The Autoregulatory Feedback Loop of MicroRNA-21/Programmed Cell Death Protein 4/Activation Protein-1 (MiR-21/PDCD4/AP-1) as a Driving Force for Hepatic Fibrosis Development*

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Background: MicroRNA-21 is important in hepatic fibrosis development, but the mechanism is unclear.

Results: MicroRNA-21 is predominantly up-regulated in activated hepatic stellate cells and could form a double negative feedback loop that links fibrogenic machinery.

Conclusion: The microRNA-21-mediated loop is a main driving force for hepatic fibrosis progression.

Significance: It suggests a mechanism for how microRNA-21 contributes to hepatic fibrosis.

Sustained activation of hepatic stellate cells (HSCs) leads to hepatic fibrosis, which is characterized by excessive collagen production, and for which there is no available drug clinically. Despite tremendous progress, the cellular activities underlying HSC activation, especially the driving force in the perpetuation stage, are only partially understood. Recently, microRNA-21 (miR-21) has been found to be prevalently up-regulated during fibrogenesis in different tissues, although its detailed role needs to be further elucidated. In the present study, miR-21 expression was examined in human cirrhotic liver samples and in murine fibrotic livers induced by thioacetamide or carbon tetrachloride. A dramatic miR-21 increase was noted in activated HSCs. We further found that miR-21 maintained itself at constant high levels by using a microRNA-21-programmed cell death protein 4/activation protein-1 (miR-21/PDCD4/AP-1) feedback loop. Disrupting this loop with miR-21 antagonomir or AP-1 inhibitors significantly suppressed fibrogenic activities in HSCs and ameliorated liver fibrosis. In contrast, reinforcing this loop with small interfering RNA (siRNA) against PDCD4 promoted fibrogenesis in HSCs. Further analysis indicated that the up-regulated miR-21 promoted the central transforming growth factor-β (TGF-β) signaling pathway underlying HSC activation. In summary, we suggest that the miR-21/PDCD4/AP-1 autoregulatory loop is one of the main driving forces for hepatic fibrosis progression. Targeting this aberrantly activated feedback loop may provide a new therapeutic strategy and facilitate drug discovery against hepatic fibrosis.

Hepatic fibrosis is a wound healing process in response to chronic liver injuries that leads to unbalanced extracellular matrix (ECM) deposition and resolution. The persistent activation of wound healing responses causes quantitative and qualitative changes in the ECM components and could finally distort liver parenchyma and vascular architecture, which could impair liver function and potentially lead to liver failure and hepatocellular carcinoma. Following liver injury, quiescent hepatic stellate cells (HSCs) transdifferentiate into myofibroblast-like cells that are characterized by the expression of smooth muscle α-actin (α-SMA) and enhanced production of ECM (1). Despite the tremendous progress in understanding the mechanisms during fibrogenesis, the driving force underlying the persistent fibrogenic activities is still only partially understood.

Extensive studies have suggested the central role of feedback networks in dictating disease progression. Strikingly, these autofeedback loops could have the capacity to promote the irreversible activation of certain signaling pathways and stabilize the cellular phenotype switch under pathological settings, thus contributing to disease progression (2). One previous study sheds light on the miR-21/PDCD4/AP-1 autoregulatory loop, which is highly activated during tumorigenesis and could drive cell transformation and proliferation (3). In addition, elevated miR-21 expression has been noted in liver and other organs undergoing fibrosis (4–6), whereas its role in promoting hepatic fibrosis progression and moreover how miR-21 is dysregulated in the liver remain only slightly understood. MiR-21 harbors several transcription factors, and its expression could be modulated by different upstream effectors under different disease settings (7–9). In regard to liver fibrosis, a role for AP-1

The abbreviations used are: ECM, extracellular matrix; α-SMA, smooth muscle α-actin; AP-1, activation protein-1; COL1A1, collagen 1A1; HSC, hepatic stellate cell; miRNA or mir, microRNA; PDCD4, programmed cell death protein 4; PTEN, phosphatase and tensin homolog; p-MEK, phosphorylated MEK; Smad, small mothers against decapentaplegic homolog; TAA, thioacetamide; antago-ctrl, scrambled antagonomir; ctrl, control; qRT-PCR, quantitative real time reverse transcription-polymerase chain reaction; CHX, cycloheximide; R-Smad, receptor-regulated Smad.

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is suggested because JNK is predominantly activated in myofibroblasts during hepatic fibrosis (10). Given that AP-1 could drive miR-21 expression and that miR-21-mediated PDCD4 down-regulation is necessary for maximal AP-1 activity (3), it is speculated that miR-21, PDCD4, and AP-1 may form a feedback loop during HSC activation and promote hepatic fibrosis progression. 

Herein, the present study demonstrates that miR-21 expression is significantly increased in fibrotic livers, predominantly occurring in myofibroblasts. The up-regulated miR-21 maintains its own high level expression by utilizing a double negative feedback circuit in which miR-21 targets PDCD4, a well known AP-1 inhibitor, for degradation; the subsequent elevated AP-1 activity in turn promotes miR-21 expression. As a result, a high level of miR-21 efficiently depresses Smad7 expression and enhances p-Smad2/Smad2 activity. Altogether, once activated, this autoregulatory paradigm could fuel HSC activation and promote the progression of liver fibrosis.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Human recombinant TGF-β1 was purchased from PeproTech (Rocky Hill, NJ). The miR-21 precursor and scrambled precursor were purchased from Ambion (Foster City, CA). Antagomirs against miR-21 were designed, and the most efficient one (5’-CACATCAGTCTGATAAGC-3’) was selected. Antagomir-21 and scrambled antagomirs with full phosphorothioate linkage were synthesized by SBS Genetech (Shanghai, China). The miRCURY LNA detection probe against miR-21 and the control scrambled probe were selected. Antagomir-21 and scrambled antagomirs with full phosphorothioate linkage were synthesized by SBS Genetech (Shanghai, China). The miRCURY LNA detection probe against miR-21 and the control scrambled probe were purchased from Exiqon (Woburn, MA). PDCD4 siRNA (sense, 5’-GCUGCUUGAAAGAGGCUATT-3’, antisense, 5’-UAUCCGCUUAGCGACGTTT-3’), c-Jun siRNA (sense, 5’-GCCCUAGCUAGCUATT-3’, antisense, 5’-UAUGCAGGUCGAUG-3’), and scrambled siRNA were designed and synthesized by Invitrogen and then verified. Dextran sulfate sodium (M, 500,000) was purchased from Sigma-Aldrich. SR11302 was purchased from Tocris (Ellisville, MO). SP600125 was purchased from Beyotime (Nantong, China) for the cellular experiments and was further synthesized by Wuxi Apptec (Tianjin, China) for the animal experiments.

**Animal Treatments**—Male ICR mice (20 ± 2 g) of the same background were obtained from the Animal Centre of Yangzhou University (Yangzhou, China). The mice were randomly divided into different groups and had free access to water and laboratory chow. Mice received intraperitoneal injections of thioacetamide (TAA; 200 mg/kg of body weight) twice weekly for 8 weeks or carbon tetrachloride (CCl4; in oil, 1:3; 4 µl/g of body weight) twice weekly for 4 weeks, whereas the control group received saline or oil intraperitoneally. For the antagonist-21 or scrambled antagonist (antago-ctrl) groups, 2 days before the first saline/TAA or oil/CCl4 injection, mice received a single dose of antagonist-21 or antago-ctrl (10 mg/kg of body weight) dissolved in saline via the tail vein. During the following 8-week TAA or 4-week CCl4 treatments, the mice received an intravenous injection of antagonist-21 or antago-ctrl (10 mg/kg of body weight) every 10 days. For the SP600125 treatment group, 24 h after every TAA injection, a single dose of 100 µl of SP600125 (5 mg/ml) was administered intraperitoneally. Mice were sacrificed at the indicated time points. All of the experiments were conducted according to the use and care guidelines for experimental animals from Jiangsu Province, China.

**Human Subjects**—This study was approved by the local Institutional Review Board, and the patients provided written informed consent. Liver samples were obtained from 10 cirrhosis patients at Gulou Hospital (Nanjing, China). The patient information is included in Table 1.

**Isolation of Primary Hepatic Stellate Cells**—Firstly, 8–12-month-old Sprague-Dawley rats were anesthetized with an intraperitoneal injection of 15 mg/kg pentobarbital. The liver perfusion buffer and liver digest medium were prewarmed at 37 °C. A warm water circulation kept the buffer flow at a constant temperature of 37 °C. The liver was perfused in situ via the vena porta with a heparin sodium buffer while the inferior vena cava was used as the outflow until the liver became pale. The perfusion was continued with collagenase (Roche Applied Science; 3 mg/ml) and Pronase (Sigma; 3 mg/ml) buffer. The liver was carefully removed and dissociated in digestion medium II (0.5 mg/ml collagenase and Pronase, 0.2 mg/ml DNase I) for further digestion. Afterward, the liver cell suspension was filtered through gauze (100 µm) and centrifuged for 5 min at 50 × g at 4 °C to obtain the parenchyma cells. The non-parenchyma cells were collected from the 50 × g supernatant and washed twice before density gradient centrifugation. A discontinuous density gradient (15%, 10%) was used as the outflow while the rotor to decelerate without the brake. The HSCs at the interface of the mounting solution and low density barrier were collected. The viability of the isolated cells was determined using trypan blue staining. The purity of isolated quiescent HSCs was determined by vitamin A autofluorescence and routinely exceeded 90%.

| Number | Gender | Age | Description                        |
|-------|--------|-----|-----------------------------------|
| 1     | M      | 43  | Nodular cirrhosis; portal chronic |
| 2     | M      | 47  | Nodular cirrhosis surrounding    |
| 3     | F      | 50  | Nodular cirrhosis                |
| 4     | M      | 71  | Nodular cirrhosis with necrosis  |
| 5     | M      | 58  | Nodular cirrhosis with inflammatory cell infiltration |
| 6     | M      | 43  | Nodular cirrhosis; chronic hepatitis B |
| 7     | M      | 40  | Nodular cirrhosis; cholestasis   |
| 8     | F      | 75  | Nodular cirrhosis; cholestasis   |
| 9     | M      | 45  | Nodular cirrhosis                |
| 10    | F      | 62  | Nodular cirrhosis; cholestasis   |

For the isolation of primary activated HSCs from fibrotic livers, although HSCs lose vitamin A during activation, the activated HSCs could also be isolated by discontinuous density gradient centrifugation according to published protocols (11, 12). The mouse liver was perfused from the left ventricle of the heart, and the isolation procedure continued as described above. To obtain enough HSCs, the cells from two mice were mixed as one sample in the end. To isolate primary murine hepatocytes, the digest medium contained collagenase but not Pronase. Because the activated HSCs lost vitamin A during activation, the purity of isolated activated HSCs was deter-
minded by α-SMA/desmin double fluorescence staining; the purity exceeded 90%.

Cell Treatments—HSC lines (rat HSC-T6 cells) (13) or isolated primary rat HSCs were cultured in DMEM containing 10% FBS (Invitrogen) at 37 °C with 5% CO2 and humidity. For HSC-T6 cells, 24 h after 50 nM antagomir-21/antago-ctrl or pre-miR-21/pre-ctrl transfection using Lipofectamine 2000, HSC-T6 cells were further stimulated with 10 ng/ml TGF-β1. For primary HSCs, during the 15-day self-activation process on plastic plates, transfections were conducted three times using 50 nM each. At the end of the process, the cells were analyzed.

Quantitative Real Time Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)—Total RNA was extracted from cells or tissues, and the complementary DNA was synthesized. Transcript levels were detected via qRT-PCR following the manufacturer’s instructions. Individual gene and microRNA expression was normalized to β-actin mRNA or small nuclear RNA (snRNA) U6 expression, respectively. The primers used are listed in Table 2.

Western Blotting—Tissue homogenate or cell lysate was prepared. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Biotrace, Randburg, S.A.). Membranes were blocked using skim milk and then incubated with diluted primary antibody at 4 °C with gentle shaking overnight. After washing five times, secondary HRP-conjugated antibody was incubated for 1 h at room temperature. Positive signal was detected with fluorography using an enhanced chemiluminescence system (7003, Cell Signaling Technology Inc., Danvers, MA). The antibodies used are listed in Table 3.

Sirius Red and HE Staining—The paraffin-embedded liver tissues in each group were stained with H&E and Sirius Red, respectively. Briefly, the paraffin-embedded liver tissues were sectioned at 5 μm. After deparaffinization and hydration, sections were stained in 0.1% (w/v) Sirius Red (Direct Red 80, Sigma-Aldrich) in a saturated aqueous solution of picric acid for 1 h. Then the slides were rinsed twice for 15 min each in 0.01 N HCl to remove unbound dye. After dehydration, the slides were mounted.

Electrophoretic Mobility Shift Assays (EMSA)—The nuclear proteins were extracted from the liver of mice receiving saline or TAA treatment using a nuclear protein extraction kit (P0028, Beyotime, Nantong, Jiangsu, China) according to the manufacturer’s instructions. For EMSAs, the biotin-conjugated AP-1 consensus binding sequence (sense, 5’-CGC TTT ATG ACT CAG CCG GAA-3’; antisense, 3’-GGG GAC TAC TGA TGG ATG CTG-5’) (GS011B, Beyotime) was used. For RNA binding reactions, 2 μl (~16 μg) of nuclear extract was added to a 10-μl reaction (final) containing 50 nM biotin-labeled oligonucleotide and 5 μg of poly(dI-dC) (GS005, Beyotime). At the same time, the unlabeled probe (GS011, Beyotime) or unlabeled mutated probe (GS011M, Beyotime) was used as a cold competitor. The binding reactions were incubated for 20 min at room temperature. Then the binding reactions were loaded on prerun 6% native PAGE gels in 0.5× Tris borate-EDTA at 6 mA and transferred onto a nylon membrane. The membrane was incubated with shaking in blocking buffer containing avidin-HRP enzyme and balanced with substrate balance buffer. The signal was detected on x-ray film using an enhanced chemiluminescence system (7003, Cell Signaling Technology Inc.)

Fluorescence in Situ Hybridization—Fluorescence in situ hybridization (FISH) was performed following published protocols (14).

Measurement of Hepatic Hydroxyproline Content—Hepatic hydroxyproline levels were biochemically measured and calculated according to the manufacturer’s instructions (Jiancheng, Nanjing, China).

| TABLE 2 |
|---|
| PCr primers used in this study |
| R, reverse; F, forward. |
| miR-21-R | CTCAGACTGTTGTCGAGTCGCAATTTCA |
| miR-21-F | TTTGAGTCAAACATC |
| miR-21-3 | ACACTCCAGCAGGTAGCTTTAC |
| U6-R | AACGCTTCACGAAATTTGGTG |
| β-actin-F | GACCTTTTCAACCACACAGTG |
| β-actin-R | GCTTATGCCCTTGATGACG |
| α-SMA(rat)-F | AACTGTCATTTGAGCTTCA |
| α-SMA(rat)-R | CTTGTTGCGAGATTTGGTG |
| Smad2(rat)-F | ACTCTTCCCGCTGACTC |
| Smad2(rat)-R | CGACCTCCCTCTCCTCTTG |
| COL1A1(rat)-F | TGGATGGCTGCACGAGTCA |
| COL1A1(rat)-R | AATGACTCCGAGAGATTTAC |
| Pdcd4(rat)-F | TGTGTTGTCACCTTGG |
| Pdcd4(rat)-R | GGGCTGTAGCTGACTG |
| α-SMA(mouse)-F | GAACCTGAAATTTCCGTA |
| α-SMA(mouse)-R | GGGCTGGTCTCGG |
| COL1A1(mouse)-F | TGCCTGTCGCTCTT |
| COL1A1(mouse)-R | AGCCATTGCTGCTGGTAC |
| c-Jun(rat)-F | GCCCTGCTGAGCTTTGT |
| c-Jun(rat)-R | GGCTGTTGACCTTGG|

| TABLE 3 |
| Primary and secondary antibodies used in Western blotting |
| Antigens | Poly/monoclonal | Manufacturer | Dilution |
| p-Smad2 | Polyclonal | Cell Signaling Technology Inc. (3101) | 1:1000 |
| Smad2 | Polyclonal | Cell Signaling Technology Inc. (3102) | 1:1000 |
| MADH7 | Polyclonal | Abcam Ltd., Cambridge, MA | (ab90086) | 1:1000 |
| c-Jun | Monoclonal | Cell Signaling Technology Inc. (9165) | 1:1000 |
| Fra-1 | Polyclonal | Santa Cruz Biotechnology, Inc., Santa Cruz, CA (sc-183) | 1:200 |
| PTEN | Monoclonal | Cell Signaling Technology Inc. (9569) | 1:1000 |
| PDCD4 | Polyclonal | Cell Signaling Technology Inc. (9535) | 1:1000 |
| p-MEK1/2 | Monoclonal | Cell Signaling Technology Inc. (9154) | 1:1000 |
| COL1A1 | Monoclonal | Santa Cruz Biotechnology, Inc. (sc-25974) | 1:200 |
| α-SMA | Polyclonal | Abcam Ltd. (ab6924) | 1:200 |
| Desmin | Polyclonal | Santa Cruz Biotechnology, Inc. (sc-7559) | 1:200 |
| HRP-linked anti-rabbit IgG | | Cell Signaling Technology Inc. (7104) | 1:1000 |
| HRP-linked anti-goat IgG | | Santa Cruz Biotechnology, Inc. (sc-2020) | 1:1000 |
| HRP-conjugated anti-GAPDH | Monoclonal | KangChen Bio-tech Inc., Shanghai, China (KG-5G5) | 1:1000 |
FIGURE 1. **Up-regulated miR-21 expression in activated HSCs during liver fibrogenesis.** A, representative FISH results on paraffin-embedded liver sections from human cirrhotic liver subjects. Scale bars, 100 μm. Panel i, miR-21; panel ii, α-SMA; panel iii, DAPI; panel iv, merge. B, representative FISH results from human subjects with higher magnification. Scale bars, 20 μm. Panel i, miR-21; panel ii, α-SMA; panel iii, DAPI; panel iv, merge. C, one of three independent FISH results on paraffin-embedded liver sections from 8-week saline/TAA-treated mice with similar results. Scale bars, 20 μm. Panel i, miR-21; panel ii, α-SMA; panel iii, DAPI; panel iv, merge. D, the miR-21 levels in the total liver of saline/TAA-treated mice were determined by qRT-PCR, and compared with the basal content before hepatic fibrosis was induced. E, miR-21 levels in the isolated activated HSCs from saline/TAA-treated mice were determined by qRT-PCR and compared with the basal content before fibrosis induction. F, c-Jun and Fra-1 protein levels were determined by Western blotting from murine fibrotic liver samples. EMSA was performed on liver nuclear extracts from the 8-week saline/TAA-treated mice. The data represent one of three cohorts with similar results. The data are expressed as the means ± S.D. of three independent experiments. Each group contained at least three samples, and the HSCs from two mice were mixed into one sample. *, p < 0.05; **, p < 0.01; ***, p < 0.001; N.S., not significant. Error bars represent S.D.
Determination of HSC Proliferation—Cell proliferation was measured with the CCK-8 kit (Dojindo). To observe cell proliferation, antagomiR-21 or pre-miR-21 and corresponding controls (50 nM) were transfected into target cells using Lipofectamine 2000. The transiently transfected cells were seeded in a 96-well plate at a cell density of $5 \times 10^3$ and then cultured at 24-h intervals for 3 days. Briefly, 10 μl of CCK-8 solution was added to each well of the plate, and the plate was incubated at 37 °C for 1 h. The absorbance was measured at 450 nm as an indicator of cell proliferation.

Determination of HSC Apoptosis—Apoptosis of HSC was induced by serum deprivation plus cycloheximide (CHX) treatment. HSC-T6 cells were pretransfected with antagomiR-21 or pre-miR-21 and corresponding controls (50 nM), and then 24 h after transfection, cells were exposed to CHX (Sigma). Following a 4-h incubation with CHX at 37 °C, apoptosis of cells was determined using an Annexin V/dead cell apoptosis kit for flow cytometry according to the manufacturer’s instructions (V13241, Invitrogen).

Statistical Analysis—The results are given as the means ± S.D. Statistical analysis was performed using a one-way analysis of variance, and $p < 0.05$ was considered significant (denoted as * in each figure; ** and *** indicate $p < 0.01$ and $p < 0.001$, respectively).

RESULTS

MiR-21 Was Predominantly Up-regulated in HSCs in Human and Murine Fibrotic Liver—Elevated miR-21 expression has been reported in human fibrotic liver (4); however, the miR-21 expression profile in different liver cells remains elusive. To shed light on this issue, 10 human cirrhotic liver samples were collected (Table 1), and FISH was performed on liver sections to locate the up-regulated miR-21. The scrambled miR-21 probe showed no positive signal on the liver sections during the experiments. The results are presented in Fig. 1A, and a higher magnification image is shown in Fig. 1B. In all 10 collected human samples, miR-21 was predominantly up-reg-
Sequestering MiR-21 by Antagomir-21 Attenuated Collagen Deposition in the Livers of TAA- and CCl₄-treated Mice—To further confirm that miR-21 participated in and promoted the progression of hepatic fibrosis, chemically synthesized antagonists were administered to knock down miR-21 expression. The miR-21 levels were determined in isolated HSCs from mice after antagonim-21/antago-ctrl treatment. Antagomir-21 significantly reduced miR-21 content in HSCs in the TAA-induced model (Fig. 2A). Furthermore, α-SMA and COL1A1 mRNA expression was down-regulated in isolated primary HSCs after miR-21 knockdown (Fig. 2, B and C). Along with the reduced profibrogenic activities in HSCs, the total collagen deposition in the liver was much reduced (Fig. 2, D and E). Consistent with the histologic results, the hydroxyproline content was significantly down-regulated after antagonim-21 treatment (Fig. 2F). Similarly, in the CCl₄-induced model, antagonim-21 greatly reduced miR-21 content in the HSCs and ameliorated hepatic fibrosis (data not shown). Accordingly, the results from animal models confirmed the involvement of miR-21 in HSC activation and in the pathogenesis of liver fibrosis.

MiR-21 Was Up-regulated during HSC Activation—To confirm the miR-21 change during HSC activation, primary quiescent HSCs were harvested and cultured on plastic plates, which mimics the in vivo activation process (20). Quiescent HSCs begin to undergo activation on plastic plates and are positive for α-SMA until day 6, and the α-SMA levels peaked at ~18 days, consistent with the reported results (21). Notably, the miR-21 level increased with the up-regulation of α-SMA expression (Fig. 3A), indicating that the miR-21 levels correlated to the activation status of HSCs.
Then the miR-21 content in HSCs was transiently increased by transfecting with precursor miR-21. Increasing the miR-21 content up-regulated the COL1A1 and \(\alpha\)-SMA mRNA levels in HSC-T6 cells (Fig. 3, D and E) and the COL1A1 and \(\alpha\)-SMA protein levels in HSC-T6 cells and primary HSCs (Fig. 3 F).

**MiR-21 Amplified the TGF-\(\beta\)/Smad Signaling Cascade by Down-regulating Inhibitory Smad7 and Up-regulating Smad2/3 Expression**—Because the TGF-\(\beta\) signaling pathway is the major profibrogenic pathway during fibrosis, to determine the role of miR-21 in the TGF-\(\beta\)/Smad signaling pathway, antagomir-21/antago-ctrl or pre-miR-21/pre-ctrl was transfected into HSCs, and the expression profiles of Smad proteins were analyzed. Antagomir-21 attenuated Smad2 phosphorylation in HSC-T6 cells and primary HSCs (Fig. 4, A and B), whereas pre-miR-21 enhanced p-Smad2 levels (Fig. 4, C and D). Smad6/7 exhibits antagonistic effects on Smad2/3 phosphorylation (22) and is a direct target of miR-21 (4, 5). Antagomir-21 increased Smad7 levels, whereas pre-miR-21 decreased Smad7 expression in both HSC-T6 cells and primary HSCs (Fig. 4, E and F), suggesting a role for miR-21 in regulating Smad2 phosphorylation by modulating the Smad7 content.

**Regulation of PTEN and p-MEK by MiR-21**—Interestingly, reduced Smad2/3 protein levels were observed when knocking down miR-21 in HSC-T6 cells (Fig. 4 A). The phosphorylation of MEK has been reported to enhance Smad2/3 expression (23). Furthermore, the phosphorylation of MEK could be depressed by PTEN, a direct target of miR-21 (24, 25). Therefore, miR-21 was hypothesized to regulate Smad2 levels by modulating the activities of PTEN and p-MEK. TGF-\(\beta\) significantly promoted the phosphorylation of MEK1/2. Antagomir-21 negated this effect, whereas PTEN was still maintained at a high level (Fig. 5, A and B). In contrast, pre-miR-21 reduced PTEN protein levels and potentiated MEK1/2 phosphorylation (Fig. 5, C and D).

**MiR-21, PDCD4, and AP-1 Formed an Autoregulatory Feedback Loop in HSCs**—Primary HSCs were harvested and cultured on uncoated plastic plates. Primary HSCs were cultured with two different AP-1 inhibitors: SR11302, an intrinsic AP-1 inhibitor, and SP600125, which interferes with c-Jun phosphorylation (26, 27). The miR-21 content was significantly down-regulated in SR11302- and SP600125-treated primary HSCs compared with the DMSO solvent-treated group (Fig. 6 A). Moreover, to exclude the off-target effects of chemical inhibi-
content enhanced HSC proliferation, whereas antagonir-21 slowed down cell growth (Fig. 6G). Furthermore, apoptosis in HSC-T6 cells was induced by serum deprivation plus CHX treatment (32). A significant increase in apoptosis was achieved in HSCs after 4-h incubation with CHX. Pre-miR-21 reduced apoptosis, whereas antagonir-21 potentiated apoptosis in CHX-treated HSCs (Fig. 6H).

Inhibiting AP-1 Activity Ameliorated HSC Activation and Hepatic Fibrosis—As noted, COL1A1 mRNA expression showed the same trend as miR-21 change when primary HSCs were co-cultured with AP-1 inhibitors (Fig. 7A). Furthermore, COL1A1 mRNA was down-regulated in primary HSCs after transfection with c-Jun siRNA (Fig. 7B). In addition, COL1A1 mRNA in primary HSCs increased after transfection with PDCD4 siRNA during culture on plastic (Fig. 7C). All these experiments were conducted in the HSC-T6 cell line as well and achieved similar results (data not shown).

To analyze whether modulating components in this feedback loop could affect hepatic fibrosis progression, mice were further treated with the AP-1 inhibitor SP600125 during TAA induction. In the SP600125-treated mouse liver, miR-21 was significantly down-regulated (Fig. 7D). Additionally, the mice treated with SP600125 exhibited much lower collagen deposition (Fig. 7E) and much lower hydroxyproline content (Fig. 7F). This autoregulatory feedback loop is illustrated in Fig. 8.

**DISCUSSION**

During activation, HSCs undergo two stages: initiation and perpetuation. Maintaining the initial activation state can lead to the perpetuation state. TGF-β plays significant roles in both stages. However, recent evidence has suggested that TGF-β may not be the only driving force in the perpetuation stage. First, anti-TGF-β therapy failed to achieve positive outcomes in the clinic (33, 34). Second, activated HSCs display decreased surface receptors for TGF-β (35). Lastly, quiescent HSCs respond to TGF-β treatment by activation of R-Smads and display a functional negative feedback regulation via Smad7 induction, whereas after activation, in the perpetuation state, the activated HSCs are fully stimulated and display strong intrinsic R-Smad activation and, more importantly, lack Smad7 up-regulation in response to TGF-β stimulation (35). This implies that other mechanisms that suppress the anti-fibrotic Smad7 expression in the perpetuation stage exist. Taken altogether, in addition to TGF-β, these findings point to other driving forces underlying the perpetuation state in HSCs.

High levels of miR-21 represent a common feature of pathological cell proliferation and cellular stress (7). MiR-21 was found to be significantly up-regulated in liver fibrosis of different etiologies (4, 36, 37). However, in vivo, it remains elusive which liver cell type exhibits up-regulation of miR-21 expression, especially given that existing reports about the expression of miR-21 in in vitro activated HSCs are not consistent (4, 38). For the first time, we report that the up-regulated miR-21 predominantly occurs in activated HSCs in both human subjects and animal models. In addition, to date, there is no study addressing how miR-21 expression is elevated during hepatic fibrosis and, moreover, how miR-21 overexpression is linked to disease progression. JNK activation, whose effects are mainly

**FIGURE 5.** Regulation of PTEN and p-MEK by miR-21. HSC-T6 cells were transfected with 50 nM antagonirs or precursors and stimulated with 10 ng/ml TGF-β for 24 h. A, the protein levels of PTEN and p-MEK in the antagonir group were measured by Western blotting. B, densitometric quantification of the Western results in the antagonir group. C, Western blot results of the PTEN and p-MEK levels in the precursor group. D, densitometric quantification in the precursor group. All Western blot data represent one of three independent experiments. The data are expressed as the means ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001; N.S., not significant. Error bars represent S.D.
mediated through AP-1 transcription factor, predominantly occurs in myofibroblasts during liver fibrogenesis (10), and AP-1 is one of the key transcription factors of miR-21. Moreover, PDCD4, as a direct target of miR-21, can inhibit AP-1 activity endogenously (3, 39). Thus, miR-21 could enhance the activity of AP-1 by down-regulating PDCD4 expression, which in turn results in the self-promoted miR-21 expression. In the present study, we proved that miR-21 was significantly up-regulated in activated HSCs during liver fibrogenesis in animal models, and this was further confirmed in human cirrhotic liver samples. Additionally, during activation in cultured primary HSCs, the miR-21 level correlated with the activation status of HSCs.

Smad7, as an intrinsic inhibitor of the TGF-β/Smad2/3 pathway, competes with R-Smads for type I TGF receptor and intervenes in TGF-β/Smad2/3 signal transduction. Overexpressing Smad7 in rat livers inhibited collagen deposition and α-SMA expression in hepatic stellate cells (22). In recent studies, the down-regulation of Smad7 in tissue fibrosis was attributed to the high level miR-21 in fibrogenic cells. Thus, miR-21 generated from the miR-21/PDCD4/AP-1 feedback loop can directly target the antifibrotic Smad7 during fibrogenesis, connecting this self-promoting feed-forward loop with the fibrogenic machinery. Furthermore, a single microRNA may have multiple targets. Besides Smad7, in the present study, we demonstrated that PTEN was also significantly down-regulated by miR-21 in activated HSCs. PTEN has been implicated in inhib-
iting the formation of functional R-Smad complexes (40). In addition, based on the present study, PTEN may also regulate Smad2/3 expression via the PTEN/p-MEK pathway. Smad2/3 is the main signal mediator of the TGF-β signaling pathway and plays a pivotal role in profibrogenic gene expression. Targeted deletion of Smad2/3 could prevent or halt the progression of hepatic fibrosis in animals (41). This implies that high miR-21 content could potentially promote fibrogenic activities by directly up-regulating R-Smad expression. Together with the reported findings that AP-1 can induce autocrine expression of TGF-β and synergize with Smad2/3 in transcription of profibrogenic genes (42, 43), the miR-21/PDCD4/AP-1 feedback loop could greatly promote fibrogenic activities in HSCs during the perpetuation stage. In addition, studies have reported that miR-21 affects PDCD4 expression and thereby affects both proliferation and apoptosis (3, 31). The effect of miR-21 on HSC activation could further be achieved by protecting the fully activated HSCs from apoptosis, thus resulting in an increased presence of these cells. Taken altogether, the miR-21/PDCD4/AP-1 feedback loop could promote HSC activation and disease progression by affecting several targets, and this may also provide a possible explanation for how HSCs could undergo self-activa-

**FIGURE 7. Attenuation of fibrogenic events by inhibiting AP-1 activity.** Isolated primary HSCs were cultured on plastic plates for 15 days during which cells were treated as indicated. A, the COL1A1 mRNA levels in primary HSCs were measured by qRT-PCR after co-culture with 10 μM SR11302, 15 μM SP600125, or DMSO vehicle. B, the COL1A1 mRNA levels in primary HSCs were measured by qRT-PCR after transfection with c-Jun/ctrl siRNA. C, the COL1A1 mRNA levels in primary HSCs were measured by qRT-PCR after transfection with PDCD4/ctrl siRNA. D–F, during 8-week TAA-induced hepatic fibrosis, the mice were treated with SP600125. D, the miR-21 levels in the liver were measured by qRT-PCR after SP600125 treatment. E, representative image of Sirius Red staining of mouse liver sections after SP600125 treatment. Scale bars, 100 μm. Panel i, saline; panel ii, TAA; panel iii, TAA + SP600125; panel iv, TAA + DMSO. F, semiquantitative analysis of the area of Sirius Red staining and the hydroxyproline content in mouse liver after SP600125 treatment. One of three independent results is presented. The data are expressed as the means ± S.D. of three independent experiments, each containing at least three samples. *, p < 0.05; **, p < 0.01; ***, p < 0.001. *Control indicates the isolated primary HSCs cultured on plastic for 15 days without treatment. Error bars represent S.D.
This finding provides additional insight into the phenotypic switch of HSCs from the quiescent state into the large ECM-producing form. Once activated, the circuit maintains the stable conversion for a double negative feedback circuit during liver fibrogenesis. Continuous production of fibrogenic ECMs could drive the pathological machinery in response to continuous production of fibrogenic ECMs. Therefore, the miR-21/PDCD4/AP-1 feedback loop could work as a major impetus behind different progressive diseases. Through coordination of hepatic fibrosis, finally leading to sustained HSC activation by modulating several targets. TjRα and TjRβ, type I and type II TGF-β receptor, respectively.

Our data herein shed further light on a possible common paradigm in shaping phenotypic outputs under different disease settings. MiR-21, which is overexpressed in almost all tumors analyzed so far (7), is also up-regulated in osteoelastogenesis (39) and fibrotic diseases. In addition, AP-1 activation is closely associated with cancer development, and inhibition of JNK activity, which is effective in cancer therapy, could also attenuate liver fibrosis (44). Therefore, the experimental outcomes from studies on different pathological processes (cancer, osteoelastogenesis, and fibrosis) suggest that the self-promoted miR-21/PDCD4/AP-1 feedback loop may be a common driving force behind different progressive diseases. Through coordinately regulating a suite of targets, this feedback loop thereby could drive the pathological machinery in response to continued pressure overload.

In summary, in activated HSCs, miR-21, PDCD4, and AP-1 form a double negative feedback circuit during liver fibrogenesis. Once activated, the circuit maintains the stable conversion of HSCs from the quiescent state into the large ECM-producing state. This finding provides additional insight into the phenotypic switch of HSCs from the quiescent state into the large ECM-producing state. In this regard, therapeutics modulating this feedback could be further developed to treat hepatic fibrosis.

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