

Ultrasound-targeted Microbubble Destruction Mediated miR-200a/PEG10 Axis Promotes Autophagy to Alleviate MAFLD

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Background: This study aimed to explore the therapeutic potential and mechanism of ultrasound-targeted microbubble destruction (UTMD) combined with the miR-200a/PEG10 axis in metabolic-associated fatty liver disease (MAFLD).

Methods: This study was performed at clinical, cellular, and animal levels. Clinically, liver tissues from 10 MAFLD patients and 10 healthy controls were collected to detect miR-200a/PEG10 expression and their correlation. A cellular MAFLD model was induced by palmitic acid (PA)/oleic acid (OA), followed by rescue experiments to assess lipid accumulation, mitochondrial function, apoptosis, and autophagy-related indicators. Animal MAFLD models were established via a high-fat diet, and corresponding interventions were administered to assess hepatic pathological injury and autophagic status.

Results: In patients with MAFLD, PEG10 expression was significantly upregulated, whereas miR-200a was downregulated, and their expression levels were negatively correlated. In both MAFLD cells and mouse models, UTMD combined with miR-200a mimics and siPEG10 significantly alleviated hepatic steatosis, reduced lipid accumulation, improved mitochondrial morphology and function, decreased apoptosis, increased the expression levels of autophagy-related proteins (Beclin1, LC3B, PINK1, and Parkin), and reduced P62 level. Rescue experiments further demonstrated that treatment with the autophagy inhibitor 3-methyladenine (3-MA) aggravated hepatocellular injury, whereas the autophagy activator rapamycin (Rapa) enhanced autophagic activity and ameliorated MAFLD pathology. These findings indicated that the combined intervention alleviated MAFLD, at least in part, through the promotion of autophagy.

Conclusion: This study identified the miR-200a/PEG10 axis as a critical regulatory pathway in MAFLD and validated UTMD as an effective gene delivery approach for its modulation. These findings provide a novel therapeutic strategy and a theoretical basis for the development of targeted interventions for MAFLD.

Key words: UTMD; miR-200a/PEG10 axis; Autophagy; Metabolic-associated fatty liver disease

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MAFLD has become the most common chronic liver diseases worldwide [1]. MAFLD from simple steatosis to non-alcoholic steato-

hepatitis, fibrosis, and potentially progressing to cirrhosis or hepatocellular carcinoma [2]. The pathogenesis of MAFLD is closely linked to metabolic syndrome, mak-

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ing MAFLD a multifactorial disease with currently limited effective treatment options [3]. Recent studies have shown that autophagy, as a crucial intracellular degradation and regeneration process, can play a therapeutic role in MAFLD by improving lipid metabolism disorders, hepatocyte injury, insulin resistance, and inhibiting oxidative stress, making it a potential target for MAFLD treatment [4]. Additionally, mitophagy plays a critical role in both physiological and pathological processes of the liver [5]. Inhibition of autophagy leads to excessive lipid accumulation in hepatocytes, triggering oxidative stress, inflammatory responses, and fibrosis [6].

MicroRNAs (miRNAs), an important class of non-coding RNA molecules, have attracted considerable attention for their roles in regulating metabolic disorders [7]. Research indicates that dysregulation of miRNAs is strongly linked to the onset and progression of MAFLD [8]. For instance, miR-34a, miR-122, and miR-21 have been demonstrated to participate in the pathological processes of MAFLD by regulating lipid synthesis, oxidative stress, and fibrosis pathways [9,10]. Among them, miR-200a is involved in regulating multiple biological processes and has shown significant regulatory roles in various metabolic diseases, including diabetes and hyperlipidemia [11,12]. Recent research has shown that miR-200a is crucial in regulating lipid metabolism and autophagy, demonstrating significant regulatory roles in multiple diseases [13,14]. In MAFLD, the expression level of miR-200a is significantly downregulated, and restoring miR-200a expression can improve hepatic lipid metabolism and inflammatory responses [15].

PEG10 (Paternally Expressed Gene 10) is an imprinted gene closely associated with cell proliferation and apoptosis, and its role in tumorigenesis and metabolic diseases has gradually garnered attention [16]. Initially identified as being closely linked to the development of liver cancer, recent studies suggest that PEG10 may influence hepatic lipid metabolism by regulating autophagy [17]. In MAFLD, the expression level of PEG10 is significantly upregulated, and its high expression is closely related to the inhibition of autophagy function [18]. Modulating the expression of miR-200a and PEG10 holds promise as a novel approach for treating MAFLD. However, issues such as the low safety and poor targeting efficiency of viral vectors have limited their application.

Ultrasound-targeted microbubble destruction (UTMD) is a safe and effective technique for enhancing gene transfection while offering targeted delivery. As a non-viral vector combined with ultrasound irradiation, UTMD-mediated microRNA transfection overcomes the limitations of viral vectors, such as low safety, poor tar-

geting efficiency, and high immunogenicity. As an emerging non-invasive therapeutic approach, UTMD has been widely applied in the targeted delivery of genes and drugs [19]. UTMD utilizes low-frequency ultrasound to stimulate microbubble cavitation in vivo, causing microbubbles to oscillate and rupture, generating microjets and shockwaves. This process generates reversible and irreversible pores in the cell membrane, allowing drugs and genes to cross the membrane and be precisely delivered into the tissue interstitium or directly into cells [20]. Recent studies have shown that UTMD can influence cellular functions by regulating the expression of specific miRNAs [21]. However, the application of UTMD in MAFLD has not yet been systematically explored.

This study aimed to investigate whether UTMD, in combination with the miR-200a/PEG10 axis, could regulate autophagy and influence the progression of MAFLD. Additionally, the therapeutic efficacy of this approach was evaluated in preclinical models of MAFLD, providing a potential breakthrough strategy for MAFLD.

Methods

Clinical sample collection

Ten patients diagnosed with MAFLD at Hohhot First Hospital were enrolled as the MAFLD group. Liver tissue samples (15-20 mg) were obtained through ultrasound- and CT-guided negative-pressure suction biopsy. Additionally, liver tissue specimens from 10 patients undergoing hepatic resection for liver trauma were selected as controls (control group). This study was approved by the Institutional Review Board/Ethics Committee of the hospital (IRB2023173), and written informed consent was obtained from all participants. Due to the invasive nature of liver biopsy and the limited availability of suitable control liver tissues from trauma surgery, the number of clinical samples was restricted. Therefore, a total of 10 MAFLD patients and 10 control subjects were included as an exploratory clinical cohort to preliminarily evaluate the expression patterns of miR-200a and PEG10 in human liver tissues. A post hoc statistical power analysis was performed using G*Power software (version 3.1). Based on the observed differences in PEG10 expression between groups and assuming an effect size of 1.2, a sample size of 10 subjects per group provided a statistical power of approximately 0.80 at a significance level of $\alpha = 0.05$, indicating that the sample size was sufficient to detect large effect sizes in this exploratory study. The baseline characteristics of the study participants are shown in [table 1](#).

Table 1 Baseline characteristics of study participants

Parameter	Control group (n = 10)	MAFLD group (n = 10)	P-value
Age (years)	65.1 ± 11.0	67.1 ± 8.7	0.658
Sex (Male/Female)	5/5	4/6	0.999
BMI (kg/m ²)	23.4 ± 2.1	27.8 ± 3.2	0.002
ALT (U/L)	23.5 ± 6.8	72.3 ± 18.5	< 0.0001
AST (U/L)	21.9 ± 5.2	54.6 ± 15.3	< 0.0001
TG (mmol/L)	1.1 ± 0.3	2.6 ± 0.8	< 0.0001

Cell culture

The L02 hepatocyte cell line was obtained from Chinese Academy of Medical Sciences. These cells were grown in DMEM medium (Gibco, USA) containing 10% FBS (Gibco, USA) and a penicillin-streptomycin mixture at 10⁵ U/L (KeyGEN BioTECH, China), and incubated at 37 °C with 5% CO₂.

Cell grouping and transfection treatment

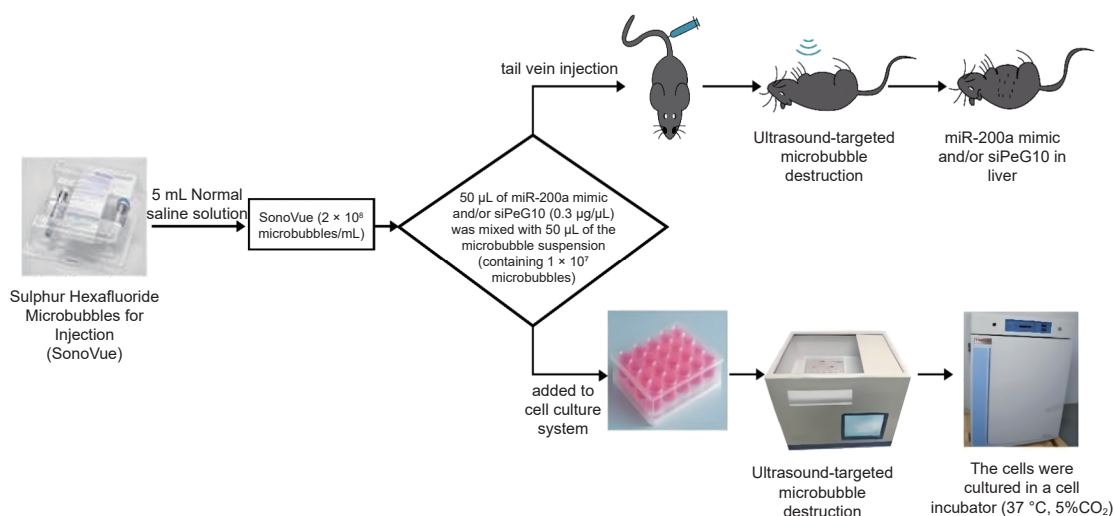
Cells in the logarithmic growth phase were selected and divided into experimental groups: control, MAFLD, miR-200a mimic, siPEG10, miR-200a mimic + siPEG10, miR-200a mimic + UTMD, siPEG10 + UTMD, and miR-200a mimic + siPEG10 + UTMD. For the rescue experiment, the groups included: MAFLD, MAFLD + 3-MA, MAFLD + Rapa, miR-200a mimic + siPEG10, miR-200a mimic + siPEG10 + 3-MA, miR-200a mimic + siPEG10 + Rapa, miR-200a mimic + siPEG10 + UTMD, miR-200a mimic + siPEG10 + UTMD + 3-MA, and miR-200a mimic + siPEG10 + UTMD + Rapa.

SonoVue microbubbles (2 × 10⁸ microbubbles/mL) were prepared according to the manufacturer's instructions (Bracco, Italy). The same batch of SonoVue was used for all experiments to ensure consistency, and all

microbubble preparations were used within 2 hours after activation to maintain optimal cavitation activity. For experimental treatments, an *in vitro* MAFLD cell model was established by culturing cells with oleic acid (OA, 1 mM) and palmitic acid (PA, 1 mM) for 48 h. Subsequently, 50 µL of miR-200a mimic (RiboBio, China) and/or siPEG10 (GenePharma, China) (0.3 µg/µL) was mixed with 50 µL of microbubble suspension (containing 1 × 10⁷ microbubbles). The mixture was then added to the culture system, followed by UTMD treatment (Fig. 1). For rescue experiments, 24 h after transfection, either the autophagy inhibitor 3-methyladenine (3-MA, 5 mM; MCE, USA) or the autophagy activator rapamycin (Rapa, 20 nM; MCE, USA) was administered. The ultrasound therapy instrument (Nu-Tek, China) was set to an output frequency of 650 kHz with an acoustic intensity of 0.5 W/cm², and ultrasound irradiation was applied for 60 s. These parameters were optimized for *in vitro* conditions to achieve maximal microbubble cavitation and gene delivery efficiency while maintaining cell viability. These ultrasound parameters were selected based on previously reported UTMD conditions for efficient microbubble cavitation and nucleic acid delivery in hepatocyte models, aiming to balance transfection efficiency with minimal cellular damage. After treatment, the cells were placed in a cell culture incubator (37 °C, 5% CO₂) for 4–6 h.

Animal model establishment

Sixty-four male C57BL/6 mice (specific pathogen-free, aged 6–8 weeks and weighing 18–22 g) were obtained from Beijing Vital River. Following 1-week acclimatization in a controlled environment (22 ± 2 °C, 50% ± 10% humidity, and a 12-hour light/dark cycle), mice were provided with a standard diet and had unre-

**Figure 1** Schematic of UTMD preparation and intervention.

stricted access to water. The experimental protocol was approved by the Animal Experiment Ethics Committee of Shenyang Medical College (Approval No. SYYXY20240319). All experimental procedures were conducted in accordance with the International Association of Veterinary Editors' Consensus Author Guidelines on Animal Ethics and Welfare, as well as relevant local and national regulations.

After the acclimatization period, the mice were randomly divided into groups: control, MAFLD, miR-200a mimic, siPEG10, miR-200a mimic + siPEG10, miR-200a mimic + UTMD, siPEG10 + UTMD, and miR-200a mimic + siPEG10 + UTMD group, with 8 mice in each group. Except the control group, the other groups were fed a high-fat diet (Research Diets) to establish the MAFLD model. All groups had free access to food and water for 16 weeks. According to the experimental grouping requirements, 50 μ L of miR-200a mimic (RiboBio, China) and/or siPEG10 (GenePharma, China) (0.3 μ g/ μ L) was mixed with 50 μ L of the microbubble suspension (containing 1×10^7 microbubbles). The mixture was then administered via tail vein injection, followed by UTMD therapy (Fig. 1). The ultrasound therapy instrument (Nu-Tek, China) was set to a frequency of 1 MHz, pulse repetition frequency of 100 Hz, sound intensity of 2 W/cm², duty cycle of 50%, and duration of 5 min. These parameters were selected to ensure sufficient ultrasonic energy penetration through the skin and abdominal wall to reach the target liver tissue, thereby inducing effective microbubble cavitation and facilitating gene delivery in the hepatic microenvironment. The selected ultrasound settings were within ranges commonly used in preclinical UTMD studies and were designed to achieve efficient microbubble destruction and gene transfer while minimizing potential tissue injury.

Immunohistochemistry

Liver tissue, fixed, embedded, and sectioned. These sections were deparaffinized, rehydrated, and underwent antigen retrieval. Following permeabilization and blocking at room temperature, the sections were incubated overnight with a PEG10 primary antibody (1:500, Proteintech, USA). A secondary antibody (1:1000, Abcam, USA) was then applied. Color development was performed using an enhanced DAB chromogenic reagent kit (Beyotime, China), followed by hematoxylin counterstaining, rinsing, and mounting. The stained sections were subsequently examined under a microscope (OLYMPUS IX51, Germany).

Flow cytometry

Cell apoptosis was detected using the Annexin V-

FITC Apoptosis Detection kit (Beyotime, China) via flow cytometry. Cells from each intervention group were digested with trypsin, washed with phosphate-buffered saline (PBS), and collected under light-protected conditions. The cells were stained with PI and Annexin V, and analyzed using a flow cytometer.

Western blot assay

Liver tissues or cells were collected and washed with PBS. RIPA protein lysis buffer (Solabio, China) was added to extract proteins. Protein concentration was measured using a BCA protein assay kit (Solabio, China). Proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Thermo, USA). The membrane was incubated in 5% bovine serum albumin at room temperature for 1 hour, followed by overnight incubation at 4 °C with the following primary antibodies: PEG10 (1:1000, Proteintech, USA), Beclin1, LC3B, P62, PINK1, Parkin (1:1000, Affinity Biosciences, USA), and GAPDH (1:3000, Affinity Biosciences, USA). After washing, the membrane was incubated with HRP-conjugated goat anti-mouse IgG (1:5000 dilution, 1:1000, Abcam, USA) at room temperature for 1 h, and protein bands were visualized using an ECL kit (Bio-Rad, USA).

qRT-PCR

Total RNA was isolated from cells or liver tissues using an RNA extraction kit (TaKaRa, China) and reverse-transcribed into cDNA (TaKaRa, China). Quantitative PCR analysis was performed with SYBR Green. U6 and GAPDH were used as internal reference genes. Primers were synthesized by Invitrogen (Beijing, China), and the corresponding sequences are listed in Table 2.

TEM

The liver tissues were fixed at 4 °C for 2 h, post-fixed with 1% osmium tetroxide (Ted Pella Inc., USA) at 20 °C for 2 h, rinsed with PBS, dehydrated through a graded ethanol series, infiltrated, embedded, and ultra-thin-sectioned. The sections were then double-stained with uranyl and lead, dried overnight at room temperature, and examined under a transmission electron microscope.

Oil red O staining

Liver tissues were dehydrated in sucrose, embedded, and sectioned into frozen slices. The sections were stained with Oil Red O (Solarbio, China), differentiated in 75% ethanol, counterstained with hematoxylin, rinsed under running water for bluing, and mounted with glycerin gelatin.

Table 2 Primer sequences

Gene	Primers sequences (5'-3')
miR-200a	AATCCTGCTTGGGTATCAGG GAGACCCAGTCTCAGGGAAA
U6	TGTCATCTCGCTCTGGTACG AAATGACCCCTTCATCACCA
PEG10	ATTTGCTTTTGCCAAGAGTCAG GGTGTGCCTTCATATTCAAACC
Beclin1	GAGGAGGACCGGATCAACG CTGGAAGAGTGTTTCGAGGCAG
LC3	TTGAAGGAGGATGTCCCATCT ACAGACACATATTGGCATGGTT
P62	CAAGGCCCGTTTCCACTAAG GAGGCGATACAGGCCGATG
PINK1	CGAGAGCTACACGTTACGG GGGTGTCGAGGGAAAAATAGG
Parkin	GACGCCATCAACACCGAGTT CTTTGTCGTTGGTTAGCTGGT
GAPDH	TGGATTGGACGCATTGGTC TTTGCACTGGTACGTGTTGAT

Dual-luciferase assay

The assay was performed strictly following the manufacturer's instructions for the Dual-Luciferase Reporter Assay kit. Cells were lysed thoroughly using the provided reporter lysis buffer, and the supernatant was collected for luminescence detection with a microplate reader. The relative activity of the target reporter gene was calculated as the ratio of Renilla luciferase activity to firefly luciferase activity.

Statistical analysis

Data analysis was performed using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA). Normally distributed data were presented as mean $\pm$ standard deviation (SD). Differences among multiple groups were evaluated using one-way analysis of variance (ANOVA). In addition, a post hoc power analysis for the clinical sample comparison was conducted using G*Power 3.1 to evaluate whether the sample size provided sufficient statistical power for detecting group differences. Exact P values were calculated for all comparisons, and statistical significance was indicated in the figures as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Results

PEG10 is highly expressed in MAFLD liver tissues, while miR-200a is lowly expressed, and miR-200a shows a negative correlation with PEG10 mRNA levels

To examine miR-200a and PEG10 expression in

MAFLD, liver tissues from MAFLD patients and healthy controls were collected. Western blot, immunohistochemical staining, and qRT-PCR were used to compare PEG10 and miR-200a levels between the healthy control (HC) and MAFLD groups. Western blot results (Fig. 2A-B) demonstrated that PEG10 was significantly higher in the MAFLD compared to the HC. Immunohistochemical staining (Fig. 2C-D) also showed stronger PEG10 staining in MAFLD liver tissues. qRT-PCR analysis (Fig. 2F) revealed that PEG10 mRNA levels were significantly lower in the MAFLD group, whereas PEG10 protein expression was markedly increased as demonstrated by Western blotting and immunohistochemistry. This discrepancy indicates that PEG10 expression may be regulated at the post-transcriptional level. Additionally, miR-200a levels were significantly reduced in the MAFLD group (Fig. 2E), and a negative correlation was observed between miR-200a and PEG10 mRNA levels in MAFLD liver tissues (Fig. 2G), indicating that miR-200a may regulate PEG10 expression through post-transcriptional mechanisms.

UTMD combined with the miR-200a/PEG10 signaling axis improves lipid metabolism, mitochondrial function, and inhibits apoptosis in MAFLD

Oil Red O staining (Fig. 3A) showed a significant lipid accumulation in the MAFLD group, which was reduced in the miR-200a mimic and siPEG10 groups, further decreased in the combined miR-200a mimic + siPEG10 group, and was found as the lowest in the miR-200a mimic + siPEG10 + UTMD group.

qRT-PCR (Fig. 3B) confirmed reduced miR-200a expression in MAFLD cells, with increased expression after miR-200a mimic or siPEG10 treatment, and the greatest increase in the combined treatment groups, including UTMD. PEG10 mRNA expression showed an opposite pattern (Fig. 3C).

TEM observations (Fig. 3D) indicated swollen mitochondria with disrupted membranes in MAFLD cells, whereas mitochondrial morphology appeared progressively more intact in the miR-200a mimic, siPEG10, combined treatment, and UTMD-enhanced groups.

Dual-luciferase assays (Fig. 3E) demonstrated decreased reporter activity in the miR-200a mimic group and increased activity in the miR-200a inhibitor group.

Flow cytometry analysis (Fig. 3F-G) revealed increased apoptosis in the MAFLD group, with reduced apoptosis after miR-200a mimic or siPEG10 treatment, further reduction in the combined group, and the lowest apoptotic rate in the UTMD-enhanced group.

UTMD combined with miR-200a/PEG10 signaling axis reversed autophagy inhibition in MAFLD

To explore the effects of UTMD combined with the

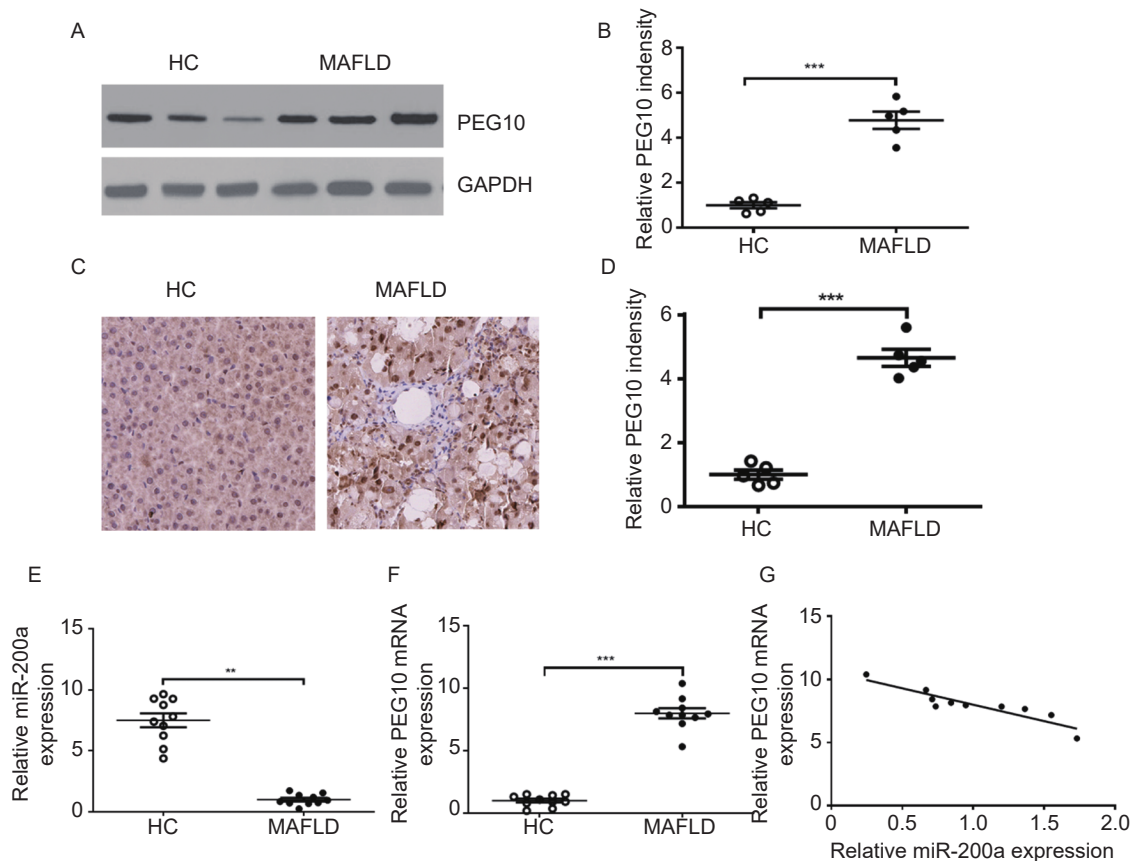


Figure 2 PEG10 expression is increased in the MAFLD group, while miR-200a expression is decreased and negatively correlated with PEG10 mRNA levels. (A-B) Western blot detection of PEG10 protein expression; (C-D) Immunohistochemical detection of PEG10 expression; (E) qRT-PCR detection of miR-200a mRNA expression levels; (F) qRT-PCR detection of PEG10 mRNA expression levels; (G) Correlation analysis of miR-200a and PEG10 expression in liver tissues of MAFLD patients. *** denotes $P < 0.001$.

miR-200a/PEG10 signaling axis on autophagy in MAFLD, we analyzed the expression of autophagy-related proteins using WB and qPCR. Western blot results (Fig. 4A-F) revealed that, compared to the control group, Beclin1, LC3B, PINK1, and Parkin levels were significantly reduced in the MAFLD group ($P < 0.05$), while P62 was significantly elevated ($P < 0.05$). In contrast, the miR-200a mimic and siPEG10 groups showed significantly higher levels of Beclin1, LC3B, PINK1, and Parkin ($P < 0.05$) and lower P62 levels ($P < 0.05$) compared to the MAFLD group. The miR-200a mimic + siPEG10 group demonstrated further increases in Beclin1, LC3B, PINK1, and Parkin ($P < 0.05$) and a further reduction in P62 ($P < 0.05$), highlighting the synergistic effect of miR-200a and siPEG10. UTMD intervention significantly enhanced Beclin1, LC3B, PINK1, and Parkin levels ($P < 0.05$) and reduced P62 ($P < 0.05$), with the most significant changes observed in the miR-200a mimic + siPEG10 + UTMD group ($P < 0.05$). qPCR results (Fig. 4G-K) aligned with the WB findings, confirming that UTMD combined with the miR-200a/PEG10 axis significantly enhances autophagy activity and improves autophagic function.

UTMD combined with the miR-200a/PEG10 signaling axis ameliorates liver pathological damage in MAFLD mice and reverses the autophagy inhibition state in MAFLD

To validate the *in vitro* results, we further constructed a MAFLD model using C57BL/6 mice to investigate the effects of UTMD combined with the miR-200a/PEG10 signaling axis on liver pathology and autophagy-related changes in MAFLD. Liver tissue morphology (Fig. 5A) showed that the MAFLD group exhibited significant hepatic steatosis, while the miR-200a mimics group, siPEG10 group, and miR-200a mimics + siPEG10 group showed improved steatosis, particularly after combined UTMD treatment, where liver morphology was significantly improved. Oil Red O staining (Fig. 5B) results revealed that the MAFLD group had significant lipid deposition in the liver, whereas the miR-200a mimics group, siPEG10 group, and miR-200a mimics + siPEG10 group showed reduced hepatic lipid deposition ($P < 0.05$), especially after combined UTMD treatment, where hepatic lipid deposition was significantly reduced ($P < 0.05$). These findings indicate that miR-200a ameliorates hepatic steatosis and lipid deposition in

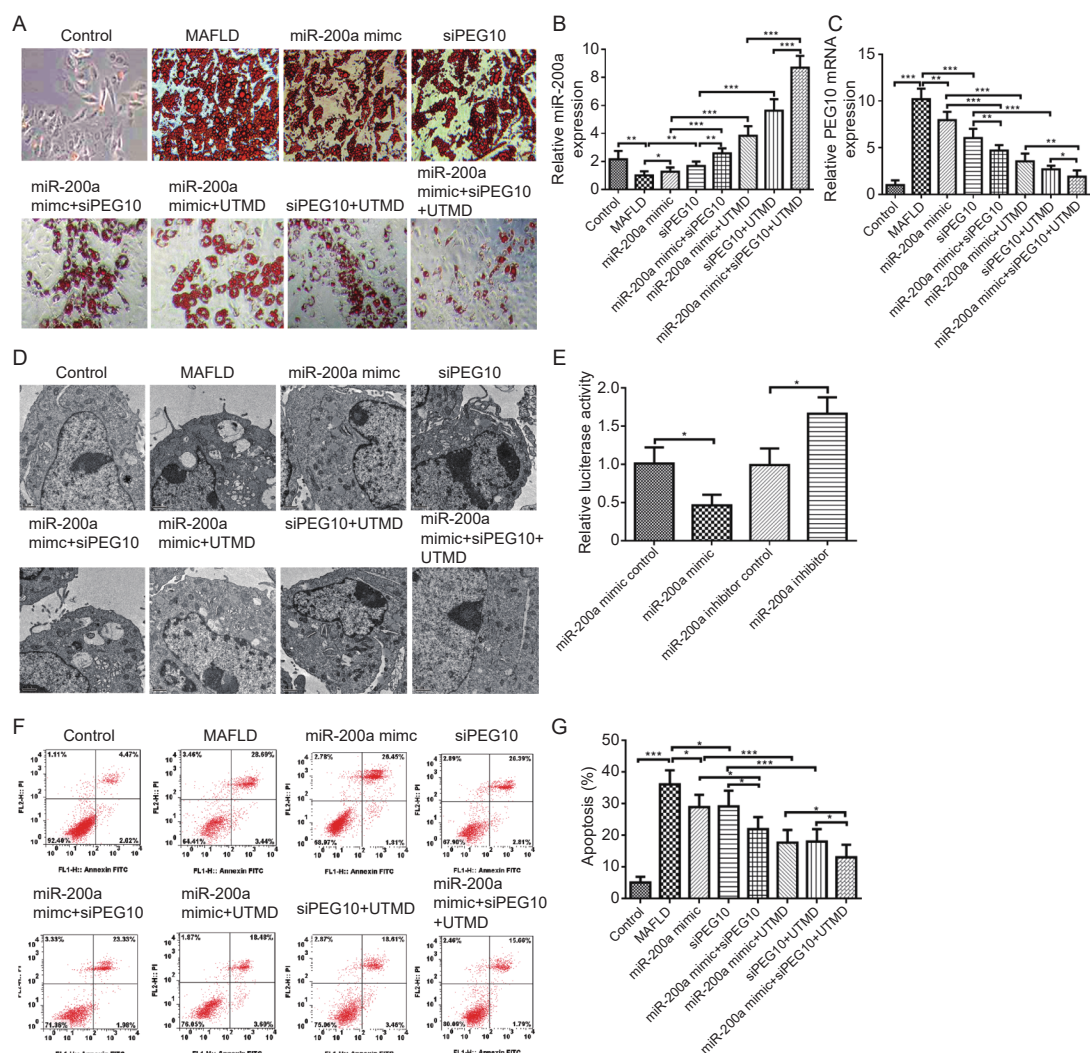


Figure 3 UTMD combined with the miR-200a/PEG10 signaling axis improves lipid metabolism, mitochondrial function, and inhibits apoptosis in MAFLD cells. (A) Oil Red O staining to detect lipid accumulation in each group; (B) qRT-PCR to detect miR-200a; (C) qRT-PCR to detect PEG10; (D) Electron microscopy to observe changes in mitochondrial morphology and structure within cells; (E) Dual-luciferase reporter assay to detect reporter gene expression levels; (F-G) Flow cytometry. * denotes $P < 0.05$, ** denotes $P < 0.01$, and *** denotes $P < 0.001$.

MAFLD by negatively regulating PEG10 expression, and the combined UTMD treatment further enhances this effect.

Immunohistochemical analysis (Fig. 5C) revealed significantly elevated PEG10 expression in the MAFLD group ($P < 0.05$). Compared to the MAFLD group, PEG10 levels were reduced in the miR-200a mimic and siPEG10 groups ($P < 0.05$), with a further decrease in the miR-200a mimic + siPEG10 group ($P < 0.05$). UTMD combined treatment led to an additional reduction in PEG10 expression ($P < 0.05$), with the most significant decrease observed in the miR-200a mimic + siPEG10 + UTMD group ($P < 0.05$). Western blot results (Fig. 5D-E) aligned with these findings, confirming that UTMD enhances the inhibitory effects of miR-200a mimic and siPEG10 on PEG10. qRT-PCR results (Fig. 5F-G) showed that miR-200a expression was significantly lower in the MAFLD group, while PEG10

mRNA levels were significantly higher. Treatment with miR-200a mimic upregulated miR-200a and downregulated PEG10 mRNA, particularly when combined with siPEG10 and UTMD, further enhancing miR-200a expression and suppressing PEG10. Additionally, Western blot analysis of autophagy-related proteins (Fig. 5H-M) indicated that P62 levels were significantly higher in the MAFLD group, while Beclin1, LC3B, PINK1, and Parkin levels were significantly lower ($P < 0.05$). Treatment with miR-200a mimic, siPEG10, or their combination significantly reduced P62 and increased Beclin1, LC3B, PINK1, and Parkin levels ($P < 0.05$). UTMD combined treatment further amplified these changes ($P < 0.05$). qRT-PCR results (Fig. 5N-R) corroborated the Western blot data, demonstrating that UTMD effectively delivers miR-200a mimic and siPEG10, enhancing mitochondrial autophagy and reversing autophagy inhibition in MAFLD.

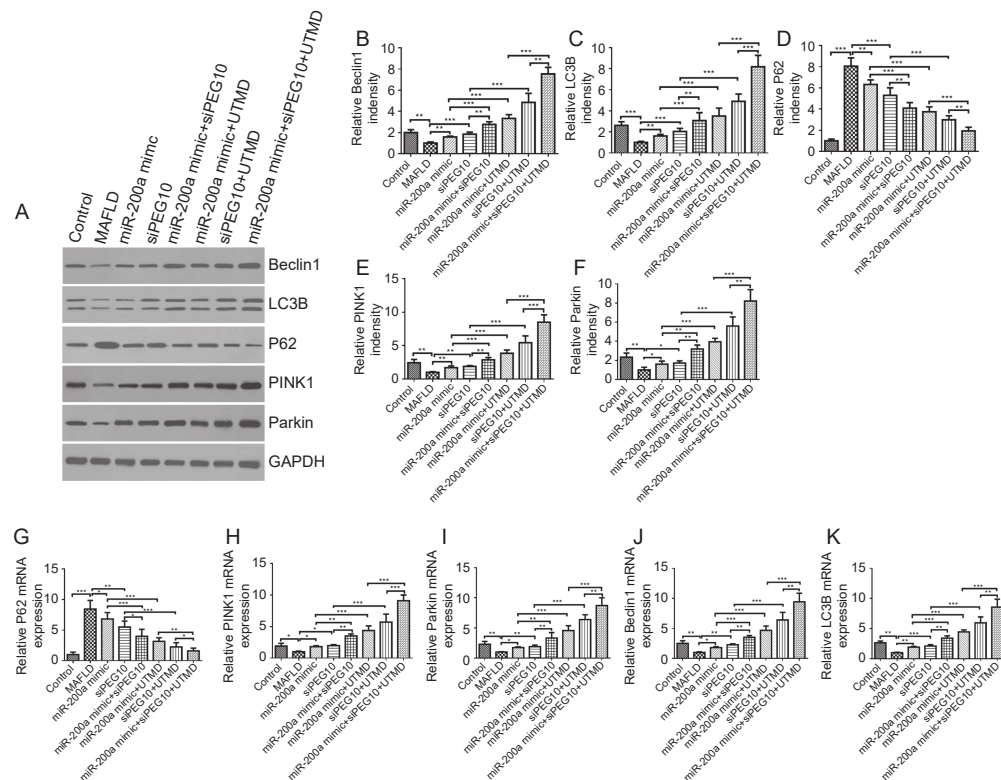


Figure 4 UTMD combined with miR-200a/PEG10 signaling axis reverses MAFLD autophagy inhibition. (A-F) WB detection of Beclin1, LC3B, P62, PINK1, and Parkin; (G-K) qRT-PCR detection of Beclin1, LC3B, P62, PINK1, and Parkin. * denotes $P < 0.05$, ** denotes $P < 0.01$, and *** denotes $P < 0.001$.

3-MA or Rapa can reverse or promote the effects of UTMD combined with the miR-200a/PEG10 signaling axis on autophagy in MAFLD

In this study, functional rescue experiments were conducted using the autophagy inhibitor 3-MA and the agonist Rapamycin (Rapa). The effects of 3-MA and Rapa treatment on the regulation of autophagy by UTMD combined with the miR-200a/PEG10 signaling axis were evaluated through WB and qRT-PCR analyses. The WB analysis results (Fig. 6A-D) showed that, compared to the control group, after 3-MA treatment, the expression of autophagy markers Beclin1 and LC3B was significantly reduced ($P < 0.05$), while the expression of P62 was significantly increased, indicating that the autophagy pathway was inhibited. At the same time, the expression of mitochondrial function markers PINK1 and Parkin was significantly decreased (Fig. 6E-F), suggesting that autophagy inhibition may affect mitochondrial quality control. In contrast, Rapa treatment exhibited a significantly different expression pattern. Rapa significantly increased the expression of Beclin1 and LC3B, indicating that Rapa restored the levels of autophagy markers by activating the autophagy pathway. Additionally, the expression of P62 was significantly reduced, confirming that Rapa promoted the clearance of P62 and enhanced the functionality of the

autophagy pathway. After Rapa treatment, the expression of PINK1 and Parkin was also significantly increased, suggesting that Rapa promoted the restoration of mitochondrial quality control by enhancing the autophagy pathway. However, compared to the MAFLD group, the miR-200 mimics + PEG10 group and the miR-200 mimics + siPEG10 + UTMD group showed increased expression of autophagy markers Beclin1 and LC3B, as well as mitochondrial function markers PINK1 and Parkin, and decreased expression of P62. The qPCR analysis results (Fig. 6G-K) further validated the WB findings. The above results indicate that miR-200 mimics combined with siPEG10 treatment can reverse the inhibition of autophagy by 3-MA and promote the activation of autophagy by Rapa. Moreover, UTMD can further deliver miR-200a mimics and siPEG10, influencing autophagy and mitochondrial function.

3-MA or Rapa reverses or promotes the effects of UTMD combined with the miR-200a/PEG10 signaling axis on MAFLD by influencing autophagy

Next, we investigated the effects of 3-MA or Rapa on the regulation of MAFLD by the UTMD combined miR-200a/PEG10 signaling axis after influencing autophagy. The results showed that compared with the control

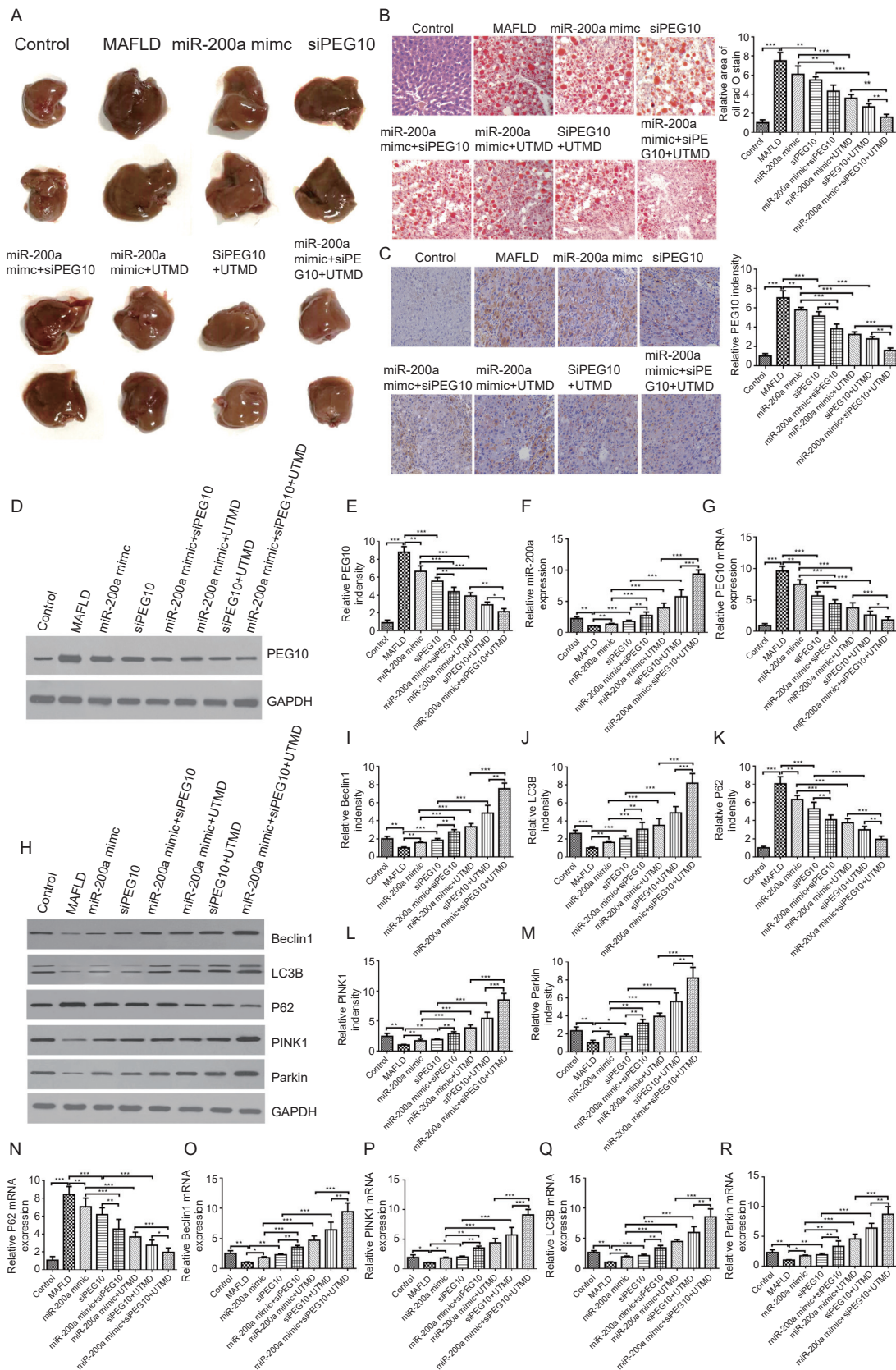


Figure 5 UTMD can deliver miR-200a mimics and siPEG10, ameliorate liver pathological damage in MAFLD mice, and reverse the autophagy inhibition state in MAFLD. (A) Liver tissue morphology; (B) Oil Red O staining of liver tissue; (C) Immunohistochemical staining to detect the expression of PEG10 in the liver; (D-E) PEG10 protein expression was analyzed by WB; (F-G) qPCR detection of miR-200a and PEG10 mRNA expression levels; (H-M) Beclin1, LC3B, P62, PINK1, and Parkin protein expression was analyzed by WB; (N-R) qRT-PCR analysis of the relative mRNA expression levels of Beclin1, PINK1, P62, LC3B, Parkin was analyzed by qRT-PCR. * denotes $P < 0.05$, ** denotes $P < 0.01$, and *** denotes $P < 0.001$.

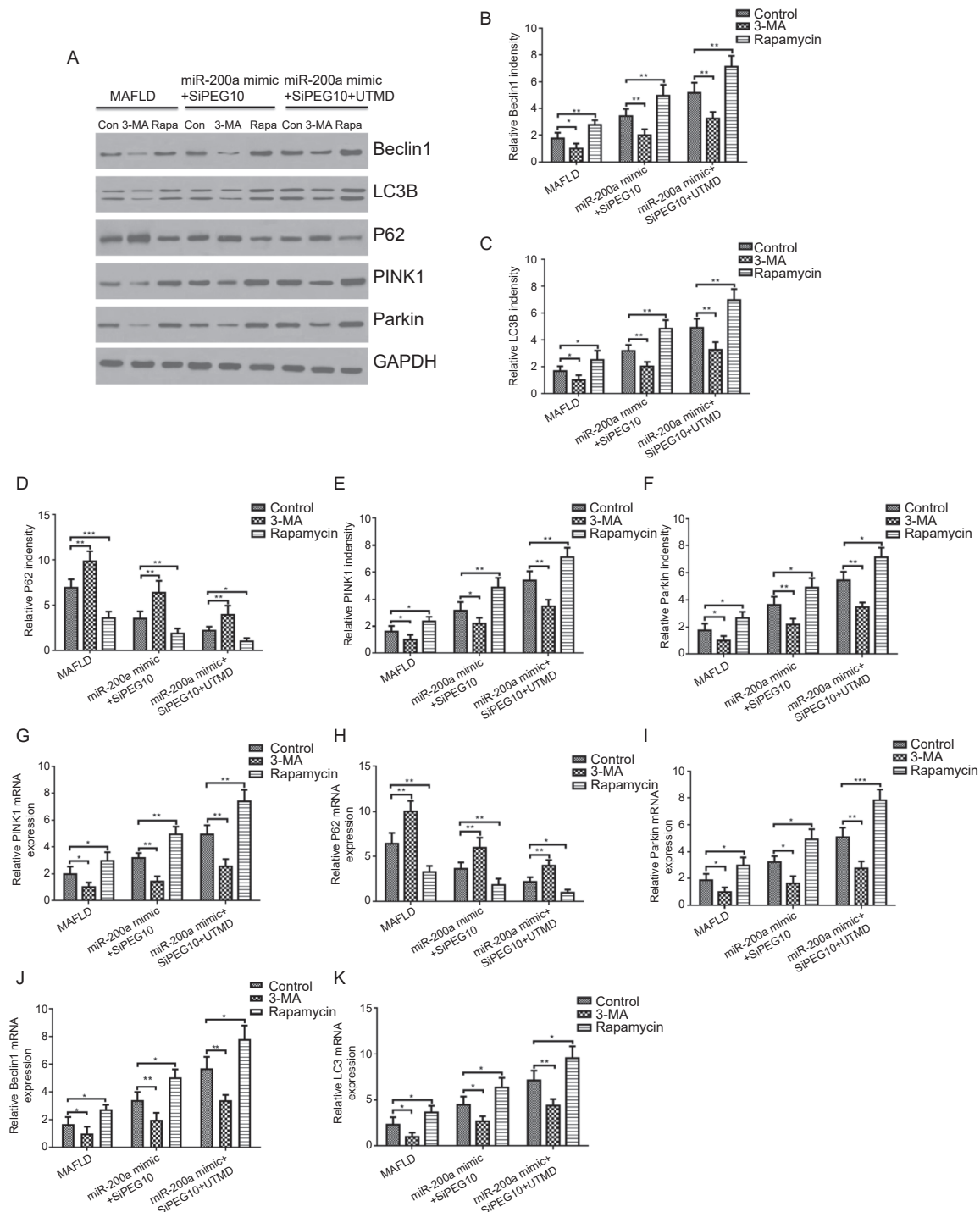


Figure 6 3-MA or Rapa reverses or promotes the effects of UTMD combined with the miR-200a/PEG10 signaling axis on lipid metabolism, mitochondrial function, and apoptosis in MAFLD cells by influencing autophagy. (A-F) WB detection of the protein expression levels of Beclin1, LC3B, P62, PINK1, and Parkin in each group; (G-K) qPCR detection of the mRNA expression levels of Beclin1, LC3B, P62, PINK1, and Parkin in each group. * denotes $P < 0.05$, ** denotes $P < 0.01$, and *** denotes $P < 0.001$.

group, cell apoptosis significantly increased after 3-MA treatment (Fig. 7A-B, $P < 0.05$), the mRNA expression of PEG10 significantly increased (Fig. 7C, $P < 0.05$), the mRNA expression of miR-200a significantly decreased (Fig. 7D, $P < 0.05$), the structure of hepatocytes showed some damage (Fig. 7E), and Oil Red O staining detected an increase in lipid droplets (Fig. 7F), indicating that

inhibition of autophagy may exacerbate the stress response of hepatocytes. In contrast, Rapa treatment showed opposite results to 3-MA treatment. However, compared with MAFLD, the miR-200 mimics + PEG10 group and the miR-200 mimics + siPEG10 + UTMD group showed reduced apoptosis, significantly decreased mRNA expression of PEG10, increased mRNA expres-

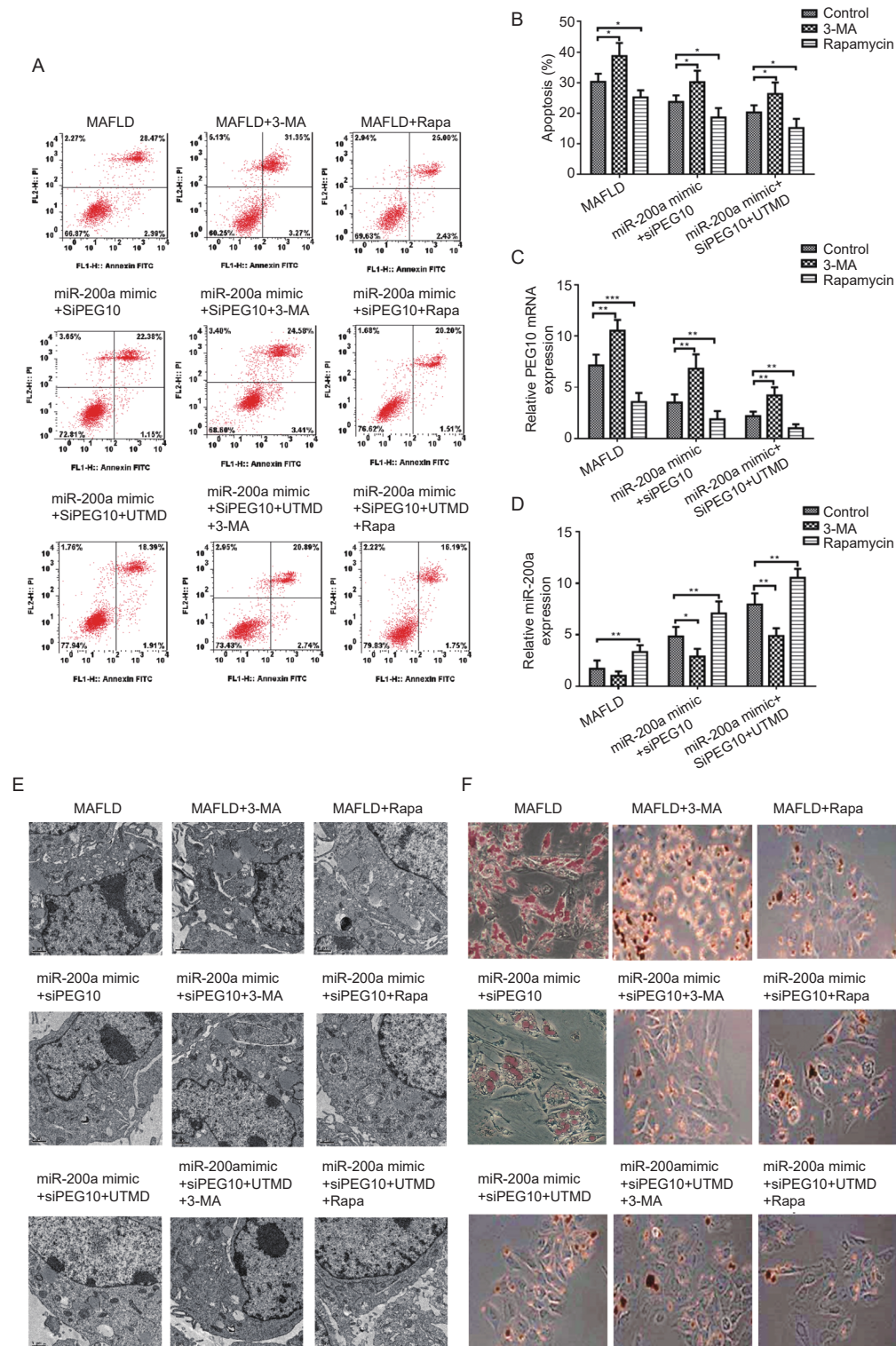


Figure 7 3-MA or Rapa reverses or promotes the effects of UTMD combined with the miR-200a/PEG10 signaling axis on MAFLD by influencing autophagy. (A-B) Apoptosis detected by flow cytometry; (C) qRT-PCR detection of PEG10; (D) qRT-PCR detection of miR-200a; (E) electron microscopy; (F) Oil Red O staining. * denotes $P < 0.05$, ** denotes $P < 0.01$, and *** denotes $P < 0.001$.

sion of miR-200a, normalized hepatocyte structure, and reduced number of lipid droplets, with the combined UTMD treatment showing better effects. The above results indicate that autophagy has a protective role in MAFLD, 3-MA reverses the improvement of MAFLD

by the UTMD combined miR-200a/PEG10 signaling axis through inhibiting autophagy, while Rapa promotes the improvement of MAFLD by the UTMD combined miR-200a/PEG10 signaling axis through promoting autophagy, further proving that the UTMD combined

miR-200a/PEG10 signaling axis can promote autophagy to improve MAFLD.

Discussion

MAFLD is one of the most common chronic liver diseases worldwide, and its pathogenesis involves various factors such as lipid metabolism disorders, mitochondrial dysfunction, and autophagy inhibition [22]. The role of miRNAs in MAFLD has gradually gained attention, and UTMD, as an efficient gene delivery method, has provided new possibilities for the treatment of MAFLD [23]. This study systematically explored the expression and regulatory mechanisms of miR-200a and PEG10 in MAFLD through *in vivo* and *in vitro* experiments, and further validated the role of the UTMD combined miR-200a/PEG10 signaling axis in improving MAFLD. The research found that the UTMD combined miR-200a/PEG10 signaling axis can promote autophagy to ameliorate MAFLD.

miR-200a, a crucial microRNA, is involved in lipid metabolism, inflammation, and apoptosis by targeting and modulating various genes. It has been documented to influence cell metabolism and apoptosis in multiple diseases [24]. Research by Torres J L et al. [15] revealed that miR-200a levels are reduced in the livers of NAFLD patients, a finding consistent with our observations in MAFLD. PEG10, a gene linked to cell proliferation, apoptosis, and metabolism, is overexpressed in several metabolic disorders [25]. According to Zhu Y et al. [26], PEG10 expression correlates positively with insulin resistance and disease severity, potentially contributing to NAFLD pathogenesis and serving as a biomarker. Our study also noted elevated PEG10 mRNA and protein levels in MAFLD, indicating its potential role in the disease. Additionally, we identified an inverse relationship between miR-200a and PEG10, suggesting that miR-200a may mitigate MAFLD progression by downregulating PEG10. *In vitro* studies using dual-luciferase assays confirmed that miR-200a regulates PEG10 transcription, with miR-200a overexpression reducing PEG10 expression and alleviating lipid metabolism issues, mitochondrial dysfunction, and apoptosis in MAFLD models. These findings underscore the protective effect of miR-200a in MAFLD through PEG10 regulation. Thus, the miR-200a/PEG10 axis is pivotal in MAFLD development and may offer a novel therapeutic target.

Autophagy is an important intracellular degradation and recycling mechanism that maintains cellular homeostasis by clearing damaged organelles and protein aggregates [27]. Research by Shi C et al. has shown that inhibition of autophagy leads to lipid metabolism disorders,

mitochondrial dysfunction, and increased apoptosis, thereby exacerbating the pathological progression of MAFLD [28]. This study similarly found that the expression of Beclin1, LC3B, PINK1 and Parkin was significantly reduced in MAFLD, while the expression of P62 was significantly increased, indicating suppressed autophagy activity. Conversely, overexpression of miR-200a and knockdown of PEG10 significantly reversed this phenomenon, upregulating the expression of Beclin1, LC3B, PINK1, and Parkin, while reducing the expression of P62. These results suggest that miR-200a promotes the activation of autophagy by inhibiting the expression of PEG10, thereby improving the pathological features of MAFLD. Mitophagy, the selective clearance of damaged mitochondria, is crucial for maintaining mitochondrial function and cellular homeostasis [29]. Research by Cao P et al. has shown that promoting mitophagy is a promising strategy for treating MAFLD [30]. Our further mechanistic studies revealed that the miR-200a/PEG10 signaling axis plays an important role in MAFLD by regulating mitophagy. This study found that overexpression of miR-200a and knockdown of PEG10 significantly improved mitochondrial function and reduced mitochondrial swelling and membrane rupture, further supporting the hypothesis that the miR-200a/PEG10 signaling axis ameliorates MAFLD by promoting mitophagy.

UTMD is a novel gene delivery technology that utilizes the disruptive effect of ultrasound microbubbles to efficiently deliver exogenous genes or drugs to target tissues. It has been widely applied in the treatment research of various diseases [31]. Li Y et al. [32] demonstrated that UTMD can rapidly trigger cell membrane perforation to mediate the delivery of miR-34a-simulate, which can be used for the treatment of ovarian cancer. This study similarly found that after treatment with UTMD combined with miR-200a mimics and siPEG10, lipid accumulation in MAFLD was significantly reduced, mitochondrial morphology and function were restored, and the apoptosis rate was significantly decreased. Therefore, the addition of UTMD not only enhanced the delivery efficiency of miR-200a and siPEG10 but also further amplified their beneficial effects on lipid metabolism and mitochondrial function. Additionally, the expression of Beclin1, LC3B, PINK1 and Parkin was reduced, while the expression of P62 was significantly increased, indicating suppressed autophagy function. After treatment with UTMD combined with the miR-200a/PEG10 signaling axis, the expression of autophagy-related proteins was significantly restored, suggesting that UTMD can promote the recovery of autophagy function by enhancing the effects of miR-200a and siPEG10, thereby ameliorating the

pathological damage of MAFLD.

Autophagy, as an important cellular self-protection mechanism, plays a crucial role in maintaining cellular environmental stability and metabolic balance [33]. This study found that autophagy activity was significantly reduced in MAFLD, while the combination of UTMD and miR-200a/PEG10 signaling axis could significantly reverse this phenomenon. Therefore, we conducted functional rescue experiments using autophagy inhibitor 3-MA and agonist Rapamycin to further validate the key role of autophagy in regulating MAFLD through UTMD combined with miR-200a/PEG10 signaling axis. 3-MA treatment significantly inhibited autophagy activity and reversed the improvement effect of UTMD combined with miR-200a/PEG10 signaling axis on MAFLD, while Rapa treatment further enhanced the efficacy of UTMD combined therapy by activating autophagy. These results indicate that autophagy plays a protective role in the pathological process of MAFLD, and the combination of UTMD and miR-200a/PEG10 signaling axis significantly improves the pathological features of MAFLD by promoting autophagy. Notably, it was found that PEG10 protein expression was significantly elevated in MAFLD liver tissues, whereas PEG10 mRNA level was reduced. This inconsistency indicates that PEG10 expression may be regulated by post-transcriptional mechanisms rather than transcriptional activation alone. MicroRNAs can modulate gene expression by affecting mRNA stability or translation efficiency. In the present study, miR-200a was significantly downregulated in MAFLD tissues and exhibited a negative correlation with PEG10 expression. Furthermore, the dual-luciferase reporter assay confirmed that miR-200a could directly target PEG10. These findings indicate that the increased PEG10 protein level in MAFLD may be partly attributed to reduced miR-200a-mediated repression, thereby enhancing PEG10 translation despite reduced mRNA level. Additionally, post-transcriptional regulation, altered protein stability, or compensatory feedback mechanisms under metabolic stress may also contribute to the discordance between PEG10 mRNA and protein levels.

Compared with other gene delivery systems, UTMD offers distinct advantages and limitations. Viral vectors provide high transfection efficiency and are widely used in preclinical studies. For therapeutic purposes, however, lentiviral vectors pose a safety risk due to insertional mutagenesis and persistent expression of site-specific nucleases, which can result in off-target mutations [34]. Additionally, viral vectors often elicit immunogenic responses and have limited cargo capacity, restricting their applicability for combination gene therapy [35]. Nanoparticle-based systems offer improved biocompatibility but often suffer from rapid clearance and passive

targeting inefficiency [36]. In contrast, microbubble cavitation in UTMD enhanced local gene delivery while minimizing systemic exposure. Zhao et al. concentrated on the biological function of miR-200a in alcoholic liver disease (ALD), and demonstrated that miR-200a could regulate the apoptosis of hepatocyte in ALD by directly target ZEB2 [37].

Although this study is the first to reveal the role of the miR-200a/PEG10 axis in MAFLD and indicated that UTMD combined with modulation of this axis could alleviate pathological damage associated with MAFLD, several limitations should be acknowledged. Firstly, the clinical sample size was relatively small, which might limit the statistical power of the analysis. Future studies with larger cohorts are therefore required to further validate these findings. Secondly, only male mice were included in the animal experiments; subsequent studies should incorporate female mice to evaluate potential sex-related differences. In addition, although the UTMD parameters used *in vitro* differed from those applied *in vivo*, standardized UTMD parameters appropriate for clinical translation remain to be established. Practical challenges for clinical application also persist, including limited ultrasound penetration and potential interference caused by respiratory motion. Furthermore, while the dual-luciferase reporter assay confirmed that miR-200a could directly target PEG10, this interaction should be further validated using techniques, such as RNA immunoprecipitation sequencing (RIP-seq). The downstream signaling pathways through which PEG10 could regulate autophagy also remain unclear and require further investigation. Moreover, the assessment of autophagy in this study relied primarily on static molecular markers, and dynamic evaluation of autophagic flux was not performed. Moreover, the number of human liver samples included in this study was limited due to the restricted availability of clinical biopsy specimens, which might affect the generalizability of the findings. Although post hoc power analysis indicated adequate power to detect large differences, larger clinical cohorts are still required to further validate these findings. Although the present study demonstrated that UTMD effectively enhanced the delivery of miR-200a mimic and siPEG10 in both cellular and animal models, several issues related to clinical translation require further investigation. The ultrasound parameters used in this study were optimized for experimental conditions and may not directly translate to clinical settings. Future studies should systematically evaluate the safety, optimal acoustic parameters, tissue penetration efficiency, and long-term effects of UTMD-mediated gene delivery in large animal models and clinical environments to further assess its feasibility for therapeutic application in

MAFLD. Although the LC3-II/p62 change pattern and 3-MA/Rapa recovery experiment supported the enhancement of autophagy flow, it still needs to be further confirmed by autophagy flow experiment in the future. Addressing the above limitations will help to promote the clinical translation of UTMD-mediated miR-200a/PEG10 targeted therapy strategies.

Conclusion

In summary, this study revealed the role of the miR-200a/PEG10 signaling axis in MAFLD and demonstrated that combining UTMD with modulation of this axis could significantly attenuate MAFLD-related pathological injury by improving lipid metabolism, restoring mitochondrial function, and relieving autophagy inhibition. These findings provide new potential therapeutic targets and strategic approaches for the management of MAFLD. Future studies should further investigate the clinical translational potential of UTMD combined with miR-200a/PEG10 modulation and evaluate its long-term safety and therapeutic efficacy.

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Authors' Contributions

Xiaohui Zhao and Xueqin Shen conceived and designed the study and drafted the manuscript, contributing equally to this work. Haiyue Zhao and Diansheng Jin acquired the data and performed the statistical analyses. Yu Zhang, Dongfang Yang, and Xiuyan Lan conducted the animal experiments and related procedures. Yang Liu reviewed and verified the data. Xin Liu oversaw the ethical approval process and screened the potentially eligible studies. Shunan Li and Xianquan Shi revised the manuscript and approved the final version for submission, also contributing equally.

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Availability of data

The data that support the findings of this study are not publicly available but are available from the corresponding author.

Declarations

Ethics approval and consent to participate

This study has been approved by the Ethics Committee of Hohhot First

Hospital. All patients signed written informed consent.

Conflict of interest

The authors declare that there is no conflict of interest.

Consent for publication

Not applicable.

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