Inhibition of SIRT2 suppresses hepatic fibrosis

Maribel Arteaga, Na Shang, Xianzhong Ding, Sherri Yong, Scott J. Cotler, Mitchell F. Denning, Takashi Shimamura, Peter Breslin, Bernhard Löscher, and Wei Qiu

1Departments of Surgery, 2Medicine, 3Pathology, 4Pharmacology, and 5Molecular/Cellular Physiology, Oncology Institute, Loyola University Chicago, Maywood, Illinois; and 6Institute of Biochemistry and Molecular Biology, RWTH Aachen University, Aachen, Germany

Submitted 12 August 2015; accepted in final form 20 April 2016

Liver fibrosis is a reversible scarring process that represents the liver’s response to chronic injury, with the accumulation of extracellular matrix (ECM) and collagen proteins (2, 8). Over time this process can result in cirrhosis of the liver, characterized by nodules formation and organ contraction (16, 31). Once cirrhosis has developed, serious complications of liver disease may occur, including portal hypertension, liver failure, and liver cancer (4, 9). The main causes of liver fibrosis include chronic HBV or HCV infection, alcohol abuse, and nonalcoholic steatohepatitis (16). There are currently no drugs available to reverse liver fibrosis or cirrhosis (20). The underlying pathogenic mechanisms of liver fibrosis are not fully known.

Hepatic stellate cells (HSCs) are the key cellular source for the genesis of ECM and hepatic fibrosis (16). Following acute or chronic liver injury, HSCs are activated and become myofibroblast-like cells (1). HSC activation can be divided into two main phases: initiation and perpetuation. The initiation phase of HSC activation refers to early changes in gene expression and phenotype, which largely result from paracrine stimulation by neighboring cells following liver injury (16). Continuous exposure to paracrine and autocrine cytokines, such as TGFβ and PDGF, causes perpetuation of HSCs and leads to increased ECM production (8). Understanding the molecular mechanisms behind HSC activation is crucial to the development of antifibrotic treatments since disrupting the key molecular pathways and genes involved in this process could inhibit fibrogenesis and allow for remodeling and regression of fibrosis and cirrhosis.

Acetylation and deacetylation have been suggested to play a role in hepatic fibrogenesis (37). Histone deacetylase (HDAC) inhibitors have been shown to have promising antifibrotic effects (37). However, the molecular mechanisms by which acetylases or deacetylases regulate hepatic fibrogenesis have remained elusive to this point. HDACs are grouped into four classes and two families: the “classical” and the silent information regulator2 (Sir2)-related protein (sirtuin) families. In mammals, sirtuins constitute a family consisting of seven genes (Sir1–Sir7). Sir1, Sir6, and Sir7 primarily localize to the nucleus, whereas Sir3, Sir4, and Sir5 are present in mitochondria (29). Sir2 is predominantly localized within the cytoplasm during most of the cell cycle. However, during the G2/M transition and in mitosis it is found in the nucleus, in complex with chromatin, contributes to gene deacetylation, and regulates gene transcription (18, 22, 38). Emerging evidence suggests that the classical HDACs, such as HDAC1 and HDAC4 (7, 26), are involved in liver fibrosis, but the role of sirtuin proteins in hepatic fibrosis remains unknown.

In this study, we found that inhibition of SIRT2 by pharmacologic inhibitors or shRNAs significantly suppressed the expression of type I collagen (COL1A1) and α-smooth muscle actin (α-SMA), markers for stellate cell activation, in primary HSCs and HSC cell lines. We further demonstrated that SIRT2 regulates the ERK/c-MYC pathway, which is critical for expression of COL1A1 and α-SMA. In addition, we found that Sirt2-knockout (KO) mice were less susceptible to the development of hepatic fibrosis induced by carbon tetrachloride (CCL4) or thioacetamide (TAA). Furthermore, we showed that SIRT2, p-ERK, and c-MYC proteins are all overexpressed in human hepatic fibrotic tissues. These data suggest a critical role for the SIRT2/ERK/c-MYC axis in promoting hepatic fibrogenesis. Inhibition of the SIRT2/ERK/c-MYC axis represents a novel strategy to prevent and to potentially treat liver fibrosis and cirrhosis.

LIVER FIBROSIS is a reversible scarring process that represents the liver’s response to chronic injury, with the accumulation of extracellular matrix (ECM) and collagen proteins (2, 8). Over time this process can result in cirrhosis of the liver, characterized by nodules formation and organ contraction (16, 31). Once cirrhosis has developed, serious complications of liver disease may occur, including portal hypertension, liver failure, and liver cancer (4, 9). The main causes of liver fibrosis include chronic HBV or HCV infection, alcohol abuse, and nonalcoholic steatohepatitis (16). There are currently no drugs available to reverse liver fibrosis or cirrhosis (20). The underlying pathogenic mechanisms of liver fibrosis are not fully known.

Hepatic stellate cells (HSCs) are the key cellular source for the genesis of ECM and hepatic fibrosis (16). Following acute or chronic liver injury, HSCs are activated and become myofibroblast-like cells (1). HSC activation can be divided into two main phases: initiation and perpetuation. The initiation phase of HSC activation refers to early changes in gene expression and phenotype, which largely result from paracrine stimulation by neighboring cells following liver injury (16). Continuous exposure to paracrine and autocrine cytokines, such as TGFβ and PDGF, causes perpetuation of HSCs and leads to increased ECM production (8). Understanding the molecular mechanisms behind HSC activation is crucial to the development of antifibrotic treatments since disrupting the key molecular pathways and genes involved in this process could inhibit fibrogenesis and allow for remodeling and regression of fibrosis and cirrhosis.

Acetylation and deacetylation have been suggested to play a role in hepatic fibrogenesis (37). Histone deacetylase (HDAC) inhibitors have been shown to have promising antifibrotic effects (37). However, the molecular mechanisms by which acetylases or deacetylases regulate hepatic fibrogenesis have remained elusive to this point. HDACs are grouped into four classes and two families: the “classical” and the silent information regulator2 (Sir2)-related protein (sirtuin) families. In mammals, sirtuins constitute a family consisting of seven genes (Sir1–Sir7). Sir1, Sir6, and Sir7 primarily localize to the nucleus, whereas Sir3, Sir4, and Sir5 are present in mitochondria (29). Sir2 is predominantly localized within the cytoplasm during most of the cell cycle. However, during the G2/M transition and in mitosis it is found in the nucleus, in complex with chromatin, contributes to gene deacetylation, and regulates gene transcription (18, 22, 38). Emerging evidence suggests that the classical HDACs, such as HDAC1 and HDAC4 (7, 26), are involved in liver fibrosis, but the role of sirtuin proteins in hepatic fibrosis remains unknown.

In this study, we found that inhibition of SIRT2 by pharmacologic inhibitors or shRNAs significantly suppressed the expression of type I collagen (COL1A1) and α-smooth muscle actin (α-SMA), markers for stellate cell activation, in primary HSCs and HSC cell lines. We further demonstrated that SIRT2 regulates the ERK/c-MYC pathway, which is critical for expression of COL1A1 and α-SMA. In addition, we found that Sirt2-knockout (KO) mice were less susceptible to the development of hepatic fibrosis induced by carbon tetrachloride (CCL4) or thioacetamide (TAA). Furthermore, we showed that SIRT2, p-ERK, and c-MYC proteins are all overexpressed in human hepatic fibrotic tissues. These data suggest a critical role for the SIRT2/ERK/c-MYC axis in promoting hepatic fibrogenesis.

EXPERIMENTAL PROCEDURES

Cells and treatments. The human immortalized stellate cell line LX2 (40) and the mouse immortalized stellate cell line JS1 (10) were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum. Mouse HSCs were isolated from C57BL/6 wild-type (WT), and Sirt2-KO mice by enzymatic digestion and Percoll density gradient centrifugation, with modifications (3, 11).
Cells were plated in six-well plates at 30–40% confluence 24 h prior to treatment with 10 μM AGK2 (R&D Systems, Minneapolis, MN) or 0.8 μM AC-93253 (Santa Cruz Biotechnology, Dallas, TX) for either 24 or 48 h. Cell viability was measured by trypan blue exclusion assay by using at least 400 cells per group. Total cell lysates were prepared for real-time PCR or Western blotting. Each experiment was repeated at least three times.

For Sir2 or c-Myc shRNA experiments, cells were infected with lentiviral pLKO.1 puro particles which contain Sir2, c-Myc, or scrambled shRNAs and selected with 0.5 μg/ml puromycin for 5 days. Lentiviral pLKO.1 puro particles against human Sir2, c-Myc shRNAs (Table 1), and control scrambled shRNA (Sigma-Aldrich, St. Louis, MO) were packaged with pCMV-DR8.2 dvpr (Addgene, Cambridge, MA) and pCMV-VSVG (Addgene). Each experiment was repeated at least three times.

For c-MYC transduction experiments, LX2 cells were infected with c-MycER retrovector particles or control LZRS-Linker viral particles; c-MYC was subsequently activated by treatment with 100 nM 4-hydroxytamoxifen for 48 h. Total cell lysates were collected 48 h after treatment with 4-hydroxytamoxifen and used for Western blotting. The c-MycER-expressing retrovector was generated by inserting the EcoRI fragment from pBabe-Puro-MycER [a kind gift from Dr. Gerard Evan (17)] into EcoRI sites of the LZRS-Linker vector. Each experiment was repeated at least three times.

For MEKDD or SIRT2 transduction experiments, LX2 cells were infected with MEKDD or SIRT2 lentiviral particles or control viral particles and selected with 9 μg/ml blasticidin for 3 days. Total cell lysates were collected 48 h after selection and used for Western blotting. The MEKDD- or SIRT2-expressing lentivector particle was generated by Gateway cloning technology using the pDONOR223-MEKDD vector (No. 31202, Addgene) or the pDONOR223-SIRT2 vector (24). Total cell lysates were collected 48–72 h after transfection and used for Western blotting. Each experiment was repeated at least three times.

Animals. All animals received appropriate humane care according to the “Guide for the Care and Use of Laboratory Animals” (http://oacu.od.nih.gov/ac_cbt/guide3.htm). The procedures for all animal experiments were approved by the Institutional Animal Care and Use Committee of Loyola University Chicago. WT (No. 000664) and Sir2-KO (No. 012772) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Sir2-KO mice were backcrossed to C57BL/6 mice for two generations (F2). The Sir2+/− mice were then generated by crossing Sir2-KO (F2) and C57BL/6 mice. The littermates of WT and Sir2+/- mice generated by crossing of Sir2+/− mice were used to ensure the same genetic background. Detailed information on the genotyping of mice can be found at the JAX website. All mice were housed in microisolator cages in a room illuminated from 7:00 AM to 7:00 PM (12:12-h light-dark cycle), and were allowed access to water and chow ad libitum.

Human tissue samples. Thirty formalin-fixed human liver tissue specimens from patients with nonalcoholic fatty liver disease (NAFLD) were obtained from the Department of Pathology of Loyola University Medical Center; these consisted of 15 with advanced fibrosis (F3-F4) and 15 with no fibrosis. The human tissue study protocol was approved by the Institutional Review Board of Loyola University Medical Center. Seven males and eight females ages 41–68 were included in the advanced fibrosis group, while seven males and eight females were included in the nonfibrotic group (age range 25–65 years old).

Toxin-induced models of acute and chronic liver injury and fibrosis. CCl4 (Sigma-Aldrich) and thioacetamide (TAA) (Sigma-Aldrich) were used to induce hepatic fibrosis. For CCl4-induced injury studies, 13 WT [8 littermates generated from heterozygous mice (C57BL/6 background) and 5 C57.129 mixed background] and 13 Sir2-KO mice [8 littermates generated from heterozygous mice (C57BL/6 background) and 5 C57.129 mixed background] received three intraperitoneal injections of 30% CCl4 (diluted in corn oil) at a dose of 1 ml/kg body wt or corn oil only three times weekly for 5 wk. For TAA-induced fibrosis studies, TAA (Deltas, 300 mg/kg) buffered saline) or vehicle solution was administered to eight WT [littermates generated from heterozygous mice (C57BL/6 background)] and eight Sir2-KO mice [littermates generated from heterozygous mice (C57BL/6 background)] in three intraperitoneal injections (100 mg/kg body wt) every other day for 1 wk or 6 wk. Animals were killed 48 h after the last dose. All of the experiments were performed on male mice 3–4 mo of age.

Alanine aminotransferase measurement. Venous blood was taken from the tail veins of 16 WT and 16 Sir2-KO littermates generated from heterozygous mice (C57BL/6 background) treated with vehicle, CCl4, or TAA for 5 wk. Blood was kept at 4°C overnight and centrifuged at 200 g for 20 min. to isolate serum. Alanine aminotransferase (ALT) levels were measured with the Infinity ALT kit (Thermo Scientific, Middletown, VA) as previously described (27).

Hydroxyproline level measurement. The total hydroxyproline content in the liver was determined as described previously (28). A commercially available hydroxyproline detection kit was purchased from Sigma-Aldrich and the hydroxyproline levels of livers from eight WT [littermates generated from heterozygous mice (C57BL/6 background)] and eight Sir2-KO mice [littermates generated from heterozygous mice (C57BL/6 background)] treated with vehicle, CCl4, or TAA were measured according to the manufacturer’s instructions.

Table 1. Sequences of shRNAs

| Sequences of shRNAs | CCGGCCCTGTGGCTAAAGCTTACATACTGGAATAGTTTCTACCTAACGCAAGGTTTTG | CCGGGCTACGTGGGATAGCTGCTGAACTTGCAAGGTTTTG | CCGGGCATGTCAGACAACTCTGAGGTTTCAAGGTTTTG | CCGGGCATGTCAGACAACTCTGAGGTTTCAAGGTTTTG |

Table 2. Sequences of RT-PCR primers

| Sequences of RT-PCR Primers | Sequences of qPCR Primers | Sequences of qPCR Primers | Sequences of qPCR Primers | Sequences of qPCR Primers |

--

Human/mus α-SMA-F 5′-GCGCGTGTGAGATAACTCGATGTC-3′
Human/mus α-SMA-R 5′-GTCACACGTATGGATACGAGGAA3′
Human Cola1-F 5′-AGGGCCGCAAGGAGGAGAC-3′
Human Cola1-R 5′-CGATACGTCATGGACACAGA-3′
Human/mus MMP2-F 5′-GATTCCGGCCCTGGTTGTA-3′
Human/mus MMP2-R 5′-GATTCCGGCCCTGGTTGTA-3′
Human c-Myc-F 5′-GGCCGGTCGGAAAGGTCTCA-3′
Human c-Myc-R 5′-CTGGTATGTTGGCTGATGT-3′
Human/mus GAPDH-F 5′-CTGTGCAAAGCTGTGGCTGTA-3′
Human/mus GAPDH-R 5′-CTGTGCAAAGCTGTGGCTGTA-3′
Human Sir2-F 5′-GCGGCACTTTTCCCTGCAAGA-3′
Human Sir2-R 5′-GCGGCACTTTTCCCTGCAAGA-3′
Mus Cola1-F 5′-GCTCTTCTTGAGGCACC-3′
Mus Cola1-R 5′-GCTCTTCTTGAGGCACC-3′
Mus Timp1-F 5′-GCAACTCTGAGCTGATTA-3′
Mus Timp1-R 5′-GCAACTCTGAGCTGATTA-3′
Mus Timp2-F 5′-TCAAGACCCAAAGCTGATGGC-3′
Mus Timp2-R 5′-TCAAGACCCAAAGCTGATGGC-3′

--
Grading of histological inflammation. The degree of histological inflammation for each biopsy site was blindly scored by a pathologist from Loyola University Chicago based on previously described criteria (12): (0 au) none; (1 au) mild, some or all portal areas; (2 au) moderate, some or all portal areas; (3 au) moderate/marked, all portal areas; (4 au) marked, all portal areas.

Enzyme-linked immunosorbency assay. Total protein was prepared from freshly isolated liver tissue. Approximately 300 mg of liver tissue was minced and homogenized in 1 ml RIPA buffer. Extracts were centrifuged at 1,000 g for 10 min, and the supernatants were collected and analyzed using the mouse inflammatory cytokines mutianalyte ELISAarray kit (Qiagen, Valencia, CA).

Western blotting. Western blotting was performed as previously described (27). Primary antibodies, including those for SIRT2, c-MYC, p-AKT (ser473), AKT, p-ERK, ERK, p-NF-κB, NF-κB, p-FOXO1, and FOXO1, were purchased from Cell Signaling (Danvers,

Fig. 1. SIRT2 inhibitors decrease fibrogenic gene expression in HSC cell lines. A: expression of α-SMA, COLA1, and GAPDH proteins in LX2 cells treated with vehicle, 10 μM AGK2, or 0.8 μM AC-93253 for 24 h. B: mRNA expression levels of α-SMA and COLA1 in LX2 cells treated with vehicle or 10 μM AGK2 for 24 or 48 h. C: mRNA expression levels of α-SMA and COLA1 in LX2 cells treated with vehicle or 0.8 μM AC-93253 for 24 or 48 h. D: representative photomicrographs of LX2 cells treated with vehicle, 10 μM AGK2, or 0.8 μM AC-93253 for 0 or 48 h. E: expression of α-SMA and GAPDH proteins in JS1 cells treated with vehicle or 10 μM AGK2 for 24 or 48 h. F: mRNA expression levels of α-Sma and Cola1 in JS1 cells treated with vehicle, 10 μM, or 20 μM AGK2 for 24 h.
Fig. 2. Knockdown or deletion of SIRT2 decreases fibrogenic gene expression in human HSCs and mouse primary HSCs. A: expression of SIRT2, α-SMA, COLA1, and GAPDH proteins in LX2 cells infected with scrambled shRNA, SIRT2 shRNA#1, or SIRT2 shRNA#2 lentiviral particles and selected by puromycin for 5 days. B: mRNA expression levels of SIRT2, α-SMA, COLA1, and MMP-2 in LX2 cells infected with scrambled shRNA, SIRT2 shRNA#1, or SIRT2 shRNA#2 lentiviral particles and selected by puromycin for 5 days. C: mRNA expression levels of α-Sma and Cola1 in mouse WT and Sirt2-KO HSCs, which were isolated and cultured for 0, 1, or 4 days after treatment. D: representative photomicrographs of mouse WT and Sirt2-KO HSCs following culturing for 4 days. E: mRNA and protein expression levels of Sirt2 in mouse WT HSCs, which were isolated and cultured for 0, 1, or 4 days after treatment. F: mRNA expression levels of α-Sma and Cola1 in mouse SIRT2-KO (on C57.129 mixed background) HSCs which were infected with control PLX304 or PLX304-SIRT2 lentiviral particles and cultured for 2 days.
MA). α-SMA antibody was purchased from Dako (Carpinteria, CA). COLA1 antibody was purchased from Rockland Immunochemicals (Limerick, PA). GAPDH antibody was purchased from Sigma-Aldrich.

**Immunohistochemical and immunofluorescence staining.** Formalin-fixed, paraffin-embedded human liver tissue samples were sectioned into 5-μm sections and processed routinely for hematoxylin and eosin staining. Sirius red [Direct Red 80 (VWR, Radnor, PA)]

---

**Fig. 3.** Inhibition of SIRT2 decreases fibrogenic gene expression through regulation of c-MYC in HSCs. A: expression of p-AKT, AKT, p-NF-κB, NF-κB, p-FOXO1, FOXO1, and GAPDH proteins in LX2 cells treated with vehicle or 10 μM AGK2 for 24 h. B: expression of c-MYC and GAPDH proteins in LX2 cells treated with vehicle, 10 μM AGK2, or 0.8 μM AC93253 for 24 or 48 h. C: expression of c-MYC and GAPDH proteins in LX2 cells infected with scrambled shRNA, SIRT2 shRNA#1, or SIRT2 shRNA#2 lentiviral particles and selected with puromycin for 5 days. D: expression of α-SMA and GAPDH proteins in mouse WT and Sirt2-KO HSCs, which were isolated and cultured for 0 or 4 days. E: expression of SIRT2, c-MYC, COLA1, α-SMA, and GAPDH proteins in LX2 cells infected with control PLX304 or PLX304-SIRT2 lentiviral particles and selected with puromycin for 5 days. F: expression of α-SMA, c-MYC, and GAPDH proteins in LX2 cells infected with scrambled shRNA, c-MYC shRNA#1, or c-MYC shRNA#2 lentiviral particles and selected with puromycin for 5 days. G: mRNA expression levels of c-MYC, α-SMA, COLA1, and MMP-2 in LX2 cells infected with scrambled shRNA, c-MYC shRNA#1, or c-MYC shRNA#2 lentiviral particles and selected with puromycin for 5 days. H: expression of α-SMA, COLA1, and GAPDH proteins in LX2 cells infected with scrambled shRNA, SIRT2 shRNA#1, or SIRT2 shRNA#2 lentiviral particles and c-MycER retroviral particles or control viral particles followed by treatment with 100 nM 4-hydroxytamoxifen for 48 h.
and/or Masson’s trichrome staining (VWR) was used to determine collagen deposition. A pathologist blinded to the treatment groups scored 10 random areas per slide for fibrosis. Immunohistochemical staining of α-SMA was performed with monoclonal antibody (Dako). Immunofluorescence staining of SIRT2, c-MYC, and p-ERK was performed as previously described (27).

Quantitative real-time polymerase chain reaction. Cellular mRNA was extracted using Zymo mini-columns (Zymo Research, Irvine, CA). Quantitative real-time polymerase chain reaction was performed as previously described (34). Primers used for RT-PCR are listed in Table 2.

Statistical analysis. Statistical analyses were carried out with GraphPad Prism V software. Data are presented as means ± SD.
Inhibition of SIRT2 suppresses fibrogenic gene expression in human and mouse HSCs. To determine the role of SIRT2 in hepatic fibrogenesis, we first treated human hepatic stellate L ox cells (40) with two specific SIRT2 inhibitors, AGK2 (23) and AC-93253 (18). We found that 10 μM AGK2 and 0.8 μM AC-93253, doses which have been shown to effectively inhibit the deacetylase activity of SIRT2 (18, 23), significantly decreased the expression of fibrogenic genes, including α-smooth muscle actin (α-SMA) and α-1 type I collagen (COLA1) (Fig. 1A). In addition, we discovered that mRNA levels of α-SMA and COLA1 were similarly decreased by treatment with either AGK2 or AC-93253 (Fig. 1, B and C), suggesting that inhibition of SIRT2 may suppress fibrogenic gene expression through transcriptional regulation. Notably, SIRT2 inhibitors did not significantly affect LX2 cell viability (vehicle/AGK2/AC-93253: 98.4% ± 2.5/96.2 ± 4.3/95.6 ± 3.8) or morphology (Fig. 1D). Furthermore, inhibition of SIRT2 by AGK2 decreased the expression of α-SMA and COLA1 in JS1 cells (10), a mouse stellate cell line (Fig. 1, E and F). These data indicate that Sirt2 inhibitors suppress fibrogenesis in human and mouse stellate cells.

To further address the specificity of inhibition of SIRT2 by its inhibitors, we knocked down SIRT2 with two shRNAs in LX2 cells. These two SIRT2 shRNAs significantly reduced SIRT2 mRNA and protein expression (Fig. 2, A and B) but not mRNA expression of other siRNAs (data not shown), indicating the specificity of the SIRT2 shRNAs. Knockdown of SIRT2 significantly reduced protein and/or mRNA expression of α-SMA, COLA1, and MMP-2 (Fig. 2, A and B). Furthermore, to investigate the effect of SIRT2 on primary stellate cell activation, we isolated primary HSCs from WT and Sirt2-KO mice. We found that Sirt2 deficiency significantly attenuated the increased mRNA expression of α-SMA and Cola1 in culture-activated HSCs (Fig. 2C), although Sirt2 deficiency does not significantly affect HSC viability (WT: 98.8 ± 0.73 vs. Sirt2 KO: 97.6 ± 0.82) or morphology (Fig. 2D). Interestingly, we also found that the expression of Sirt2 mRNA was not changed but the SIRT2 protein level in primary HSCs was increased during culturing (Fig. 2E). In addition, overexpression of SIRT2 increased mRNA expression of α-SMA and Cola1 in cultured Sirt2-KO HSCs (Fig. 2F). Overall, these results suggest that inhibition of SIRT2 suppresses HSC activation and fibrogenesis.

Inhibition of SIRT2 decreases fibrogenic gene expression through the regulation of c-MYC in HSCs. To investigate the underlying mechanisms by which inhibition of SIRT2 suppresses HSC activation and fibrogenesis, we examined the effect of SIRT2 inhibitors on the expression of potential downstream targets of SIRT2. A number of Sirt2 targets have been identified, including AKT (6), NF-κB (30), FOXO1 (13), and c-MYC (18). We found that AGK2 did not alter the expression or phosphorylation of AKT, NF-κB, or FOXO1 after 24 h of treatment (Fig. 3A). However, c-MYC expression was significantly decreased by treatment with either AGK2 or AC-93253 (Fig. 3B), indicating that SIRT2 exerts some regulatory function on c-MYC in HSCs. Furthermore, Sirt2 shRNAs decreased the expression of c-MYC protein in LX2 cells (Fig. 3C). Genetic deletion of Sirt2 also attenuated the increased expression of c-MYC protein in culture-activated HSCs (Fig. 3D). In addition, overexpression of SIRT2 increased the expression of c-MYC, α-SMA, and COLA1 in LX2 cells (Fig. 3E). These data indicate that SIRT2 is required for the expression of c-MYC in activated HSCs.

To determine whether SIRT2 regulates fibrogenic gene expression through c-MYC, we first knocked down c-MYC in HSCs using shRNAs and examined the subsequent expression of α-SMA and COLA1. We found that knockdown of c-MYC significantly decreases levels of α-SMA protein in LX2 cells (Fig. 3F). In addition, levels of α-SMA, COLA1, and MMP-2 mRNA were also decreased by c-Myc shRNAs (Fig. 3G), suggesting that transcription of these fibrogenic genes is at least partially regulated by c-MYC. Furthermore, we found that overexpression of activated c-Myc (c-MycERTM) enhanced the expression of α-SMA and COLA1 in HSCs (Fig. 3H), confirming that c-MYC indeed regulates the expression of these fibrogenic genes. We next examined whether overexpression of activated c-MYC can reverse the suppression of hepatic fibrogenesis resulting from the inhibition of SIRT2. Indeed we found that activation of c-MycERTM enhanced the expression of α-SMA and COLA1 and rescued the inhibition of α-SMA and COLA1 in SIRT2 knockdown (KD) cells (Fig. 3I). These data indicate that inhibition of SIRT2 decreases hepatic fibrogenesis through regulation of c-MYC in HSCs.

SIRT2 stabilizes c-MYC by activating ERK in HSCs. Sirt2 has been shown to stabilize N-MYC and c-MYC by directly binding to the promoter of the NEDD4 gene, the protein product of which is a ubiquitin-protein ligase, repressing

Statistical significance was calculated by the Student’s t-test, P < 0.05 was considered to be significant. Means ± SD are shown in the figures where applicable.
NEDD4 expression; NEDD4 directly binds to MYC and targets MYC for ubiquitination and degradation in neuroblastoma cells (18). We therefore examined whether inhibition of SIRT2 decreases c-MYC expression through NEDD4 in HSCs. We found that c-MYC mRNA levels were not decreased by SIRT2 inhibitors nor by deficiency in HSCs (Fig. 4A), suggesting that inhibition of SIRT2 does not alter c-MYC mRNA levels. We then examined the effect of SIRT2 inhibitors on the expression
of NEDD4 in HSCs. We found that NEDD4 mRNA expression was not affected by SIRT2 inhibitors (data not shown). Furthermore, expression of N-MYC, another Myc family member which has been shown to be regulated by NEDD4 in neuroblastoma cells, was not altered by inhibition of SIRT2 (data not shown). These data suggest that SIRT2 does not act through NEDD4 in regulating c-MYC in HSCs.

c-MYC can be regulated through protein degradation (33). We found that MG132, a proteasome inhibitor, reversed the inhibition of c-MYC by Sirt2 inhibitors (Fig. 4B), suggesting that inhibition of SIRT2 decreases c-MYC expression by inducing its degradation. ERK and AKT play important roles in the stabilization of c-MYC (33). We therefore tested whether Sirt2 stabilizes c-Myc by regulating ERK or AKT. We found that inhibition of Sirt2 affected neither the expression nor the activation of AKT in LX2 cells (Fig. 3A), suggesting that Sirt2 does not cause stabilization of c-Myc by way of AKT. We then examined whether knockdown of Sirt2 affects the activation of ERK in HSCs. Interestingly, knockdown of SIRT2 significantly decreased p-ERK in LX2 cells (Fig. 4C). To determine whether SIRT2 stabilizes c-MYC by activating ERK, we induced ERK activation by overexpressing MEKDD, a constitutively active form of MEK (14), in LX2 cells. We found that MEKDD significantly enhanced the expression of p-ERK, c-MYC, α-SMA, and COLA1 in either scrambled or SIRT2-KD LX2 cells (Fig. 4D). By contrast, inhibition of ERK by GSK-1120212 significantly decreased the expression of c-MYC, αSMA, and COLA1 in LX2 cells (Fig. 4E). In general, these data indicate that SIRT2 stabilizes c-Myc by activating ERK in HSCs.

SIRT2 binds to and deacetylates ERK in HSCs. We next investigated the manner in which SIRT2 might regulate ERK activity in HSCs. SIRT2 has been shown to regulate AKT activity by binding to and deacetylating it in hepatocellular carcinoma cells (18). We therefore reasoned that Sirt2 may bind to and deacetylate ERK in HSCs. We first examined the binding of SIRT2 and ERK in HSCs by immunoprecipitation. We confirmed SIRT2-ERK binding in LX2 cells (Fig. 4F). We then tested whether the inhibition of SIRT2 enhances the acetylation of ERK in HSCs. Indeed, we discovered that knockdown of SIRT2 or the use of SIRT2 inhibitors enhances ERK acetylation in HSCs (Fig. 4G). To study whether SIRT2’s deacetylase activity is involved in ERK activation, we examined the effect of SIRT2 inhibitors, which suppress SIRT2 deacetylase activity (41), on ERK activation. We found that AC-93253 or AGK2 rapidly decreased phosphorylation of ERK in HSCs (Fig. 4H). As ERK phosphorylation is critical for the activation of ERK (35), our data suggest that SIRT2 deacetylase activity is important for ERK activation in HSCs. Furthermore, we found that ERK inhibition abrogated the induction of α-SMA and Cola1 by overexpression of SIRT2 (Fig. 4I). In general, our data indicate that Sirt2 binds to and deacetylates ERK in HSCs, which may contribute to the activation of ERK.

Deficiency of Sirt2 suppresses CCl4- and TAA-induced hepatic fibrosis in mice. To further determine the role of SIRT2 in hepatic fibrogenesis in vivo, we treated WT and Sirt2-KO mice with 1 ml/kg CCl4 for 5 wk. Interestingly, we found that Sirt2 is expressed mainly in activated HSCs in CCl4-induced fibrotic mouse livers (Fig. 5A). There was no significant difference in the histologic appearance of livers and hepatic fibrosis between untreated WT and Sirt2-KO mice (data not shown). However, we found that CCl4-induced hepatic fibrosis was significantly decreased in Sirt2-KO mice compared with WT mice (Fig. 5, B and C). In addition, the hydroxyproline level and expression of a panel of fibrogenic genes, including α-SMA, COLA1, MMP-2, TIMP1, and TIMP2, were significantly decreased in the livers of Sirt2-KO mice compared with the livers of WT mice after CCl4 treatment (Fig. 5, D–G). Furthermore, lack of SIRT2 expression resulted in markedly decreased levels of p-ERK and c-MYC in CCl4-treated livers (Fig. 5G). However, WT and Sirt2-KO mice displayed similar histological features (Fig. 6A) and comparable levels of liver injury (Fig. 6B), apoptosis (Fig. 6, C and D), mononuclear infiltration (Fig. 6, E and F), and histological inflammation (Fig. 6, G and H) after CCl4 treatment, indicating that the effect of Sirt2 deletion on hepatic fibrosis may not be secondary to an alteration in the extent of liver injury. Finally, we also found reduced development of hepatic fibrosis in Sirt2-KO mice compared with WT mice in the TAA fibrosis model (Fig. 7, A–E), suggesting that SIRT2 promotes hepatic fibrosis independent of the type of underlying injury. These results demonstrate that SIRT2 deletion suppresses chemically induced hepatic fibrosis in mice.

SIRT2 is overexpressed in human fibrotic tissues and is colocalized with higher expression of p-ERK and c-MYC. We next examined whether SIRT2 expression is correlated with human hepatic fibrosis. We found that expression of SIRT2 is higher in human liver specimens from patients with advanced fibrosis compared with those without fibrosis (Fig. 8A). In addition, we found that SIRT2 is expressed predominantly in fibrotic tissues, which show high α-SMA expression (Fig. 8B). Furthermore, we found that expression of p-ERK and c-MYC are also elevated in hepatic fibrotic tissues and that they colocalize with α-SMA (Fig. 8, B and C).

Fig. 5. Deficiency of Sirt2 suppresses carbon tetrachloride (CCl4)-induced hepatic fibrosis in mice. A: co-staining of Sirt2 and α-SMA in the liver of a WT mouse treated with CCl4 for 5 wk. B: representative photomicrographs of sirius red staining and Masson’s trichrome staining of livers from WT (littermates generated from heterozygous mice, C57BL/6 background) or Sirt2-KO mice (littermates generated from heterozygous mice, C57BL/6 background) treated with CCl4 for 5 wk. C: quantification of sirius red staining and Masson’s trichrome staining of livers from WT (littermates generated from heterozygous mice, C57BL/6 background) or Sirt2-KO mice (littermates generated from heterozygous mice, C57BL/6 background) treated with CCl4 for 5 wk (n = 8). D: expression of Sirt2, p-ERK, ERK, c-MYC, α-SMA, COLA1, and GAPDH proteins in livers of WT (C57BL/6) or Sirt2-KO mice (littermates generated from mixed background) treated with CCl4 for 5 wk (n = 5).
C). These data indicate that SIRT2 is overexpressed in human fibrotic tissues, and that this enzyme might regulate the ERK/c-MYC pathway to promote hepatic fibrosis in human livers.

**DISCUSSION**

More effective therapeutic strategies are urgently needed for treating hepatic fibrosis and cirrhosis. In this study, we found...
that SIRT2 plays a critical role in hepatic fibrogenesis, and that the inhibition of SIRT2 suppresses hepatic fibrogenesis in vitro and in vivo models. These results suggest that inhibition of SIRT2 may be a promising strategy to inhibit fibrogenesis, which could allow for remodeling and regression of fibrosis. To our knowledge, this is the first study to demonstrate a role for SIRT2 in hepatic fibrosis. Many studies have shown common and organ-specific mechanisms of tissue fibrosis. In fact, a recent study demonstrated that blocking SIRT1 and SIRT2 inhibited renal interstitial fibroblast activation and attenuated renal interstitial fibrosis in obstructive nephropathy (25). Thus the role of SIRT2 in fibrogenesis might represent a common

Fig. 6. Deficiency of Sirt2 does not affect CCl4-induced liver injury, apoptosis, monocytic infiltration, or inflammation in mice. A: representative photomicrographs of hematoxylin and eosin staining of livers of WT (littermates generated from heterozygous mice) or Sirt2-KO mice (littermates generated from heterozygous mice) treated with CCl4 for 6 wk (n = 8). B: representative photomicrographs of sirius red staining of the livers of WT (littermates generated from heterozygous mice) or Sirt2-KO mice (littermates generated from heterozygous mice) treated with CCl4 for 6 wk. C: quantification of sirius red staining of the livers of WT (littermates generated from heterozygous mice) or Sirt2-KO mice (littermates generated from heterozygous mice) treated with CCl4 for 6 wk. D: hydroxyproline levels of livers from WT (littermates generated from heterozygous mice) or Sirt2-KO mice (littermates generated from heterozygous mice) treated with CCl4 for 6 wk (n = 8). E: measurement of ALT levels in the sera of livers from WT (littermates generated from heterozygous mice) or Sirt2-KO mice (littermates generated from heterozygous mice) treated with CCl4 for 6 wk (n = 8).

Fig. 7. Deficiency of Sirt2 suppresses thioacetamide (TAA)-induced hepatic fibrosis in mice. A: representative photomicrographs of hematoxylin and eosin staining of livers of WT (littermates generated from heterozygous mice) or Sirt2-KO mice (littermates generated from heterozygous mice) treated with TAA for 6 wk (n = 8). B: representative photomicrographs of sirius red staining of the livers of WT (littermates generated from heterozygous mice) or Sirt2-KO mice (littermates generated from heterozygous mice) treated with TAA for 6 wk. C: quantification of sirius red staining of the livers of WT (littermates generated from heterozygous mice) or Sirt2-KO mice (littermates generated from heterozygous mice) treated with TAA for 6 wk. D: hydroxyproline levels of livers from WT (littermates generated from heterozygous mice) or Sirt2-KO mice (littermates generated from heterozygous mice) treated with TAA for 6 wk (n = 8). E: measurement of ALT levels in the sera of livers from WT (littermates generated from heterozygous mice) or Sirt2-KO mice (littermates generated from heterozygous mice) treated with TAA for 6 wk (n = 8). F: measurement of ALT levels in the sera of livers from WT (littermates generated from heterozygous mice) or Sirt2-KO mice (littermates generated from heterozygous mice) treated with CCl4 for 5 wk (n = 8). G: measurement of ALT levels in the sera of livers from WT (littermates generated from heterozygous mice) or Sirt2-KO mice (littermates generated from heterozygous mice) treated with TAA for 6 wk (n = 8).

![Fig. 6](http://ajpgi.physiology.org/)

![Fig. 7](http://ajpgi.physiology.org/)
mechanism in different tissues. The role of other sirtuin family members in hepatic fibrosis remains largely unknown. Sirtuin proteins play various functions in cell- and context-specific manners (32). Therefore, it will also be interesting to delineate whether there are specific functions of each subtype of sirtuin in hepatic fibrosis.

Our data suggest that the effect of SIRT2 deletion on hepatic fibrosis is not due to the extent of liver injury (Fig. 6). In addition, we found that SIRT2 protein is predominantly expressed in HSCs in CCl4-induced fibrotic mouse livers (Fig. 5A), which further suggests a role for SIRT2 in HSCs in the process of hepatic fibrogenesis. However, it is still possible that SIRT2 expression in the liver cell types may play a role in hepatic fibrosis. SIRT2 has been shown to inhibit the inflammatory response and oxidative stress-induced cell death via the expression of antioxidant enzymes in murine macrophages (15). Therefore, it is reasonable to hypothesize that deletion of SIRT2 may affect inflammatory responses to CCl4-induced liver injury, thereby contributing to decreased fibrosis in Sirt2-KO mice. However, we found that there was comparable mononuclear infiltration and inflammation in the livers of SIRT2-KO mice compared with those of WT mice after CCl4 treatment (Fig. 6, E–H). These results suggest that SIRT2 deficiency does not inhibit inflammation in CCl4-treated mouse models. We cannot exclude the possibility that SIRT2 expression in hepatocytes or other cell types (e.g., endothelial cells)
SIRT2 PROMOTES HEPATIC FIBROSIS

G1167

might play a role in hepatic fibrogenesis. We plan to investigate this by using Sir2 cell-specific KO mice in future studies.

c-MYC plays key roles in cell proliferation, growth, differentiation, and survival (19). Emerging evidence suggests that c-MYC may play an important role in hepatic fibrosis. A recent study reported that c-MYC is upregulated in patients with advanced liver fibrosis (21). In the present study, we showed that c-MYC shRNAs significantly decreased the expression of α-SMA, COLA1, and MMP-2 mRNAs in LX2 cells, suggesting transcriptional regulation of these fibrogenic genes by c-MYC in HSCs. c-MYC is a transcription factor that activates the expression of many genes through binding to enhancer box sequences (19). It remains to be determined how c-MYC regulates the expression of α-SMA, COLA1, and MMP-2 in LX2 cells. We analyzed the promoters of human α-SMA, COLA1, and MMP-2 and did not find putative canonical c-MYC binding sites in human α-SMA, COLA1, or MMP-2 promoters (data not shown). This suggests that c-MYC might indirectly regulate the transcription of these genes. In addition, overexpression of c-MYC in hepatocytes resulted in enhanced liver damage and increased HSC activation, promoting CCl4-induced hepatic fibrosis(21), raising the possibility that germline deletion of Sir2 decreases c-MYC expression in hepatocytes, thereby inhibiting liver fibrosis. However, we found that overexpression of SIRT2 did not increase c-MYC expression in a polarized hepatocyte cell line, HepG2 (data not shown). Therefore, the regulation of c-MYC by SIRT2 might be specific to HSCs but not to hepatocytes. Inhibition of c-MYC might be an effective strategy to inhibit and potentially treat liver fibrosis and cirrhosis.

In this study, we further demonstrated that SIRT2 stabilizes c-MYC protein through activating ERK in HSCs. SIRT2 has been shown to stabilize N-MYC and c-MYC by directly binding to the promoter of the ubiquitin-protein ligase NEDD4 and repressing NEDD4 gene expression in neuroblastoma cells (18). However, we found that inhibition of SIRT2 does not suppress NEDD4 in HSCs (data not shown), suggesting that the regulation of c-MYC by SIRT2 may be context dependent. Our data show that activation of ERK, but not AKT, is responsible for the regulation of c-MYC by SIRT2 in HSCs. Emerging evidence indicates that SIRT2 regulates ERK activity. For example, one study showed that SIRT2 induces C2C12 myoblast proliferation by activating the ERK1/2 pathway (39). In addition, SIRT2 inhibitors reduced the activation of ERK in renal interstitial fibroblasts (25). We showed that inhibitors of SIRT2, which hinder its deacetylase activity (18, 23) but not its expression (data not shown), decrease the phosphorylation of ERK in LX2 cells (Fig. 4H). These results suggest that SIRT2’s deacetylase activity is important for ERK phosphorylation and activation. We also found that knockdown of SIRT2 or the use of SIRT2 inhibitors enhances the acetylation of ERK in LX2 cells (Fig. 4G), suggesting that SIRT2 deacetylates ERK. Whether the deacetylation of ERK by SIRT2 directly promotes ERK phosphorylation and activation remains unknown. However, deacetylation of AKT by SIRT1 promotes the phosphorylation of AKT and its consequent activation (36). Acetylation of AKT blocks the binding of AKT and phosphoinositide-dependent protein kinase 1 (PDK1) to phosphatidylinositol 3,4,5-trisphosphate (PIP3), thereby preventing the phosphorylation of AKT and its consequent activation (36). Whether similar acetylation-mediated mechanisms exist for the regulation of ERK is still unknown but plausible. In addition, HDAC inhibitors induced the acetylation of MAP kinase phosphatase-1 (MKP-1), a protein that dephosphorylates MAPK and inactivates MAPK pathways (5). Therefore, it is possible that SIRT2 might indirectly regulate ERK activity through deacetylating MKP-1. These questions will be addressed in future studies. Taken together, our data suggest that MAPK/ERK inhibitors should be considered as potentially beneficial in the treatment of hepatic fibrosis and cirrhosis.

In conclusion, our results show that SIRT2 promotes hepatic fibrogenesis by regulating the ERK/c-MYC pathway. Inhibition of SIRT2 may represent a novel and effective strategy to protect against the development of hepatic fibrosis. Inhibitors of ERK or c-MYC, which have been developed and are being studied in phase I and phase II clinical trials, could also prove to be useful in the treatment of liver fibrosis and cirrhosis.

ACKNOWLEDGMENTS

We thank Drs. Jiwang Zhang, Manuel Diaz, Nancy Zeleznik-Le, and Tarun Patel (Loyola University Chicago) for helpful discussions and advice, and Dr. Scott Friedman (Mount Sinai Hospital) for providing LX2 and J51 cells.

GRANTS

This work was supported in part by AASLD Liver Scholar Award (to W. Qiu), National Cancer Institute Grants R03 CA-184652 (to W. Qiu), R03 CA-195183 (to W. Qiu), and R01 CA-197128 (to W. Qiu).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


AJP-Gastroint Liver Physiol • doi:10.1152/ajpgi.00271.2015 • www.ajpgi.org

Downloaded from http://ajpgi.physiology.org/ by 10.220.33.4 on August 28, 2017
SIRT2 PROMOTES HEPATIC FIBROSIS


