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Inhibition of microRNA-24 increases liver fibrosis by enhanced menin expression in Mdr2$^{-/-}$ mice

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ABSTRACT

Background: Liver transplantation remains the primary treatment for primary sclerosing cholangitis (PSC). Mdr2$^{-/-}$ mice provide a reliable *in vivo* model of PSC and develop characteristic biliary inflammation and fibrosis. We tested the hypothesis that the tumor suppressor protein menin is implicated in the progression of liver fibrosis and that menin expression can be regulated in the liver via microRNA-24 (miR-24).

Materials and methods: Menin expression was measured in human PSC and Mdr2$^{-/-}$ mice. Twelve-week-old FVB/NJ wild-type (WT) and Mdr2$^{-/-}$ mice were treated with miR-24 Vivo-Morpholino to knockdown miR-24 expression levels. Liver fibrosis was evaluated by Sirius Red staining and quantitative polymerase chain reaction (qPCR) for genes associated with liver fibrosis, such as fibronectin 1, collagen type 1 alpha 1, transforming growth factor-β1 (TGF-β1), and α-smooth muscle actin. Studies were also performed *in vitro* using immortalized murine cholangiocyte lines treated with miR-24 hairpin inhibitor and mimic.

Results: Menin gene expression was increased in Mdr2$^{-/-}$ mice and late-stage human PSC samples. Treatment of FVB/NJ WT and Mdr2$^{-/-}$ mice with miR-24 Vivo-Morpholino increased menin expression, which correlated with increased expression of fibrosis genes. *In vitro*, inhibition of miR-24 also significantly increased the expression of fibrosis genes.

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Conclusions: Inhibition of miR-24 increases menin and TGF-β1 expression, subsequently increasing hepatic fibrosis in FVB/NJ WT and Mdr2$^{-/-}$ mice. Modulation of the menin/miR-24 axis may provide novel targeted therapies to slow the progression of hepatic fibrosis into cirrhosis in PSC patients by altering TGF-β1 expression.

Introduction

Cholangiocytes represent 3%-5% of nucleated cells within the liver and are the targets of cholangiopathies, such as primary sclerosing cholangitis (PSC).[1,2] These cholangiopathies are characterized by the classical findings of cholestatic liver injury: increased intrahepatic bile duct mass, polymorphonuclear leukocytes, and the deposition of extracellular matrix that lead to portal fibrosis and biliary cirrhosis.[3] PSC in particular is characterized by chronic inflammation and obliterative fibrosis of the intrahepatic and/or extrahepatic biliary tree.[4] This results in bile stasis and hepatic fibrosis that will progress to cirrhosis and the need for liver transplantation.[4] PSC is also associated with a 5%-10% lifetime risk for the development of cholangiocarcinoma, 160-fold higher than the general population.[5] Currently, there are no medical therapies that have been proven to alter the natural course of PSC, and liver transplantation before the onset of end-stage liver disease remains the recommended treatment strategy.[5,6] Improved understanding of the cellular mechanisms that lead to biliary proliferation and portal fibrosis is needed to develop novel therapeutic strategies to diminish disease progression.

Menin is the protein product of the *MEN1* gene, a tumor suppressor gene located on chromosome 11q13.1.[7] It is a 67 kDa nuclear protein that is ubiquitously expressed in all tissues and evolutionarily conserved, but shares little sequence homology with other proteins.[8] Several studies suggest that menin acts as a scaffold protein involved in diverse cell functions including binding and regulating transcription factor activity,[9] modifying histone proteins and chromatin structure,[10,11] and DNA repair.[12,13] Germline mutations in the *MEN1* gene cause the MEN1 syndrome, a neuroendocrine tumor syndrome that predisposes patients to neoplasms of the parathyroid glands, pancreas, and the pituitary gland.[7] In the setting of cholestatic liver injury, cholangiocytes represent a neuroendocrine cell population within the liver that respond to a variety of hormones, neurotransmitters, and growth factors that have been shown to regulate cholangiocyte proliferation and the ductular reaction associated with hepatic fibrosis.[14,15] Because of its implications in neuroendocrine signaling, we hypothesized that *MEN1* gene expression may play an important role in the progression of hepatic fibrosis.

MicroRNA-24 (miR-24) has previously been shown to bind to the 3' untranslated region (UTR) of the *MEN1* gene and regulate menin expression through a negative feedback loop in parathyroid and pancreatic tissues.[16,17] In addition, menin has been shown to interact with SMAD3 to block transforming growth factor-β1 (TGF-β1) signaling.[18] SMAD3 phosphorylation and TGF-β1 have previously been shown to contribute to hepatic fibrosis[19]; however, the role of menin in this pathway is unknown.

The multidrug resistance gene-2 knockout mouse (Mdr2$^{-/-}$) is a widely used murine model of cholestatic liver disease characterized by the development of PSC with features of biliary proliferation and portal fibrosis.[14,19,20] Mdr2$^{-/-}$ mice are deficient in a canalicular phospholipid flippase and develop liver injury due to the absence of phospholipids in bile.[21] The bile ducts of these mice are characterized by tight junction and basement membrane destruction, which creates widened intracellular spaces between biliary endothelial cells and results in bile acid leakage, periductular inflammation, and fibrosis.[22] Studies in these mice from 2 wk to 12 mo of age have shown that they develop chemical and histologic evidence of endothelial disruption, hepatic inflammation, and fibrosis.[22,23] Mdr2$^{-/-}$ mice also develop hepatic malignancies with nearly 100% incidence by 16 mo of age.[24] Unlike PSC, however, these tumors resemble hepatocellular carcinoma (HCC) rather than a primary biliary malignancy, such as cholangiocarcinoma. Using this model, we hypothesized that the miR-24/menin regulatory feedback loop could be manipulated to alter the progression of hepatic fibrosis.

Materials and methods

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Cell culture reagents and media components were purchased from Invitrogen Corporation (Carlsbad, CA). Total RNA and miRNA were isolated from cells and liver tissue using the mirVana miRNA isolation kit from ThermoFisher Scientific (Waltham, MA). Complementary DNA was generated from 1200 μg of total RNA using iScript Reverse Transcription Supermix for *q*PCR (Bio Rad, Hercules, CA). Primers for *q*PCR were purchased from Qiagen (Valencia, CA) unless otherwise indicated. The *q*PCR experiments were performed using SYBR Green PCR Master Mix from SABiosciences on the Agilent Technologies Mx3005P qPCR system.

MEN1 gene expression was quantified by *q*PCR using RNA isolated from immortalized murine cholangiocyte lines (IMCLs), mouse and human liver tissues. Liver fibrosis was evaluated by *q*PCR using mouse primers for fibronectin 1 (FN1), collagen type 1 alpha 1 (COL1α1), TGF-β1, and α-smooth muscle actin (α-SMA). Proliferation was evaluated by *q*PCR using mouse primers for Ki-67. Glyceraldehyde-3-phosphate dehydrogenase gene expression was used as a relative control. Data are expressed as relative messenger RNA levels ± standard error of the mean (SEM).

In vitro *studies*

In vitro studies were performed using our IMCLs.[19,25] Cell were cultured under standard conditions and treated with 75 nM of mirVana miR-24 inhibitor, mimic, or the standard control for 24 h according to the manufacturer's protocol. Cells were collected after treatment using TrypLE solution (Gibco) and