miR221 regulates TGF-β1-induced HSC activation through inhibiting autophagy by directly targeting LAMP2

RAN CHENG[1], HAO XU[2] and YANG HONG[3,4]

[1]The First Department of Hepatobiliary Surgery, Tangshan Gongren Hospital, Tangshan, Hebei 063000; [2]Medical Insurance Office; [3]Department of Clinical Pharmacology, The Fourth Hospital of Hebei Medical University; [4]Hebei Provincial Key Laboratory of Tumor Microenvironment and Drug Resistance, Shijiazhuang, Hebei 050000, P.R. China

Received June 4, 2021; Accepted August 19, 2021

DOI: 10.3892/mmr.2021.12417

Abstract. Liver fibrosis is a serious threat to human life and health. Activated hepatic stellate cells (HSCs) play a key role in the occurrence and development of liver fibrosis. Studies have reported that microRNAs (miRNAs/miRs) are involved in the pathological process of fibrosis, as well as its relevance in clinical diagnosis. However, the role of miR221 in hepatic fibrosis remains controversial. Remarkably, transforming growth factor-β (TGF-β1) caused HSC dysfunction in autophagic activation, characterized by an increase in P62 aggregation and LC3II expression. The present study aimed to determine whether autophagy regulates hepatic fibrosis by mediating HSC activation and explore the potential targets leading to the sequence of events associated with miR221. The expression of miR221 was quantified in a liver fibrosis model *in vivo* and *in vitro*, and its specific target gene lysosome-associated membrane glycoprotein 2 (LAMP2) was predicted by bioinformatics. The results showed that the expression levels of collagen-I (COL-I) and α-smooth muscle actin (α-SMA) were increased in miR221-overexpressing LX2 cells, while the autophagy inducer rapamycin reversed the inhibition of autophagic flux induced by miR221. Additionally, the overexpression of LAMP2 could significantly inhibit TGF-β1-induced COL-I and α-SMA expression, which was similar to the effect of the miR221 inhibitor on the regulation of TGF-β1-induced HSC activation. These results indicated that miR221 may regulate TGF-β1-induced HSC activation through inhibiting autolysosome function by directly targeting LAMP2. The molecular mechanism of miR221 in regulating TGF-β1-induced HSC activation may provide novel insight into therapies to ameliorate the pathological progression of liver fibrosis.

Correspondence to: Dr Yang Hong, Hebei Provincial Key Laboratory of Tumor Microenvironment and Drug Resistance, 12 Jiankang Road, Shijiazhuang, Hebei 050000, P.R. China
E-mail: younghong1002@163.com

Key words: liver fibrosis, microRNA221, transforming growth factor-β, lysosome-associated membrane glycoprotein 2

Introduction

As a response to chronic injury, liver fibrosis is caused by various etiologies, such as viral infection, alcohol, cholestasis and metabolic diseases (1). The pathological process of liver fibrosis is produced by the interaction of various cytokines and growth factors, and it may result from the imbalance between the synthesis and degradation of extracellular matrix (ECM) in liver tissue (2). Several studies have shown that abnormal activation of hepatic stellate cells (HSCs) promotes the progression of liver fibrosis. Transforming growth factor-β (TGF-β) is activated from the ECM and released from HSCs (3-6). In cooperation with other signaling pathways, such as reactive oxygen species (ROS) and platelet-derived growth factor (PDGF), TGF-β signaling is considered the key fibrogenic pathway that drives HSC activation (7).

Autophagy is a lysosome-dependent cellular homeostasis mechanism that regulates intracellular homeostasis (8). Efficient autophagy involves a series of intracellular signaling cascade events involving mTOR kinase activation, LC3 processing and the formation of autolysosomes (9). This selective removal of autophagic substrates is regulated by P62/SQSMT1 (P62), which is closely related to the conversion of LC3I to LC3II and the formation of autolysosomes (10). Recently, studies have shown that autophagy is implicated in a variety of liver diseases (11,12). Induction of autophagy may promote the degradation of ECM components, such as collagen, and improve the process of hepatic fibrosis (13). Therefore, it was hypothesized in the present study that the pathogenic mechanism of liver fibrosis is the key to finding effective therapeutic targets to prevent its progression and even reverse liver fibrosis. However, the regulatory mechanism of autophagy during HSC activation is still controversial.

MicroRNAs (miRNAs/miRs) are a family of single-stranded non-coding small molecules (18-25 nucleotides in length) that are involved in mediating gene expression by cleaving and destabilizing the targeted mRNAs at the post-transcriptional level (14). Evidence has shown that miRNAs are functionally implicated in various cellular biological mechanisms, such as cell growth, development, differentiation, proliferation and apoptosis (15-17), and they have relevance in clinical diagnosis (18). The differential expression of miRNAs, especially miR221, is upregulated in

liver fibrosis tissue. Roderburg *et al* (19) identified 21 downregulated and 10 upregulated miRNAs in fibrotic livers, especially miR221, which was observed to be upregulated in CCl_4-induced mice. However, the role of miR221 in hepatic fibrosis remains unclear. In previous years, miR221 has been widely reported to act as a tumor modulator involved in tumor growth and invasiveness, especially hepatocellular carcinoma (20,21). Researchers have increasingly been concerned about the biological role of miR221 in regulating hepatocellular carcinoma development (21). According to reports, astrocyte elevated gene-1 protein/miR221 can regulate the progression of liver cancer (22), and it also plays a crucial role in regulating hepatocellular carcinoma migration by targeting lysine-specific demethylase PHF2 (23). Remarkably, the pathological behavior of activated HSCs is very similar to that of hepatoma cells (24). Activated HSCs show enhanced proliferation and TGF-β1 secretion in large quantities (25), while TGF-β1 is significantly increased in patients with liver cancer (26).

In the present study, it was determined whether autophagy regulates hepatic fibrosis by regulating TGF-β1-induced HSC activation, and then the potential regulatory gene targets leading to the sequence of events associated with miR221 in HSCs were investigated.

Materials and methods

Animals and treatment. A total of 10 adult male C57BL/6 mice weighing 20-25 g (age, 6-8 weeks) were obtained from the Animal Research Center of Hebei Medical University (Shijiazhuang, China). They were housed at a temperature of 25±2˚C and 50% humidity, with free access to food and water (12 h light and dark cycles). All protocols were approved by the Hebei Medical University Institutional Animal Experimental Ethics Committee (approval no. IACUC-Hebmu-2020005) and carried out according to the provisions of the guidelines for the Care and Use of Laboratory Animals (27). The model of liver fibrosis was prepared according to a previous report by Hyun *et al* (28). Mice (n=5) were exposed to CCl_4 (Sigma-Aldrich; Merck KGaA) twice a week for 10 weeks by intraperitoneal injection to establish the *in vivo* model. In the control group for comparison, the same regimen was performed with corn oil. All liver fibrosis model mice were sacrificed by cervical dislocation, and liver samples were obtained.

Cell culture and treatment. LX2 cells (a human HSC line; Procell Life Science & Technology Co., Ltd.) were obtained from cryopreservation. LX2 cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and incubated in humidified 5% CO_2 at 37˚C. The medium was changed every 24 h. Cultured LX2 cells were used after 3 days of incubation. Then, LX2 cells ($1x10^5$) were treated with TGF-β1 (Sigma-Aldrich; Merck KGaA) in a 37˚C incubator with 5% CO_2 for 24 h to establish the *in vitro* model.

To verify the role of miR221 in HSC activation and LAMP2 expression, cultured LX2 cells were transfected with miR221 mimics, miR-negative controls (NCs), miR221 inhibitor and LAMP2 overexpression vector. After transfection, the medium was changed to fresh medium containing 10% FBS and incubated at 37˚C and 5% CO_2 for 24 h. Then, the transfected cells were treated with TGF-β1 (μM) for 24 h in a 37˚C incubator with 5% CO_2. Additionally, to assess the role of autophagy in regulating HSC activation, the autophagy inducer rapamycin (RAPA; 5 μM; Cell Signaling Technology, Inc.) was added for 1 h prior to TGF-β1 cotreatment for 24 h in a 37˚C incubator with 5% CO_2. All of the aforementioned cells were harvested for RNA/miRNA isolation, and cell lysates were collected for western blot analysis.

Cell transfection. LX2 cells were used for cell transfection. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was diluted with Optimem I (Gibco; Thermo Fisher Scientific, Inc.) to a selected optimal concentration and then transfected into LX2 cells. LX2 cells were well cultured until 60-70% confluence. LX2 cells ($1x10^5$) were transfected with 50 nM miR221 mimics (5'-ACCUGGCAUACAAUGUAGA UUU-3'), 100 nM miR221 inhibitor (5'-AAAUCUACAUUGU AUGCCAGGU-3') and their matched negative controls (25 nM NC-miR mimic, 5'-UUCUCCGAACGUGUCACGUTT-3' and 25 nM NC-miR inhibitor, 5'-CAGUACUUUUGUGUAGUA CAA-3') using Lipofectamine® 2000 in a 37˚C incubator with 5% CO_2. The cells were collected 48 h after transfection. miR221 mimics, miR-NCs and miR221 inhibitor were purchased from Guangzhou RiboBio Co., Ltd. Furthermore, LAMP2 overexpression plasmids (LAMP2 group; Shanghai GeneChem Co., Ltd.) or empty pcDNA3.1 plasmids (NC group; Shanghai GeneChem Co., Ltd.) were transfected into LX2 cells at a multiplicity of infection (MOI) of 50, according to the manufacturer's instructions. The cells were transfected for 48 h at 37˚C with 5% CO_2.

Dual-luciferase reporter assay. LAMP2 3'UTR fragments and mutants containing the miR221 binding site were synthesized and inserted downstream of the luciferase gene in the vector. Mutant (mut) LAMP2 3'UTR was constructed using a site-directed mutagenesis kit (Promega Corporation). The luciferase reporter vector was pGL3 (Shaanxi Youbio Technology Co., Ltd.). $1x10^5$ LX2 cells were transfected with wild-type (wt) LAMP2 3'UTR or mut LAMP2 3'UTR together with miR221 mimics or miR-NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After transfection for 48 h, a dual-luciferase reporter assay was performed to measure the luciferase activity (Promega Corporation). Luciferase activity was normalized against *Renilla* luciferase activity (Promega Corporation).

Reverse transcription-quantitative PCR (RT-qPCR). Primer sequences were designed and synthesized by Sangon Biotech Co., Ltd. Total RNA was extracted from liver tissues or cells by using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using a PrimeScript™ RT kit (Takara Bio, Inc.), according to the manufacturer's protocol. RT-qPCR was performed using SYBR-Green mix (Takara Bio, Inc.) on an ABI Prism 7500 real-time PCR system. Thermocycling conditions were as follows: Initial denaturation at 95˚C for 10 min, 40 cycles of 95˚C for 30 sec and 60˚C for 30 sec. Genes of each sample were amplified in triplicate. The primer sequences were as follows: LAMP2-forward (F),

5'-CUGGCUUUAAAAAAAGGAGAAAA-3' and reverse (R), 5'-ACCCATCTCACCCATTCTTG-3'; miR221-F, 5'-CCTGAA ACCCAGCAGACAA-3' and R, 5'-CAGGTCTGGGGC ATGAAC-3'; and U6-F, 5'-GCGCGTCGTGAAGCGTTC-3' and R, 5'-GTGCAGGGTCCGAGGT-3'. The $2^{-\Delta\Delta Cq}$ method (29) was used to calculate the expression of miRNA or LAMP2 normalized to U6 mRNA.

Western blotting assay. LX2 cells were treated according to the experimental design. LX2 cell lysates were collected in ice-cold RIPA buffer (Beyotime Institute of Biotechnology). Total protein was quantified using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Proteins (5 μg) were separated via SDS-PAGE using 10% gels, and electrotransferred to PVDF membranes (MilliporeSigma), which were incubated with anti-collagen-I (COL-I; cat. no. AF7001; 1:1,000; Affinity Biosciences), α-smooth muscle actin (α-SMA; cat. no. 19245; 1:1,000; Cell Signaling Technology, Inc.), LC3 (cat. no. 2775; 1:1,000; Cell Signaling Technology, Inc.), P62 (cat. no. 8025; 1:1,000; Cell Signaling Technology, Inc.), LAMP2 (cat. no. 49067; 1:1,000; Cell Signaling Technology, Inc.) and β-actin (cat. no. 4970; 1:1,000; Cell Signaling Technology, Inc.) primary antibodies at 4˚C overnight. Subsequently, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-Rabbit IgG secondary antibody (cat. no. SA00001-2; 1:2,000; ProteinTech Group, Inc.) for another 2 h at room temperature. All blots were visualized using enhanced chemiluminescence (ECL) reagents (Thermo Fisher Scientific, Inc.), and the scanned band images were semi-quantified using ImageJ software version 1.8 (National Institutes of Health).

Immunofluorescence assay. LX2 cells were treated according to the experimental design. The cells were fixed with 4% ice-cold paraformaldehyde for 20 min at 4˚C and permeabilized with 0.05% Triton X-100. Cells were treated with 3% BSA (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature and incubated with the primary antibody P62 (cat. no. 7695; 1:100; Cell Signaling Technology, Inc.) overnight at 4˚C. The next day, the cells were stained with FITC-conjugated secondary antibodies (cat. no. ZF-0311; 1:100; OriGene Technologies, Inc.) for another 1 h. After washing with PBS, the nuclei were stained with DAPI for 5 min at room temperature. Fluorescence was visualized under a microscope (IX-81; Olympus Corporation).

Bioinformatics analysis. Bioinformatics analysis was performed to determine the target gene of miR221. The possible regulatory genes of miR221 were obtained using TargetScan 3.1 (http://www.targetscan.org/vert_72/), and information on the predicted autophagy-related genes were chosen by searching the Human Autophagy-dedicated Database (HADb; autophagy.lu). Then, the targets from TargetScan and HADb were overlapped, and potential autophagy-related genes were identified, which were reported to be closely related to the degradation of autolysosome.

Statistical analysis. Each experiment was performed in triplicate. Data are expressed as the mean ± SD. The significant differences between experimental groups were determined by unpaired Student's t-test or one-way ANOVA followed by the Tukey's test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

TGF-β1-induced HSC activation is partially subject to autophagy dysfunction. Considering the potential function of TGF-β1 in inducing HSC activation, relevant markers for hepatic fibrosis were detected, including COL-I and α-SMA, which are involved in the activation of HSCs (30). The western blotting results revealed that TGF-β1 significantly induced COL-I and α-SMA expression at concentrations of 5 and 10 μM compared with the control group ($P<0.05$; Fig. 1A). Based on these results, intracellular autophagy changes were also detected by western blotting and immunofluorescence. The results showed that the conversion of LC3I to LC3II was increased in TGF-β1-treated LX2 cells. However, the expression of P62 was conversely elevated ($P<0.05$; Fig. 1B and C). Furthermore, LX2 cells were treated with the autophagy inducer RAPA (5 or 10 μM) following TGF-β1 treatment. The western blotting results showed that the levels of COL-I and α-SMA expression were decreased after RAPA treatment in TGF-β1-treated LX2 cells compared with the TGF-β1-treated group ($P<0.05$; Fig. 1D). The aforementioned results implied a potential relationship between HSC activation and autophagy deficiency in liver fibrosis.

miR221 is upregulated in liver fibrosis mice and activated HSCs. The differential expression of miRNAs, especially miR221, has been found to be upregulated in liver fibrosis tissues (14). To study the function of miR221 in activated HSCs, a liver fibrosis model was constructed *in vivo* and *in vitro* (21) and the expression changes of miR221 were verified in liver fibrosis mice and TGF-β1-treated LX2 cells. Based on the RT-qPCR results, the expression of miR221 in liver fibrosis mice was significantly increased ($P<0.05$; Fig. 2A). Furthermore, miR221 expression was significantly increased in the LX2 model group compared with the control group ($P<0.05$; Fig. 2A). To evaluate whether miR221 expression played a role in activated HSCs, miR221 mimics, miR221 inhibitor and their matched NCs were transfected into LX2 cells. The RT-qPCR results showed that miR221 was increased in LX2 cells transfected with miR221 mimics and decreased in miR221 inhibitor-transfected LX2 cells ($P<0.05$; Fig. 2B). In addition, COL-I and α-SMA expression levels were significantly increased in LX2 cells transfected with miR221 mimics compared with the NC group ($P<0.05$; Fig. 2C-E). Whereas, the expression levels of COL-I and α-SMA were decreased in LX2 cells in the miR221 inhibitor + TGF-β1 group compared with the TGF-β1-treated group ($P<0.05$; Fig. 2C-E). These data revealed that miR221 may play a crucial role in HSC activation.

miR221 inhibits autophagy in LX2 cells. To verify the role of miR221 in modulating autophagy activation or disrupting the process of autophagy initiation, intracellular autophagy functional changes were detected in miR221-overexpressing LX2 cells by using immunofluorescence. The results showed that a mass of P62 was aggregated in miR221 mimic-transfected LX2 cells, but no obvious autolysosome dysfunction was observed

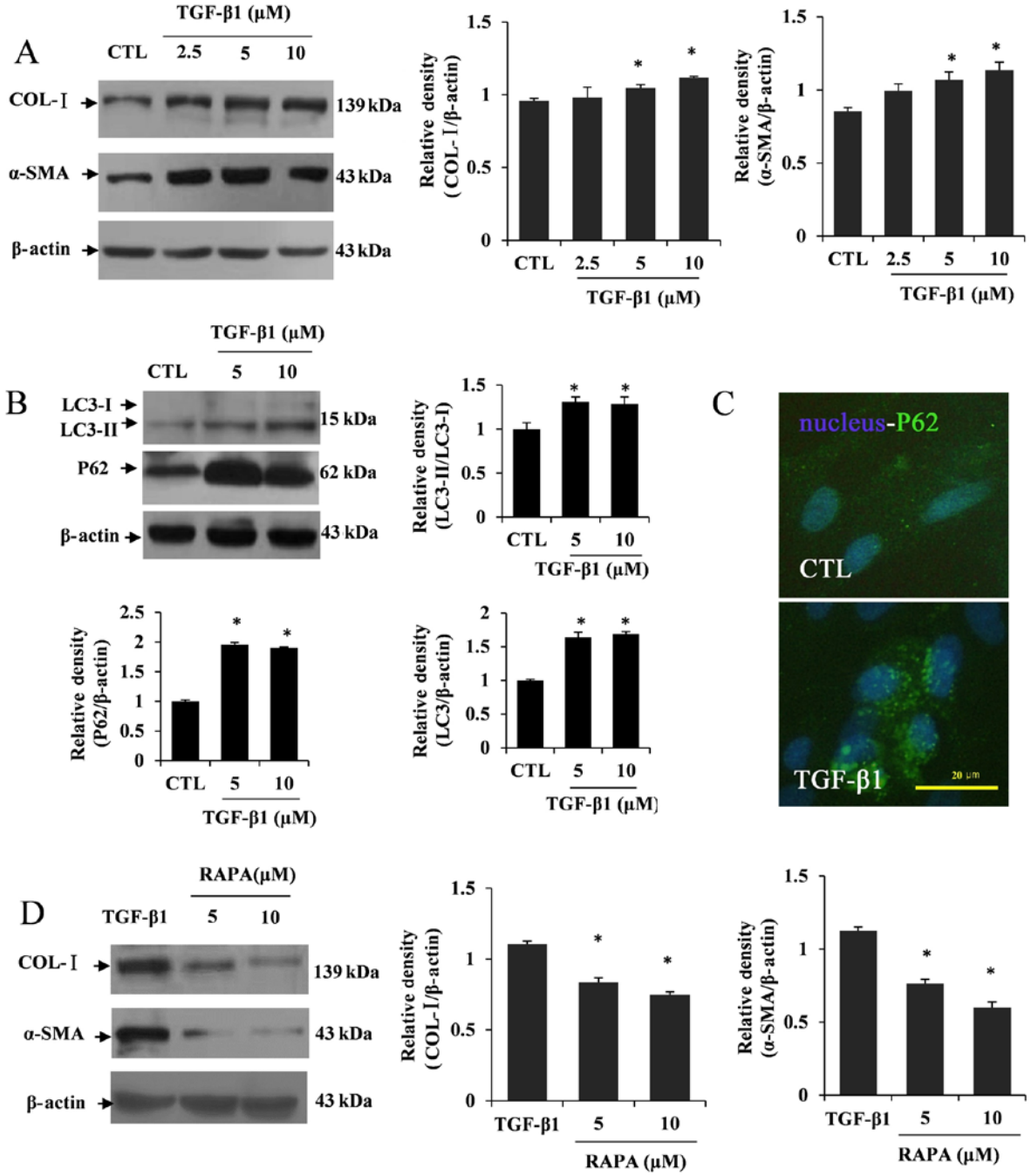

Figure 1. TGF-β1-induced HSC activation is partially subject to autophagy dysfunction. (A) Expression levels of COL-I and α-SMA were determined in LX2 cells exposed to different concentrations of TGF-β1 by western blotting. *P<0.05 vs. CTL group. (B) Expression levels of LC3 and P62 were determined in TGF-β1-treated LX-2 cells. *P<0.05 vs. CTL group. (C) The expression of P62 was detected in TGF-β1-treated LX2 cells by immunofluorescence. The cells were immunostained against antibodies P62 (green), DAPI was used to stain nuclei (blue). (D) Expression levels of COL-I and α-SMA were determined in TGF-β1-treated LX-2 cells after RAPA treatment by western blotting. *P<0.05 vs. TGF-β1-treated group. TGF-β1, transforming growth factor-β; COL-I, collagen-I; α-SMA, α-smooth muscle actin; RAPA, rapamycin; CTL, control.

in the control and NC groups (Fig. 3A). Furthermore, to study the relationship between miR221 expression and autophagy activation, miR221-overexpressing LX2 cells were treated with RAPA. The western blotting results showed that the overexpression of miR221 in LX2 cells induced the aggregation of P62 expression and resulted in low conversion of LC3I to LC3II. Whereas, LC3-I to LC3-II conversion was significantly increased in the NC + RA and miR221 mimics + RA groups compared with the NC and miR221 mimics groups, respectively (P<0.05; Fig. 3B-D). Furthermore, P62 aggregation was significantly decreased in the miR221 mimics + RA group compared with the miR221 mimics group (P<0.05), although there was no significant difference between the NC + RA and NC groups (Fig. 3B, E and F). Thus, RAPA appeared to upregulate the autophagic flux in LX2 cells. These findings implied potential cross-talk between miR221 expression and autophagic flux.

miR221 specifically regulates autophagy by targeting LAMP2 expression. To explore the molecular mechanism of

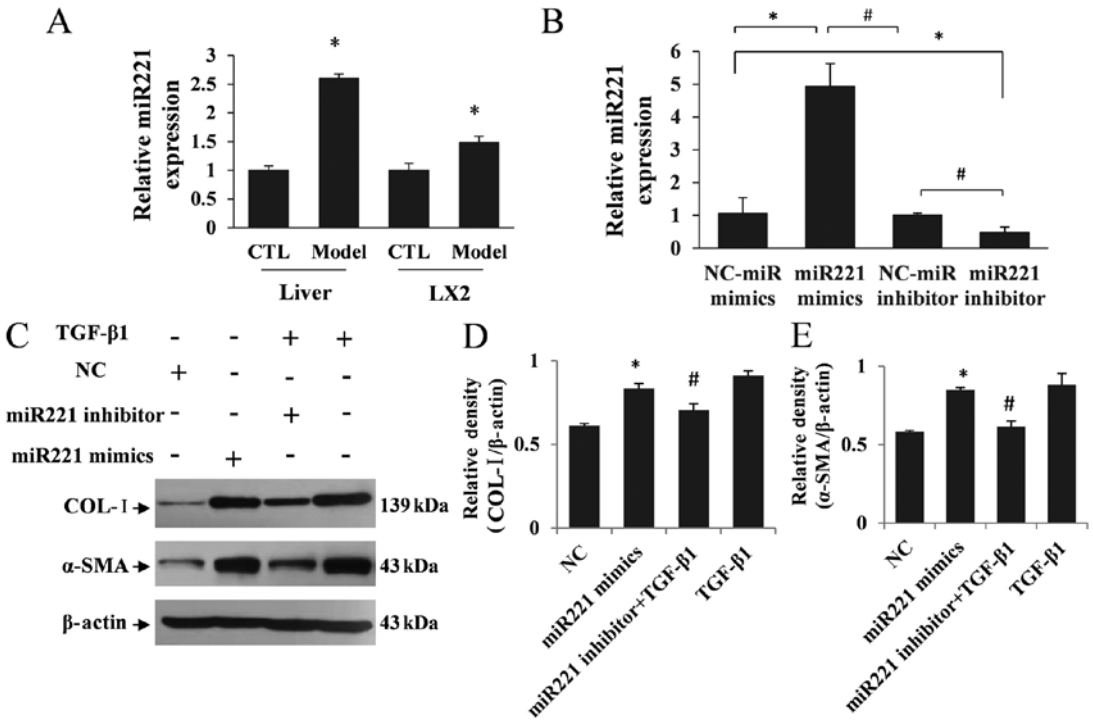

Figure 2. miR221 is upregulated in liver fibrosis mice and activates hepatic stellate cells. (A) Expression of miR221 in liver fibrosis mice or in LX-2 cells was detected via RT-qPCR. *P<0.05 vs. CTL group. (B) LX2 cells were transfected with miR221 mimics, miR221 inhibitor and their matched NCs (NC-miR mimics and NC-miR inhibitor), and the expression of miR221 was detected via RT-qPCR. *P<0.05 vs. NC-miR mimics group; #P<0.05 vs. NC-miR inhibitor group. (C-E) Expression levels of COL-I and α-SMA were determined in miR221 mimic- or miR221 inhibitor-transfected LX2 cells following TGF-β1 treatment by western blotting. NC-miR mimics denoted as NC. *P<0.05 vs. NC group; #P<0.05 vs. TGF-β1-treated group. miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; TGF-β1, transforming growth factor-β; COL-I, collagen-I; α-SMA, α-smooth muscle actin; CTL, control.

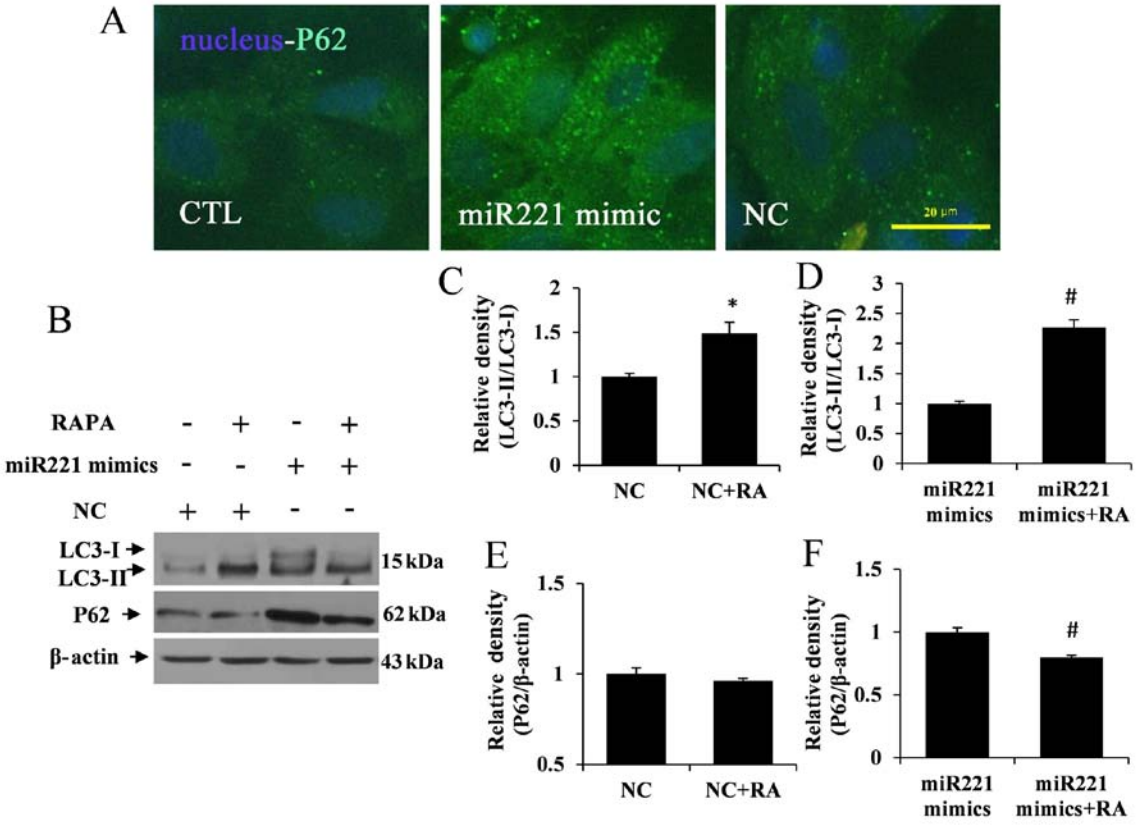

Figure 3. miR221 inhibits autophagy in LX2 cells. (A) The expression of P62 was detected in LX2 cells transfected with miR221 mimics using an immunofluorescence assay. The cells were immunostained with antibodies against P62 (green), DAPI was used to stain nuclei (blue). (B-F) Expression levels of LC3 and P62 were determined by western blotting in LX2 cells transfected with miR221 mimic or miRNA-NC following RAPA treatment. *P<0.05 vs. NC group; #P<0.05 vs. miR221 mimic-treated group. miR, microRNA; NC, negative control; RAPA, rapamycin.

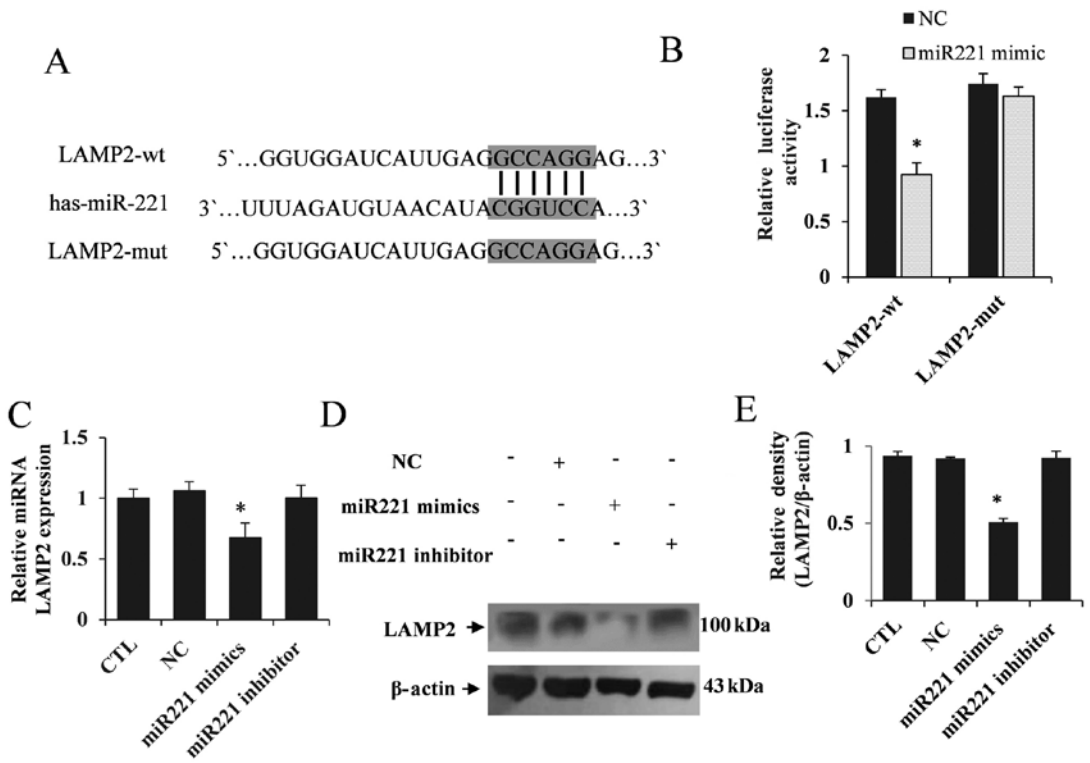

Figure 4. miR221 specifically regulates autophagy by targeting LAMP2 expression. (A) Bioinformatics online analysis of the predicted miR221 binding sites of LAMP2. (B) Dual-luciferase reporter assay was performed to detect fluorescence activity of LAMP2. (C-E) Expression of LAMP2 was detected via reverse transcription-quantitative PCR or western blotting analyses in LX2 cells transfected with miR221 mimics or miR221 inhibitors. *P<0.05 vs. NC group. miR, microRNA; LAMP2, lysosome-associated membrane glycoprotein 2; NC, negative control; wt, wild-type; mut, mutant; CTL, control.

miR221 in regulating autophagy, bioinformatics tools were used to predict potential target genes. Among these potential targets, it was found that LAMP2, a crucial gene in the regulation of autophagy activation, was a potential target of miR221 (Fig. 4A). The dual-luciferase reporter assay showed that miR221 significantly inhibited the fluorescence activity of LAMP2-wt, but did not regulate LAMP2-mut (P<0.05; Fig. 4B). These results confirmed the targeted regulatory effect of miR221 on LAMP2. Furthermore, LX2 cells were transfected with miR221 mimics, NC or miR221 inhibitor for 48 h, and RT-qPCR and western blotting analyses were performed to determine LAMP2 expression. It was demonstrated that LAMP2 expression was significantly decreased in the LX2 cells transfected with miR221 mimics, but the miR221 inhibitor had no effect on LAMP2 expression (Fig. 4C-E).

miR221 regulates TGF-β1-induced HSC activation by targeting LAMP2 expression. In rescue experiments, LAMP2 expression was significantly upregulated in LAMP2-overexpressing LX2 cells compared with the NC group (P<0.05; Fig. 5A). Based on these findings, the expression of autophagic marker proteins, including LC3II and P62, were examined by western blotting. As a result, overexpression of LAMP2 considerably upregulated autophagic flux following TGF-β1 treatment in LX2 cells, as indicated by the decreased expression of LC3II and P62 compared with the NC group (P<0.05; Fig. 5B-E). Additionally, the expression levels of COL-I and α-SMA were determined to assess the regulatory function of LAMP2. The western blotting results showed a concomitant decrease in the expression levels of COL-I and α-SMA in LAMP2-overexpressing LX2 cells following TGF-β1 treatment (P<0.05; Fig. 5B, F and G). These results indicated that miR221 may regulate TGF-β1-induced HSC activation by targeting LAMP2 expression.

Discussion

Liver fibrosis is caused by a variety of chronic pathogenic factors, especially an imbalance in ECM synthesis and degradation, which lead to abnormal liver structure and hepatocellular function (2). To further explore potentially effective targets for liver fibrosis therapy to solve this problem, elucidating the mechanism of HSC activation is of importance. Autophagy is a lysosome-dependent cellular homeostasis mechanism that regulates intracellular homeostasis. Although the specific mechanism mediating HSC activation is still unclear, some conclusions have been made, indicating that the different effects of autophagy on the fate of HSCs depend on the degree and extent of autophagy (31). In the present study, TGF-β1 activated HSCs and simultaneously caused P62 aggregation, which was involved in autolysosome degradation, as indicated by the impaired processing of LC3. These results implied that autolysosome function, but not autophagy initiation, was blocked. Therefore, it was hypothesized that its pathogenic mechanism is related to the abnormal regulation of autophagy. Moreover, induction of autophagic flux by RAPA reduced TGF-β1-induced COL-I and α-SMA expression. These results showed that TGF-β1 was able to impair autophagy function in LX2 cells, thereby further showing that TGF-β1 induced the

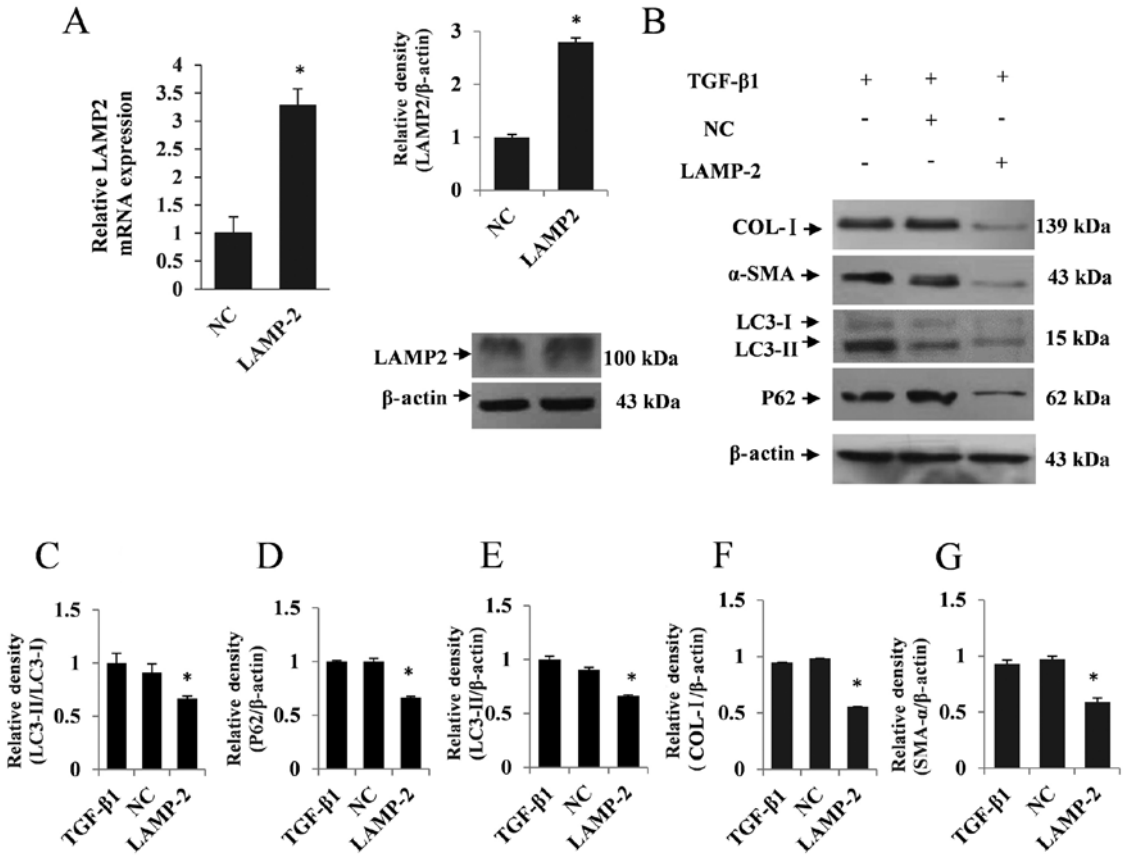

Figure 5. miR221 regulates TGF-β1-induced hepatic stellate cell activation by targeting LAMP2 expression. (A) Expression of LAMP2 in LX2 cells transfected with LAMP2 overexpression vector was determined via reverse transcription-quantitative PCR or western blotting. *P<0.05 vs. NC group. (B-G) Expression levels of COL-I, α-SMA, LC3 and P62 were determined by western blotting in LX2 cells transfected with LAMP2 overexpression vector following TGF-β1 treatment. *P<0.05 vs. TGF-β1-treated group. miR, microRNA; TGF-β1, transforming growth factor-β; LAMP2, lysosome-associated membrane glycoprotein 2; NC, negative control; COL-I, collagen-I; α-SMA, α-smooth muscle actin.

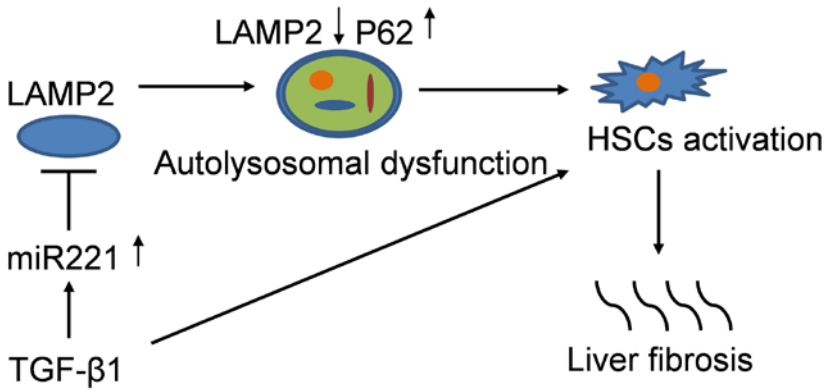

Figure 6. miR221 regulates TGF-β1-induced HSC activation through inhibiting autophagy by targeting LAMP2. TGF-β1 activated HSCs, and simultaneously induced HSC autolysosome dysfunction. The upregulation of miR221 resulted in decreased LAMP2 expression and inactivation of autophagy-lysosomal pathway in TGF-β1-treated LX2 cells. miR221 could induce hepatic fibrosis via mediating autophagy by targeting LAMP2. miR, microRNA; HSC, hepatic stellate cell; TGF-β1, transforming growth factor-β; LAMP2, lysosome-associated membrane glycoprotein 2.

activation of HSCs, which was accompanied by dysfunction of autophagy.

miRNAs play a vital role in the regulation of the expression of a large number of genes at the posttranscriptional level. Various microRNAs have been proven to be involved in epigenetic changes and multiple pathological mechanisms, such as cancer or fibrosis (32). The differential expression of miRNAs, especially the upregulation of miR221, has been observed in liver fibrosis tissue. Roderburg *et al* (19) identified 21 downregulated and 10 upregulated miRNAs in fibrotic livers, especially miR221, which was upregulated in CCl_4-induced mice. Thus, the present study sought to study the function of miR221 in activated HSCs and elucidate its potential role in regulating autophagy function. miR221-overexpressing cell models of liver fibrosis were constructed in the exploration of the possible molecular physiological and pathogenic mechanisms of

miR221 in hepatic fibrosis. Based on western blotting results, expression levels of the hepatic fibrosis-relevant markers COL-I and α-SMA were increased in miR221-overexpressing LX2 cells, while the miR221 inhibitor decreased the induction of COL-I and α-SMA expression following TGF-β1 treatment. The results revealed that liver fibrosis progression was associated with increased miR221 expression. Similar studies have reported that miR221 is increased in liver tissues of patients with hepatocellular carcinoma, and is involved in the progression of hepatic diseases (20,33).

Previous studies have confirmed the role of miR221 in regulating autophagy (34,35). TGF-β1-induced HSC activation abnormally decreases autophagy (36), which seems to be consistent with the present results showing increased LC3II and P62 expression, indicating disruption of autophagy. To investigate the possible involvement of miR221 in this process, intracellular autophagic flux was detected in miR221-overexpressing LX2 cells by immunofluorescence. Results showed that a mass of P62 was aggregated in LX2 cells, and the empty vector did not affect P62 expression. Western blot analysis was performed, and the results indicated that the autophagy inducer RAPA reversed the inhibition of autophagic flux induced by miR221. Therefore, the aforementioned findings revealed that miR221 partially regulated hepatic fibrosis by inhibiting autophagy.

Autophagy maintains cell viability by targeting the degradation of misfolded proteins in autolysosomes. To explore the molecular target of miR221 in regulating autophagy, the present study used bioinformatics tools to predict potential target genes. Based on these predicted results, the possible regulatory genes of miR221 were obtained using TargetScan and HADb. Among these potential autophagy-related gene targets, miR221 was found to regulate LAMP2 gene expression, which has been reported to be closely related to the degradation of autolysosome (37). LAMP2 is a crucial gene regulating cellular autolysosome lysosome fusion during autophagy (38), and was found to be a potential target of miR221 in the current study. The dual-luciferase reporter assay verified that miR221 significantly inhibited the fluorescence activity of LAMP2, but did not regulate the mutant LAMP2. miR221 likely binds to LAMP2 and then recruits and activates autolysosome function. To further verify the regulation of LAMP2 by miR221 in activated HSCs, LX2 cells were transfected with miR221 mimics for 48 h. RT-qPCR and western blotting results showed that LAMP2 expression was significantly decreased in miR221-overexpressing LX2 cells. However, there were no significant differences in LAMP2 expression following transfection with the miR221 inhibitor.

The present study demonstrated that autophagic degradation capacity of lysosomes was impaired due to the elevated expression of P62 in TGF-β1-treated LX2 cells. Consistent with these findings, Babuta *et al* (39) reported the causal role of miR155 in regulating alcohol-induced LC3-II and p62 accumulation via targeting LAMP2 *in vitro* and *in vivo*. Although LAMP2 has been shown to mediate autophagy activation (8), the role of LAMP2 in hepatic fibrosis is still controversial. Considering the potential target gene of TGF-β1-induced HSC activation in hepatic fibrosis, we intended to verify the functional role of miR221 in regulating HSC activation by targeting LAMP2. In rescue experiments, LAMP2-overexpressing LX2 cells were constructed, treated with TGF-β1, and the expression levels of COL-I and α-SMA were observed to determine the regulatory function of LAMP2. The results showed that LAMP2 was significantly increased in LAMP2-overexpressing LX2 cells, while COL-I and α-SMA were downregulated following TGF-β1 treatment. Based on these findings, the expression of autophagic marker proteins was examined by western blotting. Overexpression of LAMP2 considerably upregulated autolysosome activity in TGF-β1-treated LX2 cells, as indicated by decreased expression of LC3-II and P62. These results indicated that upregulated LAMP2-dependent autolysosome activity blocked TGF-β-induced hepatic fibrosis, which had a similar fibrosis protective effect in other disease models (40). The current study attempted to explore miR221 intervention pathways for regulating TGF-β signals. However, it does not exclude the involvement of miR221 in other mechanisms of onset of activating HSC (41). In future research, we will verify the differential expression of miRNA in liver fibrosis by microarray data and further elucidate the possible mechanism of HSC activation. Therefore, inhibiting TGF-β1-induced HSC activation by regulating LAMP2-dependent autolysosome activation could be a possible therapeutic strategy that exerts antifibrotic effects in hepatic fibrosis.

Overall, these results demonstrated that miR221 played a regulatory role in hepatic fibrosis and could regulate TGF-β1-induced HSC activation through inhibiting autophagy by targeting LAMP2 (Fig. 6). The molecular mechanism of the involvement of miR221 in regulating the autophagy-lysosomal pathway may represent a novel therapeutic approach for liver fibrosis.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RC and YH contributed to the conception and design of this study. RC and HX were responsible for the details of the experimental procedures. RC completed the manuscript and YH revised the manuscript. RC, HX and YH contributed to data analysis and interpretation. RC, HX and YH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All protocols were approved by the Hebei Medical University Institutional Animal Experimental Ethics Committee (approval no. IACUC-Hebmu-2020005; Shijiazhuang, China) and carried out according to the provisions of the guidelines for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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