Short Hairpin RNA Modulates Transforming Growth Factor β Signaling in Life-Threatening Liver Failure in Mice

YOSHIAKI MIZUGUCHI,*,† SHIGEKI YOKOMURO,* TAKUYA MISHIMA,† YASUO ARIMA,* TETSUYA SHIMIZU,* YUTAKA KAWAHIGASHI,† TOMOHIRO KANDA,† HIROSHI YOSHIDA,* TOSHIHIRO TAKIZAWA,† and TAKASHI TAJIRI*

Departments of *Surgery for Organ Function and Biological Regulation, and †Molecular Anatomy and Functional Morphology, Nippon Medical School, Tokyo, Japan

Background & Aims: **Transforming growth factor β (TGF-β) receptor II (TGF-βRII), which is essential for TGF-β signaling and is involved in the causation or participates in the pathway of various human disorders, is consequently considered a key target for therapeutics and analysis of the pathophysiology associated with disruption of the TGF-β system. In the liver, TGF-β plays an essential role in hepatocyte apoptosis, growth inhibition, and progression of fibrogenesis. There is a critical need to introduce technology involving the TGF-β system, such as RNA interference (RNAi), which has high potential for in vivo therapeutics and analytical activities.** ***Methods:*** **Here, we investigated the effect of short hairpin RNA targeting TGF-βRII, using hepatocyte injury in human and mouse cell lines and liver injury mouse models.** ***Results:*** **We demonstrated that short hairpin RNA targeting TGF-βRII can be used to silence TGF-βRII genes in mouse and human cell lines, and physiologic and morphologic changes in hepatocytes suffering from acute injury are spared by RNAi-mediated gene silencing of the target gene and by suppressing downstream signal transduction. Furthermore, short hairpin RNA targeting TGF-βRII protected mice from life-threatening acute liver failure.** ***Conclusions:*** **Our study suggests the potential use of TGF-βRII silencing by RNAi as an analytical tool for TGF-β signaling and gene-specific therapy in human disorders.**

Disruption of transforming growth factor β (TGF-β) signaling underlies many human disorders.[1] TGF-β signaling involves receptor serine/threonine kinases at the cell surface and their substrates, the SMAD proteins, which move into the nucleus, where they activate target gene transcription in association with DNA-binding partners. In the liver, TGF-β plays an essential role in hepatocyte apoptosis, growth inhibition, and progression of fibrogenesis. The pathophysiology underlying acute liver failure (ALF) involves over-efficient apoptosis and inhibition of hepatocyte regeneration associated with TGF-β signaling.[2,3] In the TGF-β-signaling pathway, there is direct evidence that mutation inactivates TGF-β receptor II (TGF-βRII) and causes gastrointestinal cancers and various other carcinomas.[1,4,5] Furthermore, TGF-βRII is considered a key target for interfering with TGF-β signaling.[6] Hence, TGF-βRII is an attractive target for therapeutics and analysis of pathophysiology in many human disorders. RNA interference (RNAi) is a process of sequence-specific posttranscriptional gene silencing initiated by double-stranded RNA.[7] RNAi is recognized both as a powerful tool for mammalian gene function analyses[8] and as having therapeutic potential in the clinical setting.[9] However, the potential of in vivo RNAi for therapeutic and analytic activities in disorders by suppressing elements such as TGF-βRII in the TGF-β signaling pathway has not yet been established or documented. Therefore, a critical requirement for introducing RNAi technology in the TGF-β system is the confirmation of RNAi in targeting the TGF-β gene or genes in the TGF-β-signaling pathway and how it sequentially affects downstream signal transduction, target gene expression, and morphology and physiology. To approach this problem, we investigated the effect of the TGF-βRII short hairpin RNA (shRNA) coding plasmid (shTGF-βRII) using hepatocyte injury in human and mouse cell lines and liver injury mouse models.

Abbreviations used in this paper: ALF, acute liver failure; EGFP, enhanced green fluorescent protein; IFN, interferon; LPS, lipopolysaccharide; rh, recombinant human; RNAi, RNA interference; shNS, nonspecific shRNA coding plasmid; shRNA, short hairpin RNA; shTGF-βRII, TGF-βRII shRNA coding plasmid; TGF-β, transforming growth factor β; TGF-βRI, TGF-β receptor I; TGF-βRII, TGF-β receptor II; TNF-α, tumor necrotic factor α.

0016-5085/05/$30.00
doi:10.1053/j.gastro.2005.08.013

Materials and Methods

Constructing shTGF-βRIIs

To construct vectors that express mouse and human TGF-βRII shRNA, we used the p*Silencer* (Ambion, Austin, TX) plasmid, which contains the human H1 promoter, and the pPUR-tRNA (Clontech, Palo Alto, CA) plasmid, which contains the human $tRNA_{VAL}$ promoter, respectively. Two complementary oligonucleotides encoding mouse TGF-βRII shRNA with a loop motif were synthesized, annealed, and ligated into the linearized p*Silencer* vector between the *Bam*HI and *Hin*dIII linker sequences. Similarly, human shTGF-βRIIs were synthesized between the *Sac*I and *Kpn*I linker sequences of pPUR-tRNA. The coding sequences and secondary structures[10] for mouse and human shTGF-βRIIs can be found in Figures 1 and 2, respectively.

Mice, Culture Cells, and shRNA Coding Plasmid Treatment

The Ethics Review Committee for Animal Experimentation of the Nippon Medical School, Tokyo, Japan, approved our experimental protocols. Male BALB/c mice, aged 6–7 weeks and weighing 20–25 g, were purchased from the Nihon Seibutsuzairyo Centre (Tokyo, Japan). Mouse BNL CL.2 cells were cultured with Dulbecco's modified Eagle medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS). Human HuCC-T1 cells were cultured with RPMI 1640 (Gibco) supplemented with 10% FCS. Plasmids encoding shRNA were delivered into mice using a modified hydrodynamic injection method,[11] in which 100 μg plasmid dissolved in 2.5 mL PBS was injected into mice via a tail vein over 6–8 seconds. Control experiments were performed in vivo using a nonspecific shRNA coding plasmid (shNS) that lacked significant homology to the genome (Ambion), followed by treatment with hepatotoxic reagent or an equal volume of PBS, followed by no treatment with hepatotoxic reagent (vehicle-treated mice). Cell transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were transfected 3 times at 16- to 18-hour intervals. Control experiments in vitro were performed with a GFP shRNA-coding plasmid (Ambion) or shNS followed by treatment with or without hepatotoxic reagent.

Real-Time PCR

Real-time polymerase chain reaction (PCR; ABI Prism 7300, Applied Biosystems, Foster City, CA) was carried out

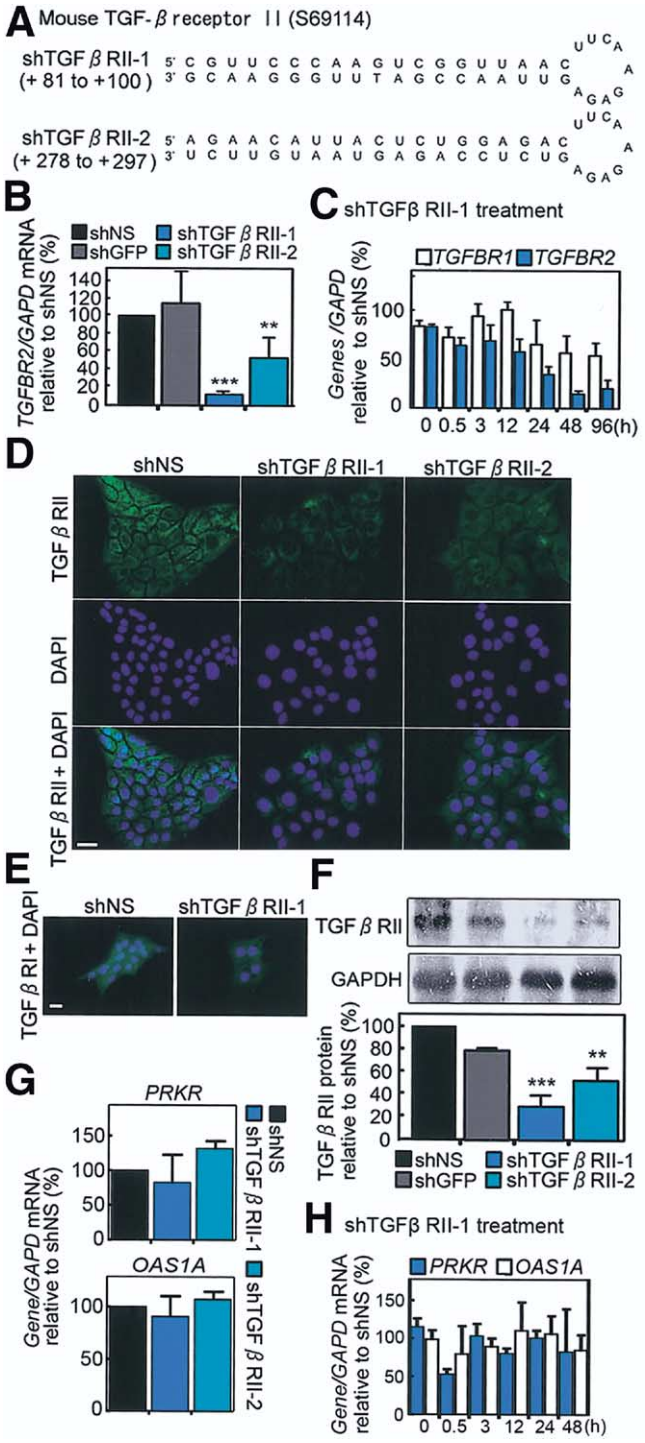

Figure 1. Silencing of mouse TGF-βRII by shTGF-βRII in BNL CL.2 cells. Cells were transfected 3 times with shNS, shGFP, shTGF-βRII-1, or shTGF-βRII-2 (n = 6 per group). Values are given as means ± SD. $^{**}P < .01$; $^{***}P < .001$ compared with shNS (*solid bar*). (*A*) Predicted structures of the coding sequence for shRNAs targeting mouse TGF-βRII with secondary structure prediction. (*B* and *C*) Real-time PCR analysis of the silencing effects on *TGF-βR2/GAPD* (*B*) and *TGF-βR1/GAPD* (*C*) mRNA expression. (*D*) Immunofluorescence staining assessment of the silencing effects on TGF-βRII protein expression. Representative double staining with TGF-βRII-specific antibody (*top*), DAPI staining of DNA (*middle*), and overlaid images with TGF-βRII-specific antibody and DAPI (*bottom*; *scale bar* indicates 50 μm). (*E*) Representative double fluorescence staining (TGF-βRI + DAPI) of cells (*scale bar* indicates 50 μm). (*F*) Western blotting assessment of the silencing effects on TGF-βRII protein expression. Blotting was performed with TGF-βRII- or GAPDH-specific antibodies. (*G* and *H*) Real-time PCR analysis of the effect of shTGF-βRII treatment on mRNA expression by IFN-responsive genes (*PRKR/GAPD* and *OAS1A/GAPD*).