liver damage, HSCs transdifferentiate into myofibroblastic cells by inflammatory cytokines released mainly from Kupffer cells (KCs). Transformed HSCs are distinct from quiescent HSCs in terms of their proliferative activity, contractility facilitated by *α-smooth muscle actin* (*α-SMA*) expression, and production of collagens and other extracellular matrices. Since these properties contribute to the pathogenesis of liver fibrosis and cirrhosis, the molecular mechanism underlying myofibroblastic transformation of HSCs has been a research focus in this field.

Septins are a family of cytoskeletal/scaffold proteins ubiquitously expressed in eukaryotic cells. Among the 13 septin genes shared by mice and humans, *Sept4* is unique in that it is expressed in specific cells such as Bergmann glial cells in the cerebellum, dopamine neurons in the midbrain, and spermatozoa. Loss of *Sept4* causes disparate phenotypes in these systems [3,4].

Abbreviations: HSCs, hepatic stellate cells; RT-PCR, reverse transcription polymerase chain reaction; α-Sma, α-smooth muscle actin; Dkk, Dickkopf; Fzd, Frizzled; LRP, low density lipoprotein receptor-related protein; Lef/Tcf, lymphoid enhancer-binding factor/transcription factor; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; CCl_4, carbon tetrachloride; DIV, day in vitro; sFRPs, secreted frizzled-related proteins; TGF-β, transforming growth factor β; SOCS7, suppressor of cytokine signaling 7; NCK, non-catalytic region of tyrosine kinase adaptor protein

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We previously reported that *Sept4* is expressed exclusively in quiescent HSCs in the liver, it is strongly downregulated through transdifferentiation of HSCs in vitro, and Sept4 protein is undetectable in HSCs in human specimens with liver fibrosis. Since *Sept4*$^{-/-}$ mice are prone to liver fibrosis, the presence of Sept4 contributes to the suppression of myofibroblastic transformation of HSCs by unknown mechanisms [5]. In this study, we examined the accelerated transdifferentiation of HSCs in *Sept4*$^{-/-}$ mice attempting to identify the unknown molecular network underlying liver fibrosis and the physiological role of Sept4 in HSCs.

By comparing the transcriptomes of HSCs collected from *Sept4*$^{+/+}$ and *Sept4*$^{-/-}$ mice, we found reproducible differences. Here, we focus on *Dickkopf2* (*Dkk2*) and its homologues. We found that *Dkk2* is expressed specifically in HSCs, that it is upregulated through myofibroblastic transformation in vivo and in vitro, and that the *Dkk* upregulation is diminished in *Sept4*$^{-/-}$ HSCs. Previous studies from other groups have shown that the canonical Wnt pathway promotes fibrosis of the liver, kidney, lung, and other tissues [6–9], which is countered by Dkks that interfere with the interaction between Wnt proteins, the cognate membrane-bound receptor Frizzled (Fzd), and LRP5/6 (low-density lipoprotein receptor-related protein 5/6) [10–12]. On the basis of the above information and our own data, we conclude that pro-fibrotic transformation of HSCs in the absence of Sept4 is, at least in part, due to the reduced upregulation of *Dkk* genes and the resulting disinhibition of the canonical Wnt pathway.

2. Materials and methods

2.1. Animals

Characterization of *Sept4*$^{-/-}$ mice with a C57BL/6 J background has been reported previously [3–5]. The protocol for animal handling was reviewed and approved by the Animal Care and Use Committee of Kyoto University.

2.2. Liver cell fractionation and cell culture

We fractionated HSCs from *Sept4*$^{+/+}$ or *Sept4*$^{-/-}$ male mice using a standard method with some modifications [5,13]. In brief, we dispersed liver cells by infusing 0.025% Pronase E (Kaken Pharmaceutical, Tokyo, Japan) and 0.025% collagenase (Wako, Osaka, Japan) in SC-2 solution (137 mM NaCl, 5.4 mM KCl, 0.57 mM NaH_2PO_4, 0.85 mM Na_2HPO_4, 10 mM HEPES, 4.2 mM $NaHCO_3$, and 3.8 mM $CaCl_2$ [pH 7.25]). The collected liver cells were centrifuged on an 8.0% Nycodenz (Nycomed Pharma, Oslo, Norway) cushion. The density-separated HSCs were grown on uncoated dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics in 5% CO_2 at 37 °C. The purity of HSCs was consistently more than 98%, as assessed by the presence of lipid droplets and autofluorescence of vitamin A. We isolated hepatocytes as previously described [14]. In brief, the liver cells dispersed by infusing 0.03% collagenase in SC-2 solution were centrifuged at 50 ×*g* for 1 min. The cell pellet was washed twice by resuspending in GBSS-B solution (137 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, 0.28 mM $MgSO_4$, 0.84 mM NaH_2PO_4, 0.22 mM KH_2PO_4, 5.5 mM glucose, 2.7 mM $NaHCO_3$, and 1.5 mM CaCl [pH 7.25]) and pelleting at 50 ×*g* for 1 min. We grew the fractionated hepatocytes using the same method as for HSCs, except that the dishes were coated with type I collagen.

2.3. Experimental liver fibrosis induced by carbon tetrachloride

As previously reported [5], we injected carbon tetrachloride (CCl_4) (1.0 μl/g body weight, 25% [v/v] in olive oil) or vehicle alone intraperitoneally into 6-week-old, male *Sept4*$^{+/+}$ and *Sept4*$^{-/-}$ mice, biweekly for 10 weeks.

2.4. Total RNA extraction and DNA microarray analysis

We prepared total RNA from HSCs at the 3rd day in vitro (DIV3) using a phenol–chloroform extraction method (TRIzol; Invitrogen, Carlsbad, CA) and monitored the quality of total RNA from the elution profile of 18S and 28S ribosomal RNA [15] using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). We amplified RNA (aRNA) samples using a T7-RNA polymerase system (TargetAmp 1-Round Aminoallyl aRNA amplification kit; Epicentre, Madison, WI) and labeled the aRNA samples using Cy5 Mono-Reactive Dye Pack (GE Healthcare, England). We hybridized DNA microarray chips (3D-Gene Mouse Oligo chip 24 k; Toray, Tokyo, Japan) with Cy-5-labeled aRNA samples at 37 °C for 16 h and then washed the chips thoroughly. We scanned the hybridized chips and acquired chip image data using ProScanArray (Perkin Elmer, Waltham, MA). The scanned image data were processed using Genepix Pro 4.0 software (Molecular Devices, Sunnyvale, CA) to extract gene expression data, which were normalized using MATLAB software (Mathworks, Natick, MA) and a quantile normalization method [16]. For the above procedures, we followed the manufacturers' protocols with some modifications. We have registered the microarray data with NCBI's Gene Expression Omnibus (GEO) database under the accession number GSE24588.

2.5. Quantitative reverse transcription polymerase chain reaction analysis

Total RNA was extracted from liver samples or fractioned liver cells using TRIzol and reverse transcribed into cDNA using Omniscript RT Kit (QIAGEN, Germantown, MD) with random primers (Invitrogen). We conducted quantitative reverse transcription polymerase chain reaction (qRT-PCR) with gene-specific PCR primers (Table 1) and SYBR Green I Master reaction mix on a LightCycler 480 II (Roche Diagnostics, Basel, Switzerland). We quantified the relative abundance of each gene product against *18S* as internal controls with the LightCycler 480 software (ver. 1.5).

2.6. Immunoblot analysis

We homogenized and sonicated cells or tissues in lysis buffer (50 mM Tris–HCl, 2% sodium dodecyl sulfate, and 10% glycerol), and centrifuged the homogenates at 15,000 ×*g* for 10 min. After measuring the protein concentration and adding bromophenol blue (final concentration, 0.1%) and 2-mercaptoethanol (5%), each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene fluoride (PVDF) membrane (Hybond-P, GE Healthcare), and probed with primary antibodies (anti-Dkk2, anti-desmin, anti-FLAG M2; SIGMA-Aldrich, St. Louis, MO; anti-albumin, BETHYL; Montgomery, TX, anti-PECAM-1, anti-β-actin; Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and chemiluminescence reaction

Table 1
Primers used quantitative reverse transcription polymerase chain reaction.

Primer	Forward	Reverse
α-Sma	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
18S	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGGCCTCACTA
Col1α1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
Col3α1	CACCCTTCTTCATCCCACTC	TCTCCAAATGGGATCTCTGG
Dkk1	CTCATCAATTCCAACGCGATCA	GCCCTCATAGAGAACTCCCG
Dkk2	CTGATGCGGGTCAAGGATTCA	CTCCCCTCCTAGAGAGGACTT
Dkk3	CTCGGGGGTATTTTGCTGTGT	TCCTCCTGAGGGTAGTTGAGA
Smad7	GCATCTTCTGTCCCTGCTTC	CCGGTCTTCCTTTCCTTTTC