The miR-455-3p/HDAC2 axis plays a pivotal role in the progression and reversal of liver fibrosis and is regulated by epigenetics

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Abstract
Histone deacetylases (HDACs), especially HDAC2, play a role in alleviating liver fibrosis; however, the specific upstream regulation mechanism is unknown. Herein, TargetScan was used to predict the potential upstream targets of HDAC2, and the role of miR-455-3p was explored. The dual luciferase reporter assay showed that miR-455-3p binds to the 3’ UTR of HDAC2 mRNA. Additionally, miR-455-3p was downregulated in the liver tissues of patients with cirrhosis and mice with liver fibrosis, as well as in primary HSCs isolated from fibrotic mouse livers and TGF-β-treated LX-2 cells. In contrast, it is highly expressed in the reversal stage of hepatic fibrosis and MDI-cultured LX-2 cells. Our functional analyses showed that miR-455-3p overexpression facilitated apoptosis and reduced the expression of pro-fibrotic markers and the proliferation of activated LX-2 cells. On the contrary, miR-455-3p inhibition converted inactivated LX-2 cells into activated, proliferative, fibrogenic cells. Interestingly, restoration of HDAC2 expression partially blocked the function of miR-455-3p. Downregulated miR-455-3p expression can be restored by DNA methyltransferases in activated LX-2 cells. Methylation-specific PCR, bisulfite sequencing PCR, and chromatin immunoprecipitation assays indicated that the methylation level of miR-455-3p promoter CpG islands was elevated in TGF-β-treated LX-2 cells and that miR-455-3p was downregulated in activated LX-2 cells by DNA hypermethylation, which is mediated by DNMT3b and DNMT1. In conclusion, miR-455-3p acts as a liver fibrosis suppressor by targeting HDAC2, and its deficiency further aggravates the reversal phase of fibrosis. Thus, the epigenetics mediated miR-455-3p/HDAC2 axis may serve as a novel potential therapeutic target for clinical treatment of hepatic fibrosis.

Abbreviations: α-SMA, α-smooth muscle actin; 3’ UTR, 3’ untranslated region; a-LX-2 cells, activated LX-2 cells; aHSCs, activated hepatic stellate cells; BSP, bisulfite sequencing PCR; ChIP, chromatin immunoprecipitation; COL1α1, type 1 collagen; DNMTs, DNA methyltransferases; ECM, extracellular matrix; HDACs, histone deacetylases; HF, hepatic fibrosis; i-LX-2 cells, inactivated LX-2 cells; iHSCs, inactivated hepatic stellate cells; miRNAs, microRNAs; MSP, methylation-specific PCR; q-LX-2 cells, quiescent LX-2 cells; qHSCs, quiescent hepatic stellate cells.

Hongmei You and Ling Wang contributed equally to this work.

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1 | INTRODUCTION

Hepatic fibrosis is a vital pathological feature and a common cause of a variety of chronic stimuli, such as viral hepatitis, alcohol abuse, autoimmune diseases, metabolic diseases, and cholestatic liver diseases. If the stimuli exist for a long time, much of the extracellular matrix (ECM), including type I collagen (COL1α1), is deposited in the liver, which disrupts the normal architecture of the liver. However, because of its unique regenerative capacity, the liver possesses a marvelous ability to resorb scars when the damage ceases. Thus, reversibility is one of the most important concepts in experimental and clinical hepatic fibrosis. In most cases, the underlying cause of liver damage cannot be confirmed and eliminated; therefore, therapeutic measures of liver fibrosis are restricted to addressing complications such as portal hypertension, hepatocellular carcinoma, and liver failure. Thus, better understanding of the therapies for chronic liver fibrosis is needed.

Hepatic stellate cells (HSCs) account for 5%-8% of cells in the liver and are located in the space of Disse between hepatic epithelial cells and sinusoidal endothelial cells. Under normal circumstances, HSCs are quiescent (qHSCs) and store vast amounts of vitamin A lipid droplets. When the liver is attacked by hepatic toxins or viral infections, the damaged hepatocytes and immune cells signal the HSCs, which then differentiate into activated proliferative, contractile, aged hepatocytes and immune cells. Underlying function of miR-455-3p in the activation and apoptosis of aHSCs play a pivotal role by eliminating the cell type responsible for producing the fibrotic scar. In addition, iHSCs downregulate the expression of fibrogenic genes and are more promptly activated into myofibroblasts than qHSCs. Therefore, the targeting of HSCs by inducing apoptosis and inhibiting their activation is important to establish therapeutic strategies to reverse liver fibrosis.

Histone deacetylases (HDACs) control the acetylation levels of core histones to modulate the expression of genes in various pathological processes, and histone acetyltransferases and HDACs are the two key mediators that maintain the balance between acetylated and deacetylated status of main histones. To date, according to their similarity to yeast orthologs, HDAC proteins have been divided into four sub-families: class I, II, and IV HDACs, and the class III or SIRT family. Class I comprises HDAC1, HDAC2, HDAC3, and HDAC8, which are ubiquitously tissue distributed and mainly localized in the nucleus of the cell. HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10 are members of class II HDACs, and all of them display a tissue-specific location in mammals and are mainly distributed in the cytoplasm. Our previous research team has studied the expression of class I, II, and IV HDACs in mouse liver tissues with liver fibrosis at the progression or reversal phase. Of note, HDAC2 level was increased in the progression stage, but greatly decreased in the reversal stage. Further research showed that HDAC2 deficiency could suppress TGF-β-induced proliferation and HSC activation by repressing Smad7 expression. However, how the upstream regulation mechanism of HDAC2 works in liver fibrosis remains to be identified.

MicroRNAs (miRNAs) are short, endogenous, non-coding RNAs that control gene expression by interacting with the 3′ untranslated region (3′ UTR) of target mRNAs and triggering either the inhibition of translation or degradation of mRNA. Many studies have shown that miRNAs are essential for proliferation, development, and differentiation in a variety of cell types and are related to the development of many diseases, including fibrosis. A single miRNA has the potential to target multiple mRNAs; similarly, one single mRNA can be targeted by multiple miRNAs. This special mechanism enables miRNAs to regulate multiple mRNAs in the same disease. Thus, to locate the upstream target of HDAC2, we utilized TargetScan, an open website for forecasting. Among the predicted upstream targets of HDAC2, miR-455-3p was considered as it has a significant role in renal fibrosis, pulmonary fibrosis, cardiac fibrosis, and hepatic fibrosis. The dual luciferase reporter assay verified this prediction and showed that miR-455-3p targeted the 3′ UTR of HDAC2 mRNA. However, the expression level and function of miR-455-3p in the reversal stage of liver fibrosis and inactivated LX-2 cells is unknown, and whether it functions by inhibiting HDAC2 in liver fibrosis and the mechanism of miR-455-3p expression changes needs further research. In this study, we hypothesized that miR-455-3p targets HDAC2 to perform its function. Hence, we explored the underlying function of miR-455-3p in the activation and apoptosis of hepatic stellate cells in the early phase and reversal stage of liver fibrosis.

Many studies have concentrated on the assessment of genetic factors correlated with hepatic fibrosis and its associated
complications. However, little is known about epigenetic changes, especially DNA methylation, which is catalyzed by DNA methyltransferases, including DNMT3a, DNMT3b, and DNMT1. Our laboratory and other groups have verified that DNA methylation plays an important role in regulating HSC activation. In addition, parallel to protein-coding genes, DNA hypermethylation may lead to the downregulation of miRNAs, and DNA hypomethylation may result in the upregulation of miRNA. For instance, abnormal hypermethylation of the miR-148a coding region decreases the expression of miR-148a in human pancreatic and hepatocellular carcinogenesis.

In this study, we aimed to investigate the role of miR-455-3p in the progression and reversal of liver fibrosis in vivo and in vitro by targeting HDAC2 and investigated whether the changes in the expression of miR-455-3p is related to epigenetic modifications.

2 MATERIALS AND METHODS

2.1 Murine models

We used male C57BL/6J mice (6-8-week-old, 18-22 g weight), purchased from the Experimental Animal Center of Anhui Medical University. All animal experimental protocols were reviewed and approved by the Animal Care and Use Committee of Anhui Medical University, China. After a week of adjustment, mice were randomly divided into three groups: vehicle-treated, CCl4-treated, and liver fibrosis recovery groups (n = 6 per group). To establish a mouse liver fibrosis model, mice were first injected with 10% CCl4 in olive oil (0.1 mL/kg/mouse) intraperitoneally, twice a week for 4 weeks. During the next 4 weeks, the injection was continued but its frequency halved to once a week. Throughout the 8 weeks, vehicle-treated, CCl4-treated, and liver fibrosis recovery groups were injected at the same frequency and same dose of CCl4. In the first four weeks, but the injection was stopped in the later four weeks to trigger spontaneous reversal. Finally, 24 hours after the last injection of olive oil and CCl4, the vehicle-treated and CCl4-treated model groups, three groups of mice were sacrificed at the same time, and serum, liver tissues, and primary HSCs were collected for further study.

2.2 Human liver samples

Human liver samples were obtained from the First Affiliated Hospital of Anhui Medical University (Anhui, China). This study was approved by the First Affiliated Hospital of Anhui Medical University, and all volunteers were informed of consent. Eight liver fibrosis tissue samples were collected from patients with cirrhosis caused by non-alcoholic steatohepatitis (NASH), and eight healthy liver tissue samples were obtained from patients with hepatic hemangioma resection. All tissues were immediately frozen in liquid nitrogen and stored at −80°C for subsequent experiments.

2.3 ALT/AST activity assay

We detected serum ALT and AST activities using alanine aminotransferase/aminotransferase Assay Kits (Nanjing Jiangcheng Bioengineering Institute) following the manufacturer’s instructions. The absorbance was analyzed at 510 nm using a Multiskan MK3 (Biotek, USA).

2.4 Isolation of primary HSCs

Primary HSCs were isolated from mouse liver fibrosis as described previously. Briefly, the abdominal cavity was opened after the mice were anesthetized, and then the liver was perfused with digestion buffer (1×PBC dissolved with type IV collagenase [Sigma-Aldrich, St. Louis, USA], 4.76 mM CaCl2 [Sigma-Aldrich, St. Louis, USA], and pronase [Sigma-Aldrich, St. Louis, USA]). After that, the liver tissue was separated and shredded with tweezers to obtain a cell suspension. The cells were centrifuged in Nycodenz mixture (Sigma, GER) at a density of 1.040-1.060 g/mL, and then, a 1-mL layer of Hank’s buffer (Gibco, USA) was added onto the cell-Nycodenz mixture. Subsequently, primary HSCs were gathered in the middle layer. The medium was separated and centrifuged to obtain the final primary HSCs to perform the follow-up experiments.

2.5 Histopathology and immunofluorescence staining

The middle portion of the left lobe of the liver of each C57BL/6J mouse was excised, sectioned, and then perfused in 4% paraformaldehyde for 48 hours. Then, the tissues were soaked in paraffin, sliced into 5-μm-thick sections and stained with hematoxylin and eosin for morphological analysis. For immunofluorescence staining, sections were blocked with 10% BSA solution to avoid non-specific staining. Then, the sections were incubated with rabbit polyclonal primary antibodies for HDAC2 (1:100) and mouse monoclonal primary antibodies for α-SMA (1:100). The sections were incubated with primary antibodies overnight at 4°C, followed by a mixture of anti-rabbit FITC (green, 1:200) and anti-mouse Cy-3 (red, 1:200) conjugated secondary antibodies for 2 hours at room temperature. The α-SMA and HDAC2 expression levels were visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB) staining. The stained sections were...
then examined with an inverted fluorescence microscope (OLYMPUS IX83, Tokyo, Japan).

### 2.6 Cell culture

LX-2 cells (human hepatic stellate cell line) were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 mg/mL streptomycin, and 100 U/mL penicillin and incubated in an atmosphere of 5% CO₂ at 37°C.

The quiescent LX-2 cells (q-LX-2) were stimulated with 10 ng/mL TGF-β for 24 hours to generate activated LX-2 cells (a-LX-2). In addition, a-LX-2 reverts to the inactivated state (i-LX-2) by MDI treatment with 10% FBS for 48 hours. MDI is an adipogenic differentiation mixture composed of 0.5 mM isobutylmethylxanthine (Sigma, GER), 1 µM dexamethasone (Sigma, GER), and 167 nM insulin (Sigma, GER). The same procedure was used for the control group but without the treatment.

### 2.7 Transient transfection of LX-2 cells

After LX-2 cells were grown to 70%- 80% confluency, they were resuspended by trypsinization, evenly spread in a 6-well plate, incubated with DMEM for 24 hours. Human-derived mimics of miR-455-3p, and the negative control were transfected into a-LX-2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States) according to the manufacturer’s instructions at a final concentration of 50 nmol/L. For the next 6 hours, the cells were cultured in Opti-MEM (Invitrogen, USA) at 37°C in an atmosphere of 5% CO₂. Then, the culture medium was changed to 10% FBS and stimulus was applied using 10 ng/mL TGF-β. After 24 hours, the cells were harvested for western blotting, RT-qPCR, and other experiments. All experiments were performed thrice. Table 1 lists the sequences used in transient transfection analysis.

### 2.8 TUNEL staining

To visualize apoptotic bodies, cell slides were first fixed in 10% buffered formalin at room temperature for 25 minutes and then supplemented with 0.2% Triton X-100 solution in PBS after washing twice with PBS at room temperature for 5 minutes. Subsequently, the cell slides were covered with 100 µL equilibration buffer and equilibrated for 10 minutes. rTdT reaction mix was added to the slides, and the slides were covered with coverslips. Next, the coverslip was removed and action was terminated using saline-sodium citrate. Then, the cell slides were immersed in 0.3% H₂O₂ for 3- 5 minutes. After washing three times with PBS, the slides were immersed in 50 µL of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, Servicebio, China) detection solution at 15°C-25°C for 60 minutes. Finally, slides were incubated with DAPI (Bi Yuntian Biological Technology, China) for 10 minutes. TUNEL-positive cells were visualized using a fluorescence microscope.

### 2.9 Dual Luciferase reporter assay

HDAC2 sequences containing the target sites for miR-455-3p were synthesized and cloned into pSI-Check2 reporter vector downstream to firefly luciferase gene (pSI-Check2-HDAC2-wildtype). A mutant version of HDAC2 (pSI-Check2-HDAC2-mutant) was also generated with the deletion of complementary sites. The reporter vector, miR-455-3p mimics, or negative control, were co-transfected into HEK-293T cells using Lipofectamine 3000 (Invitrogen, CA). Activity of firefly and Renilla luciferase genes were measured using the Dual-Luciferase system (Promega, USA) according to the manufacturer’s protocol and detected by GloMax Multi Jr (Promega, USA). Activity of firefly and Renilla luciferase genes were measured using the Dual-Luciferase system (Promega, USA) according to the manufacturer’s protocol and detected by GloMax Multi Jr (Promega, USA). Activity of firefly and Renilla luciferase genes were measured using the Dual-Luciferase system (Promega, USA) according to the manufacturer’s protocol and detected by GloMax Multi Jr (Promega, USA). In detail, HEK 293T cells were prepared for transfection and seeded into 96-well plates and cultured until

| Gene       | Sense (5’-3’)                  | Antisense (5’-3’)               |
|------------|--------------------------------|--------------------------------|
| Human      |                                |                                |
| HDAC2      | GCAAAUACUAUGCUUGACAATT          | UUGACAGCAUAGUAUUUGCTT           |
| DNMT3a     | GCGUCACACAGAGAAUCUATT           | AUAGCUUCUGUGUGAGCCTT            |
| DNMT3b     | GCUACCUGAGCUCUAAGATT            | UCUUUGAGGACAGGUAGCTT            |
| DNMT1      | GGAUGAGUCCAUAAGGAAATT           | UUCCUUGAAGGACUCAUCCTT           |
| Scrambled-RNAi | UUCUCGAACAGUGUCACGUTT         | ACGUGACAGCGUCCGGAGAATT          |
50%-70% cell confluency. 10 µL of DMEM was mixed with 0.16 µg of the target plasmid and 5 pmol has-miR-455-3p/ Negative control; and stored at room temperature (solution A). Then, 10 µL of DMEM was mixed with 0.3 µL transfection reagent (solution B) for 5 minutes. Mix solution A and solution B incubated for 20 minutes at room temperature. And exchanged before transfection. Fresh medium was added before transfection. And then the transfection mixture was added to 96-well plates and incubated at 37°C and 5% CO2. Fresh medium was replaced 6 hours after transfection of the plasmids into 293T cells. Forty-eight hours after transfection, the cells were collected for further detection. 1×Passive Lysis Buffer was added to 96-well plates (100 µL/well) to disperse the cells. Shake the 96-well plates slowly at room temperature for 15 minutes and then suck the cell lysate into centrifuge tubes for centrifuging at 12 000 rpm at 4°C for 10 minutes. Remove the supernatant into a black 96-well plate. In each well, 100 µL of Luciferase Reaction Reagent II was mixed with the supernatant and the Firefly luciferase value was detected. Likewise, 100 µL of stop & Glo® Reagent (Luciferase Assay Reagent, Progema) mixed with the contents of each well and the Renilla luciferase value was determined.

2.10 | RNA extraction and quantitative real-time PCR (RT-qPCR)

Total RNA from mouse liver tissues and LX-2 cells was isolated using TRIzol reagents (Invitrogen, USA) and quantified using the Thermo Scientific NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA). Then, first-strand of cDNA was synthesized with Thermoscript RT-PCR synthesis kit (Fermentas, USA) according to the manufacturer's instructions; and amplified according to the following procedure: pre-denaturation at 94°C for 5 minutes, then at 94°C for 20 seconds, 69°C for 20 seconds, 72°C for 20 seconds, and 87°C for 10 seconds for 55 cycles. RT-qPCR of mRNAs of α-SMA, Col1α1, HDAC2, and β-actin were performed using Thermoscript RT-qPCR kits (Fermentas, USA) in an ABI Prime step-one plus real-time PCR System (Applied Biosystems, USA). β-actin was used for normalization. Relative expression levels were calculated according to the standard 2^ΔΔCt method. The primer sequences (Sangon Biotech, China) are listed in Table 2. All experiments were performed in triplicates.

2.11 | Western blot

Cultured LX-2 cells and primary HSCs were lysed using RIPA lysis buffer (Beyotime, China) and total protein was extracted. The concentration of extracted protein was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Total protein of 30-50 µg of each sample was separated using an SDS-PAGE gel (10%), then transferred onto PVDF membranes (Millipore Corp, Billerica, MA, United States). Blocking solution comprising 5% skimmed milk was used to block the transferred membranes. Subsequently, membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies in this study included antibodies against α-SMA (Bioss, China), Col1α1 (Bioss, China), HDAC2 (Abcam, UK), c-myc (Cell Signaling Technology, USA), CyclinD1 (Cell Signaling Technology, USA), Bax (Cell Signaling Technology, USA), Bcl-2 (Cell Signaling Technology, USA), DNMT1 (Abcam, UK), DNMT3a (Abcam, UK), and DNMT3b (Abcam, UK). The next day, membranes were incubated with secondary antibodies at room temperature for 1 hour. Signals were captured with a Bioshine ChemiQ image system. ImageJ software (NIH, Bethesda, MD, USA) was used to quantify the signal intensities of each western blot.

**Table 2** Primes used in RT-qPCR

| Gene   | Forward Primer (5’-3’) | Reverse Primer (3’-5’) |
|--------|------------------------|------------------------|
| Mouse  |                        |                        |
| HDAC2  | GGACAGGCTTGGTTTGTTCAA  | CTCCACCGAGCATCAAT      |
| Col1α1 | TGTAACCTCCTCACCCCA     | TCGTCTGTTCAGGTTGG      |
| α-SMA  | CGGGCTTTGTGTTGTGATG    | CCCCCATGAGTTGGAAAA     |
| β-actin| GCCAACAGTGCTGCTGG      | CTCAGGAGGGAATGTCTTG    |
| Human  |                        |                        |
| HDAC2  | GTACAGTCAAGGAGGCGCAA   | CTTCATGGATGACCTCTGCC   |
| Col1α1 | TCTAGACATGTTCAGCTTTGAC | TCTGACGGAATGATTGTG     |
| α-SMA  | TGTAACGACGCTCAGCTAT    | CTTACAGGAGCCAAGGCAAT   |
| β-actin| GCCAACACAGTGCTGCTGG    | CTCAGGAGGGAATGTCTTG    |
2.12 | Methylation-special PCR (MSP)

DNA samples were treated with Wizard® DNA Clean-Up System (Promega, Co. Madison, USA) according to the manufacturer’s protocols. Unmethylated cytosine was converted to uracil using Methylamp™ DNA Modification Kit (Epigentek, Inc USA) in purified DNA samples. Primers for the methylated and unmethylated promoter regions of miR-455-3p were as follows:
FIGURE 1  The expression of HDAC2 in vivo and in vitro. A, The procedure of mouse HF and HF recovery model used for C57BL/6j mice. B, Pathology observation of H&E, Masson staining, Sirius Red staining (100×). C, Serum ALT and AST levels of mice in vehicle group, HF group, and HF reversal group. D, The protein levels of α-SMA, COL1α1, and HDAC2 in mouse liver tissues detected by western blotting. E, The mRNA levels of α-SMA, COL1α1, and HDAC2 in mouse liver tissues detected by RT-qPCR. The protein (F) and mRNA (G) levels of α-SMA, COL1α1, and HDAC2 in LX-2 cells. H, The mRNA levels of α-SMA, COL1α1, and HDAC2 in primary HSCs isolated from mouse fibrotic livers. I, The mRNA levels of α-SMA, COL1α1, and HDAC2 in liver tissues obtained from patients with cirrhosis. The data represent the mean ± SEM, (n = 6).

*$$P<.05$$, **$$P<.01$$, ***$$P<.001$$ versus vehicle; #$$P<.05$$, ##$$P<.01$$, ###$$P<.001$$ versus CCl4.

| Forward (5′-3′) | Reverse (3′-5′) |
|-----------------|-----------------|
| miR-455-3p-U    | GGTGTTTTTGGTTTTT | CAACCTTTTAAACCT |
|                 | GTAGGAAGTAGT     | AACTAACAC       |
| miR-455-3p-M    | TGGTTTTGGTTGGGT  | GACCTTTAAACCCTA |
|                 | AGGAAGTGC        | ACTACACG        |

2.13 | Chromatin immunoprecipitation (ChIP) assay

q-LX-2 and a-LX-2 cells were cultured to around 80%-90% density. Subsequently, ChIP assay was performed with the SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) #9003 (Cell Signaling Technology, USA) according to the manufacturer’s protocol. Rabbit anti-DNMT3a, rabbit anti-DNMT3b, rabbit anti-DNMT1 antibodies, and normal rabbit IgG were utilized to immunoprecipitate cross-linked protein-DNA complexes. Finally, the immunoprecipitated DNA was subjected to PCR analyses to quantitate the content of putative binding sites within the promoter of miR-455-3p.

2.14 | Flow cytometry

LX-2 cells were cultured, collected, and resuspended in binding buffer (400 μL) for analysis by flow cytometry. Then, 5 μL Annexin V-FITC and PI were added (5 minutes, 2°C-8°C, dark). A flow cytometer (BD Biosciences) was used to detect the cell suspension within 1 hour. All samples were assayed in triplicate.

2.15 | CCK-8 assay

The proliferation of LX-2 cells was detected by Cell Counting Kit-8 (CCK-8) analysis. One-hundred microliters of LX-2 cell suspension was seeded in 96-well culture plates at a density of approximately 5×10³ cells per well. After the cells were attached and a series of operations were performed (TGF-β induction in the presence or absence of MDI treatment and transient transfection), 10 μL of CCK-8 (Sigma, United States) was added for 4 hours. The absorbance (A) was detected at 490 nm wavelength. Cell viability = the A of treatment group wells/the A of control wells * 100%. All experiments were repeated at least three times.

2.16 | Statistical analysis

The data are expressed as the mean ± SEM. One-way analysis of variance followed by the Newman–Keuls post-hoc test (Prism 5.0 GraphPad Software, Inc, San Diego, CA, USA) was used to analyze the results. These results provided a summary of the data from at least three experiments. Differences with P values < .05 were considered statistically significant.

3 | RESULTS

3.1 | Successful establishment of mouse models of HF and its reversal, and upregulation of HDAC2 expression in vivo and in vitro

The procedure used for C57BL/6j mouse treatment is shown in Figure 1A. To verify the successful construction of mouse HF models caused by CCl4 and mouse models of HF spontaneous regression, histopathological analysis was performed. H&E staining showed that liver tissues from mice treated with CCl4 (10% in olive oil) presented significant liver fibrosis, including hepatic steatosis, infiltration of inflammatory cells, and structural disorder of hepatic lobules compared to the vehicle-treated group. Collagen deposition was observed by Masson trichrome staining and Sirius red staining (Figure 1B). The results of double immunofluorescence showed that HDAC2 was ubiquitous in the mouse liver, and high expression of HDAC2 (green) in α-SMA-positive cells (red) in fibrotic areas implied that HDAC2 could be expressed in hepatic stellate cells (S1A). Moreover, the gross appearance of liver tissues displayed hepatomegaly after treatment with CCl4. In contrast, the liver tissues from spontaneous recovery mice showed smooth surface and normal lobular architecture, including radiating hepatic cords and central veins (Figure 1B). In addition, a dramatic increase in serum ALT and AST levels was observed in CCl4-induced fibrotic mice compared to the vehicle group which reverted to the baseline level in the reversal group (Figure 1C). In addition, western blotting and RT-qPCR analyses showed that the protein and mRNA levels of α-SMA and COL1α1 were significantly increased in mouse fibrotic liver tissues (Figure 1D,E), TGF-β-induced the activation of LX-2 cells (Figure 1F,G), and primary HSCs isolated from mice
treated with CCl₄ (Figure 1H). The expression of α-SMA and COL1α1 (S1B) was increased in LX-2 cells cultured with TGF-β as determined by immunofluorescence staining. After the termination of CCl₄ treatment for 4 weeks, the protein and mRNA levels of both α-SMA and COL1α1 decreased and gradually returned to normal in mouse liver tissues and
primary HSCs isolated from mouse recovery liver tissues (Figure 1D,E,H). With the addition of MDI medium, a-LX-2 cells can revert to a quiescent phenotype. The downregulation of α-SMA and COL1α1, as shown by immunofluorescence (S1B), indicated the successful reversal of LX-2 cells. In addition, the mRNA levels of α-SMA and COL1α1 in liver tissues obtained from patients with cirrhosis were significantly increased (Figure 1I). Together, these data indicated the fibrotic progression and regression models were successfully established in vivo and in vitro. In our preliminary research, we found an upward trend of HDAC2 in mouse fibrotic liver tissues obtained from patients with cirrhosis were significantly increased (Figure 1I). Together, these data indicated that the fibrotic progression and regression models were successfully established in vivo and in vitro. In our preliminary research, we found an upward trend of HDAC2 in mouse fibrotic liver tissues obtained from patients with cirrhosis were significantly increased (Figure 1I).

### 3.2 miR-455-3p is a direct upstream target of HDAC2

To investigate the upstream regulatory target of HDAC2, TargetScan, a public prediction platform, was used which predicted a large number of target miRNAs. Among them, miR-455-3p, which has important roles in proliferation and apoptosis in cancers and fibrotic diseases, attracted our attention. As shown in Figure 2A, the sequence information of the binding sites between 3’ UTR of HDAC2 mRNA and miR-455-3p predicted by TargetScan, indicated that there were two potential consequent pairings of the target region in HDAC2 mRNA 3’ UTR: a site spanning 105-111 (conserved) nucleotides and a site at position 7510-7516 (poorly conserved) of HDAC2 3’ UTR. Based on the predicted results, we speculated that miR-455-3p inhibits the expression of HDAC2 by binding to the predicted binding sites of HDAC2, thus playing a regulatory role. To further explore the relationships between the two, we tested the expression of miR-455-3p in liver tissues, LX-2 cells, and primary HSCs. As shown in Figure 2B,E, miR-455-3p level was dramatically decreased in mouse fibrotic liver tissues but rebounded to a certain extent in the recovery liver, and was decreased in human liver tissues obtained from patients with cirrhosis. In TGF-β-treated LX-2 cells, miR-455-3p level was notably decreased compared to q-LX-2 cells and MDI medium-induced inactivation of LX-2 cells (Figure 2C). As shown in Figure 2D, the level of miR-455-3p was notably decreased in primary HSCs isolated from mouse fibrotic livers relative to primary HSCs isolated from the vehicle and reversal groups. In addition, we performed transient transfection trials in LX-2 cells to investigate the functional relationship between miR-455-3p and HDAC2. Figure 2F shows the successful transfection of miR-455-3p mimics and the corresponding inhibitor by detecting the expression of miR-455-3p in LX-2 cells transfected with miR-455-3p mimics or inhibitor. As shown in Figure 2G,H, miR-455-3p mimics remarkably reduced HDAC2 protein and mRNA expression levels in a-LX-2 cells and the use of the miR-455-3p inhibitor markedly upregulated HDAC2 expression in i-LX-2 cells. These data demonstrated a negative correlation between miR-455-3p and HDAC2. To establish the effect of miR-455-3p on the 3’ UTR of HDAC2 mRNA, a dual luciferase assay was performed. Then, an HDAC2 fragment with wild-type or mutant complementary binding sites was inserted into the luciferase reporter gene psiCHECK-2, with the miR-455-3p mimics group compared to the negative control. As shown in Figure 2I, upon co-transfection of HEK-293T cells with a HDAC2-WT reporter vector and miR-455-3p using Lipofectamine 3000, luciferase activity was significantly decreased. Binding sites of miR-455-3p were identified within the HDAC2 sequences (Figure 2J). These results revealed that miR-455-3p targeted the 3’ UTR of HDAC2 and regulated its expression specifically.

### 3.3 The functional roles of miR-455-3p in a-LX-2 and i-LX-2 cells

Given that miR-455-3p binds to the 3’ UTR of HDAC2, we explored its function in LX-2 cells. First, to investigate whether ectopic expression of miR-455-3p in a-LX-2 cells influenced their activation, proliferation, and apoptosis, LX-2 cells were treated with TGF-β (10 ng/mL) for 24 hours and transfected with miR-455-3p mimics or miR-NC (negative control). Twenty-four hours after transfection, the expression levels of genes related to LX-2 cell activation (α-SMA and COL1α1), cell cycle-related proteins (C-myc
and CyclinD1), and apoptosis-associated proteins (Bax and Bcl2) were examined. As expected, forced ectopic expression of miR-455-3p noticeably decreased the protein and mRNA levels of α-SMA and COL1α1 compared to the miR-NC group in α-LX-2 cells (Figure 3A,B) and downregulated their viability (Figure 3C). Furthermore, the protein
expression levels of cell-cycle-related-proteins, C-myc and CyclinD1, were notably attenuated in miR-455-3p-mimics-transfected group compared to the miR-NC-transfected group (Figure 3D). These results indicated that excessive expression of miR-455-3p in a-LX-2 cells inhibits their activation and proliferation. The effect of forced expression of miR-455-3p on the survival of a-LX-2 cells was further investigated by flow cytometry. As shown in Figure 3E,F, overexpression of miR-455-3p increased the ratio of apoptotic cells and the apoptosis-associated protein; the ratio of Bax/Bcl2 was elevated in miR-455-3p mimic-transfected group compared to the miR-NC transfected group. Thus, these results revealed that ectopic expression of miR-455-3p inhibits activation and proliferation of a-LX-2 cells and promotes apoptosis in vitro.

We further explored the functional role of miR-455-3p silencing in i-LX-2 cells; miR-455-3p inhibitor and negative control transfection were utilized. As shown in Figure 3G,H, α-SMA and COL1α1 at both the protein and mRNA levels were significantly upregulated in the miR-455-3p inhibitor-transfected group, as analyzed by western blotting and RT-qPCR. Meanwhile, silencing miR-455-3p expression increased cell viability significantly compared to the miR-455-3p-NC group (Figure 3I). In addition, the inhibition of miR-455-3p led to increased production of cell-cycle-associated proteins (C-myc and CyclinD1) (Figure 3J). These data implied that the knockdown of miR-455-3p enhanced the activation and proliferation of i-LX-2 cells.

3.4 | miR-455-3p regulates the activation and apoptosis of LX-2 cells by targeting HDAC2

Our results indicate that miR-455-3p has a specific effect on LX-2 cells and can bind to the 3’ UTR of HDAC2. To test whether the role of miR-455-3p was by targeting HDAC2 and whether HDAC2 restoration attenuates the effects of miR-455-3p, we co-transfected a-LX-2 cells with pEX-3-HDAC2 plasmids and miR-455-3p mimics. The i-LX-2 cells were treated with HDAC2 siRNA and miR-455-3p inhibitor. The cells were collected 24 hours after transfection. As shown in Figure 4A, the protein levels of α-SMA and COL1α1 were significantly downregulated in the mim-miR-455-3p+pEX-3-NC transfected group compared to that in the mim-NC+pEX-3-NC group. Interestingly, the expression levels of α-SMA and COL1α1 rebounded to normal in the mim-miR-455-3p+pEX-3-HDAC2 co-transfected group. Similarly, the content of cell-cycle-related proteins, C-myc and CyclinD1 was also decreased in the mim-miR-455-3p+pEX-3-NC transfected group compared to that in the mim-NC+pEX-3-NC group, whereas with the addition of pEX-3-HDAC2, the expression level was significantly increased. In addition, an increasing ratio of Bax/Bcl2 was observed in the mim-miR-455-3p+pEX-3-NC transfected group compared to mim-NC+pEX-3-NC group, but expression level of Bax/Bcl2 decreased after pEX-3-HDAC2 transfection. Flow cytometry (Figure 4B) and TUNEL staining results (Figure S2A) were similar to those of western blotting. CCK-8 assay revealed that the viability of a-LX-2 cells was significantly decreased in the mim-miR-455-3p+pEX-3-NC transfected group compared to that in the mim-NC+pEX3-NC group, and the viability increased again in the mim-miR-455-3p+pEX-3-HDAC2 co-transfected group (Figure 4C). These data indicated that pEX-3-HDAC2 plasmids could block the action of miR-455-3p mimics, at least in part, in a-LX-2 cells. To further study the relationship of miR-455-3p with HDAC2, we co-transfected HDAC2 siRNA and miR-455-3p inhibitor in i-LX-2 cells. As shown in Figure 4D, the protein expression levels of α-SMA, COL1α1, and cell-cycle-related proteins (C-myc and cyclinD1) were significantly upregulated in inh-miR-455-3p+scrambled-RNAi transfected group compared to that in the inh-miR-NC+scrambled-RNAi transfected group. Interestingly, while HDAC2 was knocked down, the protein levels decreased. CCK-8 assay suggested that the viability of i-LX-2 cells was noticeably increased in inh-miR-455-3p+scrambled-RNAi transfected group compared to inh-miR-NC+scrambled-RNAi transfected group, but was inhibited again in the inh-miR-455-3p+HDAC2 RNAi transfected group compared to inh-miR-455-3p+scrambled-RNAi group (Figure 4E). These data indicate that HDAC2 is a downstream target of miR-455-3p in LX-2 cells. Thus, the function of miR-455-3p on LX-2 cells could be partially blocked by the restoration or loss of HDAC2, indicating that
miR-455-3p regulates the activation and apoptosis of LX-2 cells by targeting HDAC2, at least partly.

3.5 The dynamic expression change of miR-455-3p is associated with epigenetic modification in LX-2 cells and can be restored by DNMTs-RNAi

Many studies in our group and other laboratories have shown that DNA methylation plays an important role in the regulation of HSC activation. The three major DNA methyltransferases that catalyze DNA methylation are DNMT3a, DNMT3b, and DNMT1, all of which have been shown to be upregulated in activated HSCs. Excess expression of DNMTs resulted in aberrant DNA methylation patterns. In the present study, we detected significant elevation of DNMT3a, DNMT3b, and DNMT1 levels in a-LX-2 cells, but a decline in i-LX-2 cells (Figure 5A).

To investigate whether the downregulated expression of miR-455-3p in a-LX-2 cells was associated with promoter methylation, we performed a prediction on the website. As shown in Figure 5B, the red and blue regions indicate two predicted CpG island sites in the miR-455-3p gene promoter. The CpG islands of most nonimprinted genes are thought to remain unmethylated in normal status but may be methylated when damaged. Through the interaction between methyl CpG binding proteins, histones, and HDACs, methylation of CpG islands occur, which contributes to changes in chromatin that cause transcriptional silencing. To further investigate whether the downregulated expression of miR-455-3p was caused by aberrant promoter methylation, DNMT3a-RNAi, DNMT3b-RNAi, and DNMT1-RNAi were utilized to knockdown DNMT3a, DNMT3b, and DNMT1 in LX-2 cells, respectively. As shown in Figure 5C, the expression of DNMTs decreased, indicating that the transfection trial was performed successfully. In addition, in TGF-β-treated LX-2 cells, the expression of miR-455-3p was elevated in the DNMT3b-RNAi- and DNMT1-RNAi-transfected group but not in the DNMT3a-RNAi-transfected group, and there was no significant change in the expression of miR-455-3p between q-LX-2 cells transfected with DNMTs-RNAi and scrambled-RNAi. MSP analysis indicated that the miR-455-3p gene was mainly amplified by miR-455-3p unmethylated primer (miR-455-3p-U) in q-LX-2 cells, whereas it was mainly amplified by miR-455-3p methylated primer (miR-455-3p-M) in TGF-β-induced a-LX-2 cells (Figure 5D), which means that TGF-β induced ectopic hypermethylation of the miR-455-3p promoter in LX-2 cells and hypermethylation occurs in a-LX-2 cells. To verify the reliability of the MSP results, the BSP method was used to detect the methylation levels of the miR-455-3p promoter. As shown in Figure 5E, when LX-2 cells were activated using TGF-β, the methylation level of the miR-455-3p promoter region was increased compared to q-LX-2 cells. The BSP results further confirmed the above MSP conclusion. Furthermore, the ChIP assay revealed that the miR-455-3p promoter could be pulled down by anti-DNMT3b and anti-DNMT1 antibodies. However, anti-DNMT3a and negative control anti-IgG antibody failed to pull down the miR-455-3p promoter (Figure 5F). The ChIP assay results suggest a direct binding of DNMT3b and DNMT1 with the miR-455-3p promoter in LX-2 cells. Thus, all the above results showed that decreased miR-455-3p expression in a-LX-2 cells was related to epigenetic modification and miR-455-3p promoter methylation was mainly mediated by DNMT3b and DNMT1.

4 DISCUSSION

Liver fibrosis, because of its rapidly rising morbidity, high mortality, and no effective treatment measures, is a challenging illness. Nevertheless, the prolonged lifespan and reduced incidence of hepatocellular carcinoma can be a criterion for successful liver fibrosis therapies. After chronic injury to the liver, the injured hepatocytes undergo apoptosis, Kupffer cells activate, and lymphocytes infiltrate the liver parenchyma, which drives qHSCs activation into aHSCs, ultimately leading to the increased production and deposition of ECM proteins which triggers fibrotic progression. The prospect of liver fibrosis reversibility is remarkable, and experiments with antiviral drugs have set up an explicit proof of concept for this possibility in humans. In animal model trials, HSCs have been identified as a vital target. When liver injury is attenuated, some aHSCs revert into iHSCs, while others undergo apoptosis. It is unknown whether the master switches to push aHSCs toward qHSC reversion or death, but a previous finding established that a significant number of stellate cells reacquire features of quiescence. Interestingly, the previously injured liver is more susceptible...
to fibrogenesis than a healthy liver, because iHSCs are more prone to aHSCs transdifferentiation after pro-fibrogenic stimulus. Hence, through apoptosis of aHSCs to reduce the number of fibrogenic cells or by inducing aHSCs to revert into an inactivated state with restored features of quiescence, liver fibrosis recovery can occur.\textsuperscript{2,6,55}

HDAC2, a member of class I HDAC proteins, maintains the deacetylation level of histones. A considerable number of
Downregulation of miR-455-3p expression is mediated by DNMT3b and DNMT1. A. Protein levels of DNMT3a, DNMT3b, and DNMT1 in LX-2 cells. B. The CpG islands of miR-455-3p promoter were predicted. The red and blue region represented CpG islands where methylation occurs. C, miR-455-3p expression level was measured by RT-qPCR in LX-2 cells transfected with DNMTs-RNAi and the efficiency of DNMT3a, DNMT3b, and DNMT1 loss-expression was detected by western blotting. D. The methylation level of miR-455-3p promoter in quiescent and activated LX-2 cells was detected by MSP assay. M: methylation; U: unmethylation. E. Methylation of miR-455-3p in the promoter region was detected by BSP. Yellow indicates methylation, blue indicates unmethylation. F. ChIP assay showing the interaction of DNMT3a, DNMT3b, and DNMT1 with the potential binding sites on the miR-455-3p promoter. $P < .01, @@ @ P < .001 versus LX-2 cells transfected with scrambled RNA. **P < .01, as indicated.
hypermethylation occurs in α-LX-2 cells and hypomethylation occurs in i-LX-2 cells. The ChIP assay indicated that the methylation of miR-455-3p is attributed to DNMT3b and DNMT1.

In summary, our findings in the present study demonstrated that miR-455-3p plays a pivotal role during LX-2 cell activation and reversion by targeting HDAC2, and epigenetic modification is involved in regulating its level. The overexpression of miR-455-3p offsets TGF-β-treated LX-2 cell activation and promotes the inactivation of cells by targeting HDAC2. In miR-455-3p inhibitor-treated i-LX-2 cells, we observed the opposite phenomenon. Thus, our data indicated a crucial role of miR-455-3p by targeting HDAC2 in regulating the state of hepatic stellate cells and may serve as a potential therapeutic drug candidate for liver fibrosis.

Furthermore, the decreased expression of miR-455-3p in α-LX-2 cells is attributed to DNMT3b and DNMT1-induced methylation of the miR-455-3p promoter.

MiRNAs regulate diverse biological functions in the pathogenesis of liver diseases, which makes them attractive therapeutic targets for various types of liver diseases, and have been tested in preclinical models. Based on our expression exploration and functional research of miR-455-3p in liver fibrosis, we suggest that miR-455-3p has the potential to act as a promising biomarker and therapeutic drug for the treatment of hepatic fibrosis. In other words, the upregulated expression of hepatic miR-455-3p during liver fibrosis may be a defensive response to relieve fibrosis. In addition, a recent study demonstrated that miR-455-3p-enriched exosomes derived from human umbilical cord mesenchymal stem cells inhibit

**FIGURE 6** Schematic depicting the mechanisms underlying the miR-455-3p/HDAC2-mediated prevention of liver fibrosis. miR-455-3p was selectively downregulated in hepatic fibrosis mediated by DNMT3b and DNMT1. Excess expression of miR-455-3p could alleviate liver injury by targeting HDAC2 to increase the acetylation of antifibrotic genes, which would lead to increased expression of “good” genes. However, the silencing of miR-455-3p exacerbates the process of liver fibrosis in vitro. Briefly, miR-455-3p/HDAC2 may serve as a new potential therapeutic target in the treatment of liver fibrosis.
the activation and cytokine production of macrophages challenged with lipopolysaccharide in IL-6-induced acute liver injury.\textsuperscript{74} miR-455-3p has been shown to play an important role in Alzheimer’s disease (AD) pathogenesis.\textsuperscript{75} Aging is the most important risk factor for AD and many other aging-related human diseases.\textsuperscript{76,77} Fibrotic diseases are a major cause of morbidity and mortality in the aging process of humans worldwide. Treatment of fibrosis is a potential strategy for slowing the aging process and prolonging lifespan.\textsuperscript{78,79} These results indicate that miR-455-3p may be a potential therapeutic target for liver diseases and aging-related human diseases.

In the current study, we demonstrated that HDAC2 is a direct target and essential mediator of miR-455-3p in LX-2 cells. We detected the expression of miR-455-3p in the progression and reversal of liver fibrosis in vivo and in vitro for the first time. We found that overexpression of miR-455-3p inhibits activation and promotes apoptosis in a-LX-2 cells. In addition, knockdown of miR-455-3p accelerated the activation and proliferation of i-LX-2 cells. In all, we explored the mechanisms of decreased miR-455-3p expression in a-LX-2 cells. MSP, BSP, and ChIP assay results indicated that the decreased expression of miR-455-3p in a-LX-2 cells is attributed to DNMT3b and DNMT1-induced methylation of the miR-455-3p promoter. Thus, our data illustrated in Figure 6 indicates a crucial role of miR-455-3p in regulating the state of hepatic stellate cells by targeting HDAC2 and may serve as a potential therapeutic approach for liver fibrosis.

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CONFLICT OF INTEREST
The authors have declared that no competing interest exists.

AUTHOR CONTRIBUTIONS
HMY and LW conceived and carried out experiments, analyzed the data, and wrote the manuscript. FTB, HWM, and XYP provided technical assistance during the experiment. JYL, YFZ, AW, and NNY isolated primary HSCs. CH and JL participated in the design of this study and revised the manuscript. All authors contributed to the writing of the manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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