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MiR-124 protects human hepatic L02 cells from H_2O_2-induced apoptosis by targeting Rab38 gene

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ABSTRACT

Background: Hepatic ischemia reperfusion injury (IRI) is an inevitable clinical problem for liver surgeons. Because microRNAs (miRNAs) participate in various hepatic pathophysiological processes, this study aimed to explore the role and potential mechanism of miR-124 in hepatic IRI.
Methods: A liver IRI model was established in rats. The differential expression of miRNAs was detected using microarrays, and the expression of miR-124 was measured by qRT-PCR. A hydrogen peroxide (H_2O_2)-induced oxidative stress apoptosis model was also established. Cell apoptosis was detected by flow cytometry, and viability was detected by CCK8. The expression of Rab38 was detected by Western blotting and qRT-PCR, and a luciferase reporter assay was used to verify the expression of the miR-124 target gene.
Results: The miRNA spectrum changes dramatically after hepatic IRI in rats, and miR-124 is significantly down-regulated after liver IRI. MiR-124 decreases the H_2O_2-induced apoptosis of human hepatic L02 cells by up-regulating the activation of the AKT pathway. Rab38 is a target gene of miR-124 and is involved in H_2O_2-induced apoptosis. Interference with the expression of the Rab38 gene can protect hepatic L02 from H_2O_2-induced apoptosis by increasing the phosphorylation of AKT. These protective effects of miR-124 are attenuated by over-expression of Rab38.
Conclusions: Many miRNAs are involved in hepatic IRI in rats, and miR-124 is significantly decreased in this model. MiR-124 significantly decreases the H_2O_2-induced apoptosis of human hepatic L02 cells by targeting the Rab38 gene and activating the AKT pathway.

1. Introduction

The occurrence of ischemia reperfusion during liver operations, such as partial hepatectomy and liver transplantation, is inevitable in clinical settings [1]. This injury may induce pathological disorders in hepatocytes, including damage to their structure and function, which result in postoperative organ dysfunction or even liver failure [2].

MicroRNAs (miRNAs) are a class of small regulatory RNAs that regulate the expression of messenger RNAs by binding to their 3′-untranslated regions (UTRs). Further research has shown that miRNAs regulate approximately 30% of human protein-coding genes [3]. MiRNAs modulate a variety of biological processes including cellular differentiation and proliferation, metabolism and apoptosis [4]. Importantly, circulating microRNA-1 is considered as a potential novel biomarker for acute myocardial infraction [5], the systemic delivery of miR-26a inhibited tumorigenesis in murine liver cancer [6], which revealed that miRNAs might be new biological markers in the diagnosis of diseases and could become targets for new drug research, which would most likely generate new treatments for human diseases.

Some recent studies confirmed that miRNAs play important roles in hepatic ischemia reperfusion injury (IRI). For example, Farid analyzed liver allograft samples ($n = 45$) 1 h after reperfusion and confirmed that the expression of miR-122 was negatively correlated with the degree of warm ischemia injury [7]. MiRNA-223, which regulates the expression of ephrin A1, acyl-CoA synthetase long-chain family member 3 and ras homolog gene family member B, was strongly up-regulated in hepatic IRI [8]. MiR-146a was down-regulated in the early stage of liver IRI and was associated with the Toll-like receptor 4 signaling pathway [9]. Therefore, the

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study of miRNAs and their related target genes may provide new therapeutic strategies for hepatic IRI.

In this study, we focused on miR-124 due to its expression level after hepatic IRI and further studied its function in the human hepatic L02 cell model of H_2O_2-induced apoptosis. Our data suggest that miR-124 might be a novel therapeutic for hepatic IRI.

2. Materials and Methods

2.1. Establishment of a rat model of 70% hepatic IRI

Male Sprague-Dawley (SD) rats (180–200 g) were purchased from the Animal Center of Sun Yat-sen University (Guangzhou, China). All animals were treated in a humane manner in accordance with the institutional animal care instructions. The study protocol was approved by the Animal Ethics Review Committee of Sun Yat-sen University. SD rats were randomly divided into IRI and sham (SH) groups (n = 4 rats/group). The rats were then anesthetized with chloral hydrate (3 mL/Kg, intra-peritoneally) and underwent 70% hepatic IRI by clamping the left and middle liver arteries, portal vein and bile duct. After 1 h ischemia, the clamp was removed and the liver was reperfused for the indicated times (6 h, 9 h, 12 h). For the SH group, the rats underwent abdominal surgery without liver ischemia. At the indicated times, the rats were sacrificed, and the left liver tissues were gathered.

2.2. Microarrays

Total liver samples from IRI and SH rats were examined by the Kangchen Corporation (Kangchen, Shanghai, China). Total RNA was harvested using TRIzol reagent (Invitrogen, USA) and the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. The RNA was quantified using a NanoDrop 1000 (NanoDrop, Wilmington, DE) and the samples were labeled with the miRCURY™ Hy3™/Hy5™ Power Labeling Kit (Exiqon, Vedbaek, Danmark) and hybridized to the miRCURY™ LNA Array (v.18.0) (Exiqon). After normalization, the statistical significance of the differentially expressed miRNAs was analyzed using a t-test. Fold changes >1.5 and P-values <0.05 were considered significant.

2.3. Cell culture and transfection

Human hepatic L02 cells (purchased from the Shanghai Cell Band of the Chinese Academy of Sciences) were cultured in RPMI-1640 medium containing 10% FBS, 1% penicillin (100 IU/mL) and 1% streptomycin (100 g/mL) at 37 °C in 5% CO_2. The miR-124 mimics, miR-124 inhibitor, Rab38 siRNA and negative control (NC) sequences were obtained from RIBOBIO (Guangzhou, China). Cells were transfected using a Lipofectamine RNAiMAX Kit (Invitrogen) according to the manufacturer's protocol.

2.4. Viability detection by CCK8

Transfected hepatic L02 cells in FBS-free medium were treated with H_2O_2 (0–350 μM) for 6 h, 10 μL of CCK8 (Dojindo, Kumamoto, Japan) was added to the cells, and the viability of the cells was measured at 490 nm using an ELISA reader (BioTek, Winooski, VT, USA) according to the manufacturer's instructions.

2.5. Evaluation of cell apoptosis by flow cytometry

Transfected hepatic L02 cells were treated with 200 μM H_2O_2 for 6 h, and cell apoptosis was measured by flow cytometry according to the manufacturer's instructions (KeyGen, NanJing, China). The cells were collected and resuspended in 500 μL of binding buffer and 5 μL each of annexin V-FITC and PI was added. The analyses were conducted within 30 min. The PI3K inhibitor (LY294002) was purchased from Cell Signaling Technology (Beverly, USA).

2.6. Reverse transcription and quantitative real-time PCR

Total RNA was extracted from transfected cells or liver samples by TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) of the liver samples was performed using 100 ng of total RNA, multiplex RT primers and the TaqMan MicroRNA Reverse Transcriptase Kit (Life Technologies, Gaithersburg, MD, USA). Quantitative real-time PCR (qRT-PCR) was performed using the TaqMan Universal PCR Master Mix, No AmpErase UNG (Life Technologies) and the LightCycler 480 system (Roche, Switzerland). cDNA of the transfected cells was synthesized following the manufacturer's protocol (Takara, Dalian, China). qRT-PCR was performed using a standard SYBR green PCR kit (Takara) and PCR-specific amplification was performed using a LightCycler 480 real-time PCR machine. The relative expression of the target genes (miR-124, u6, Rab38 and GADPH) was calculated using the $2^{-\Delta\Delta}$Ct method [10].

2.7. Western blot analysis

Protein samples were prepared and separated on a 10–12% SDS–PAGE gel. The proteins were transferred to PVDF membranes (Millipore, Boston, USA), which were then blocked with bovine serum albumin. Primary antibodies against human Rab38, p-AKT, AKT (Cell Signaling Technology, Beverly, USA) and β-actin (Santa Cruz, Biotechnology, Inc, Santa Cruz, USA) were incubated overnight at 4 °C. The membranes were washed with TBST and incubated with goat anti-rabbit secondary antibody for 1 h at room temperature. Finally, the membranes were exposed using enhanced chemiluminescence for 2 h.

2.8. Luciferase reporter assay

The 3′-UTR of Rab38, containing the Rab38-miR-124 response element, was cloned into the XhoI/BamHI site of a pLUC control Luciferase vector (Hapk, Shenzhen, China). HEK293 cells were seeded in a 96-well plate (2×10^4 cells per well) and transfected with Rab38-UTR-pLUC/Mu-Rab38-UTR-pLUC and the miR-124 mimics/miRNA NC using FugeneHD transfection reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Forty-eight hours later, the luciferase activity was measured using the dual luciferase reporter assay system (Promega).

2.9. Rexcue experiments

2 μg of pcDNA3.1-Rab38 plasmid, containing Rab38 cDNA, was cotransfected with miR-124 mimics in hepatic L02 cells using FugeneHD transfection reagent. Cell apoptosis was measured by flow cytometry.

2.10. Statistical analysis

A paired Student's t-test was used, and a P-value of less than 0.05 was considered statistically significant.

3. Results

3.1. MiR-124 is down-regulated during hepatic ischemia reperfusion injury

To measure miRNA expression during hepatic IRI, a hepatic IRI rat model (1-h ischemia/6-h reperfusion) was established. We

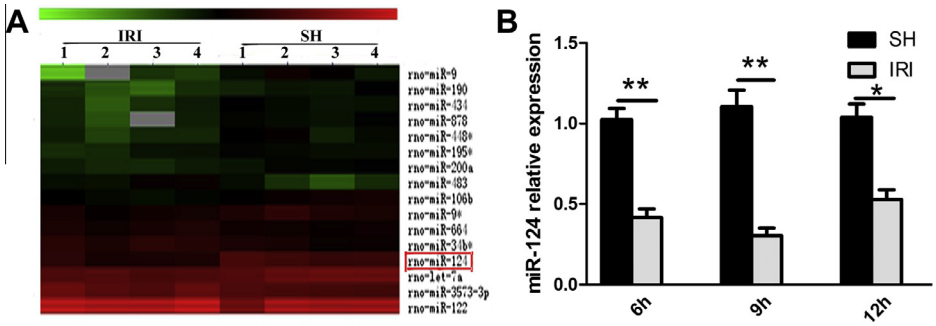

Fig. 1. Differentially expressed miRNAs during hepatic ischemia reperfusion. (A) Hierarchical clustering of differentially expressed miRNAs in the SH (sham group, $n = 4$) vs. IRI (1-h ischemia/6-h reperfusion, $n = 4$) groups; red indicates high relative expression and green indicates low relative expression. Fold changes >1.5 and P-values <0.05 were considered significant, and miR-124 is significantly down-regulated after hepatic ischemia reperfusion in rats (fold changes = 2.51, $P = 0.004$). (B) The expression of miR-124 during hepatic IRI by quantitative real-time PCR (qRT-PCR) analysis at each time point (6 h: 1-h ischemia/6-h reperfusion, 9 h: 1-h ischemia/9-h reperfusion, 12 h: 1-h ischemia/12-h reperfusion) compared with each SH group ($n = 4$). MiR-124 levels were normalized to that of the vector. $^{*}P < 0.05$, $^{**}P < 0.01$.

performed miRNA microarrays on the liver samples and found 5 up-regulated and 11 down-regulated miRNAs, miR-124 was down-regulated at least 2.5-fold compared with the SH group, with a P-value of 0.004 (Fig. 1A). We then examined the expression levels of miR-124 at different reperfusion time points. As shown in Fig. 1B, we confirmed that miR-124 was significantly down-regulated when compared with the SH control group at 6 h, 9 h, and 12 h after reperfusion.

3.2. MiR-124 decreased the H_2O_2-induced apoptosis of hepatic L02 cells by activating AKT signaling pathway

ROS is a critical factor that leads to hepatic IRI [11,12]. An in vitro H_2O_2-induced apoptosis model was used to mimic hepatic IRI. Transfected hepatic L02 cells were treated with H_2O_2 (0–350 μM) for 6 h, and cell viability was measured using CCK8 (Fig. 2A), we found that miR-124 significantly increased cell viability in different

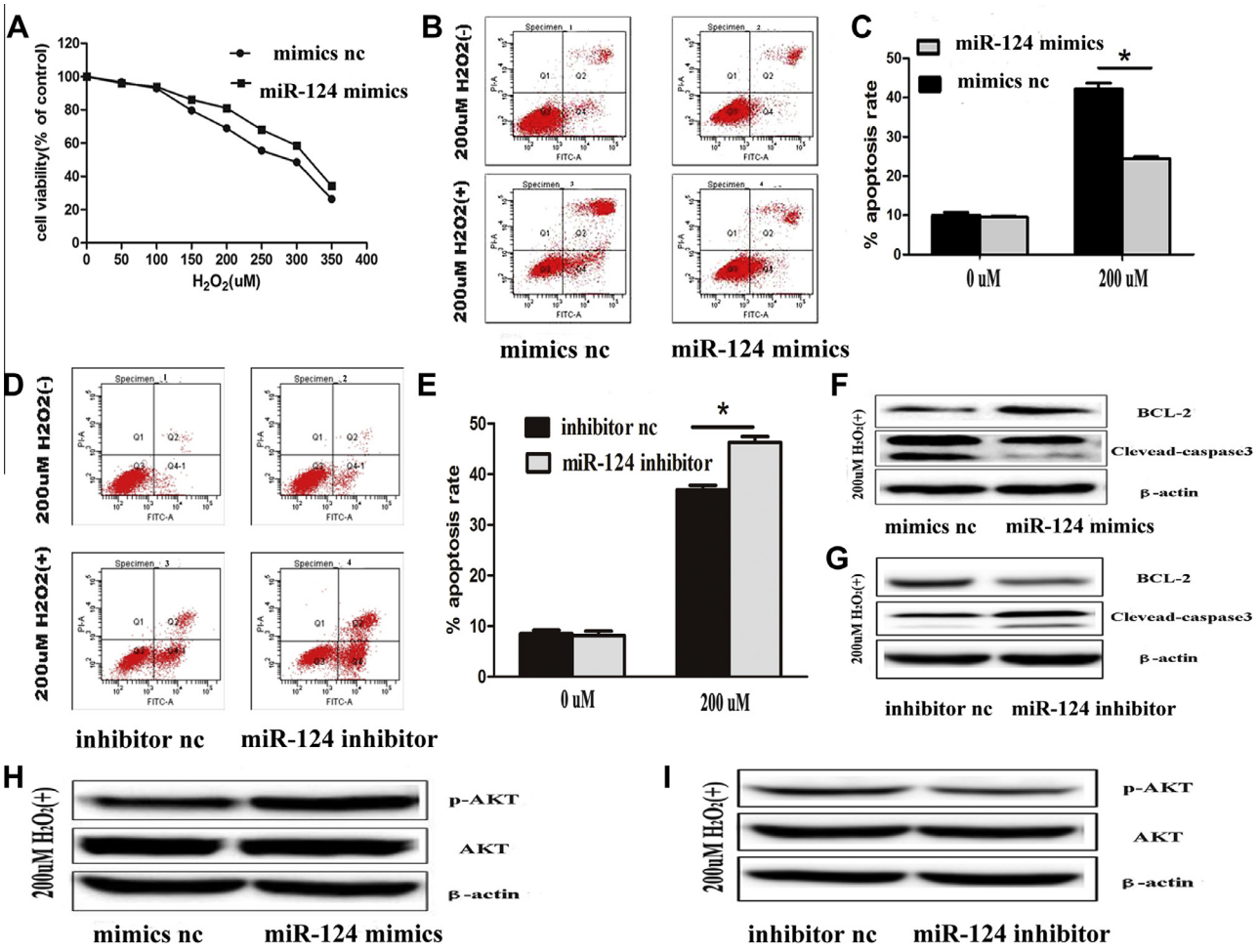

Fig. 2. MiR-124 decreased the H_2O_2-induced apoptosis of hepatic L02 cells by activating AKT signaling pathway in vitro. (A) Transfected hepatic L02 cells were treated with H_2O_2 (0–350 μM) for 6 h, and cell viability was measured using CCK8 ($n = 3$). (B–E) The cell apoptosis rate after treatment with 200 μM H_2O_2 was detected by flow cytometry ($n = 4$). (F, G) Apoptosis-associated protein was detected by Western blot ($n = 3$). (H, I) The activation of AKT was examined by Western blot ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$.

concentrations (150, 200, 250, 300, and 350 μM) compared with the NC group. Based on these results, the concentration of 200 μM H_2O_2 was chosen for following experiments. As shown in Fig. 2B and C, the apoptosis rates of the miR-124 mimics and NC groups were 39.05% ± 3.20% and 24.75% ± 2.91%, respectively, after treatment with 200 μM H_2O_2, which indicated that miR-124 had a protective effect against the H_2O_2-induced apoptosis of hepatic L02 cells, whereas transfection of miR-124 inhibitor aggravated the H_2O_2-induced apoptosis of hepatic L02 cells (Fig. 2D and E). As shown in Fig. 2F and G, the level of the anti-apoptosis protein Bcl-2 was significantly increased after miR-124 mimics transfection and reduced after miR-124 inhibitor transfection, and the pro-apoptosis protein cleaved caspase-3 was significantly decreased after miR-124 mimics transfection and increased after miR-124 inhibitor transfection.

To investigate the mechanisms responsible for the miR-124-mediated protection, the oxidative stress apoptosis signaling pathway was analyzed. A Western blot analysis showed that the AKT pathway was activated in the miR-124 group (Fig. 2H). In contrast, miR-124 inhibitor suppressed the activation of AKT (Fig. 2I). Moreover, after pretreatment with LY294002 (20 μM, 30 min), the anti-apoptotic effect of miR-124 was abrogated (data not shown).

3.3. Rab38 is a target gene of miR-124

To identify the miR-124 target gene, bioinformatic tools (Targetscan and miRBase) were used. As shown in Fig. 3A, Rab38 is the theoretical target gene of miR-124. The expression of the candidate target gene Rab38 after treatment with miR-124 mimics or miR-124 inhibitor was detected at the protein and mRNA levels by Western blotting and qRT-PCR, respectively (Fig. 3B and C).These data showed that the expression of Rab38 was significantly decreased after miR-124 mimics transfection and increased after miR-124 inhibitor transfection both at the protein and mRNA levels.

To further validate whether Rab38 is a direct target gene, we fused the 3′-UTR region of Rab38 to a luciferase system. As shown in Fig. 3D, miR-124 obviously suppressed the luciferase activities of the 3′-UTR segment of Rab38, but not those of the construct containing a mutant binding site (Rab38 3′-UTR-MUT), compared to the NC group.

3.4. MiR-124 protects hepatic L02 cells from H_2O_2-induced apoptosis by targeting Rab38

To delineate the function of Rab38 in hepatic L02 cell apoptosis induced by H_2O_2, cultured hepatic L02 cells were treated with H_2O_2 (0–350 μM) for 6 h, and the expression of Rab38 protein was up-regulated after H_2O_2 stimulation (Fig. 4A). As shown in Fig. 4B, C and D, Rab38 siRNA remarkably protected hepatic L02 cells from H_2O_2-induced apoptosis. We further confirmed that Rab38 down-regulation protected hepatic L02 cells from H_2O_2-induced apoptosis through the AKT pathway (Fig. 4 E).

To further confirm Rab38 involved in miR-124-mediated protection, we performed rescue experiment. The anti-apoptotic effect of miR-124 was significantly attenuated when Rab38 was over-expressed (Fig. 4F and G).

4. Discussion

Hepatic IRI is a universal problem that occurs during liver surgery, especially in liver resections and transplantations [13]. Multiple genes have been reported to be involved in the pathophysiological processes of hepatic IRI [14]. Because miRNAs can regulate the expression of endogenous genes, we hypothesized that miRNAs might be involved in hepatic IRI. Using microarray analysis and qRT-PCR, we found that many miRNAs are involved in hepatic IRI, and miR-124 was consistently down-regulated in ischemia reperfusion livers, suggesting that miR-124 is an ischemia reperfusion-related miRNA in this organ. Intriguingly, miR-124 protects against focal cerebral ischemia [15], and some studies have confirmed that miR-124 participates in other liver

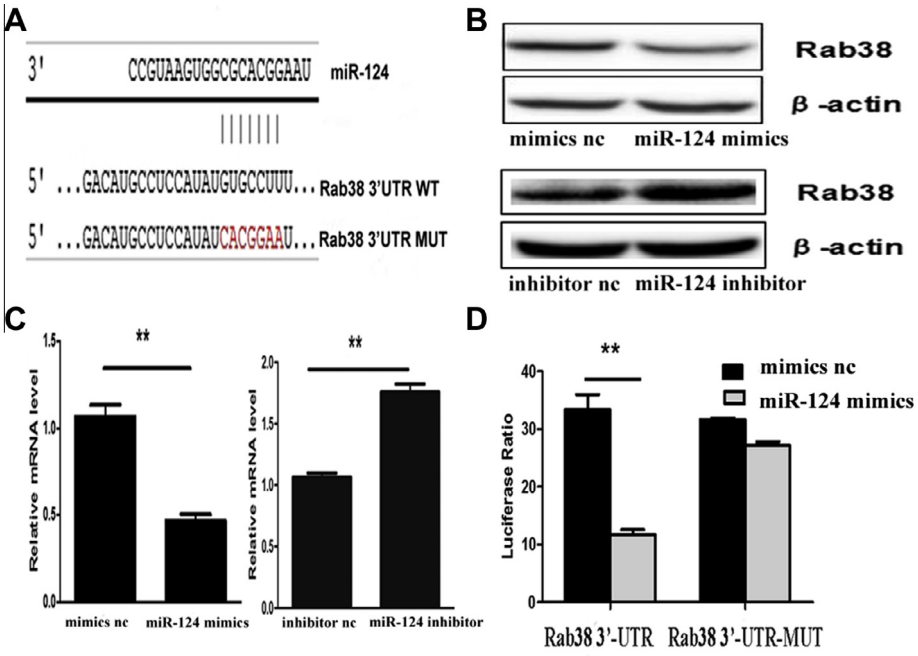

Fig. 3. Rab38 is a target gene of miR-124. (A) miR-124-binding sequences in the 3′-UTR of Rab38 and mutated sites in 3′-UTR of Rab38. (B, C) The expression of Rab38 was detected by Western blotting and qRT-PCR, respectively, after treatment with miR-124 mimics or miR-124 inhibitor ($n = 3$). (D) MiR-124 suppressed the luciferase activities of constructs containing the 3′-UTR segment of Rab38 ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$.

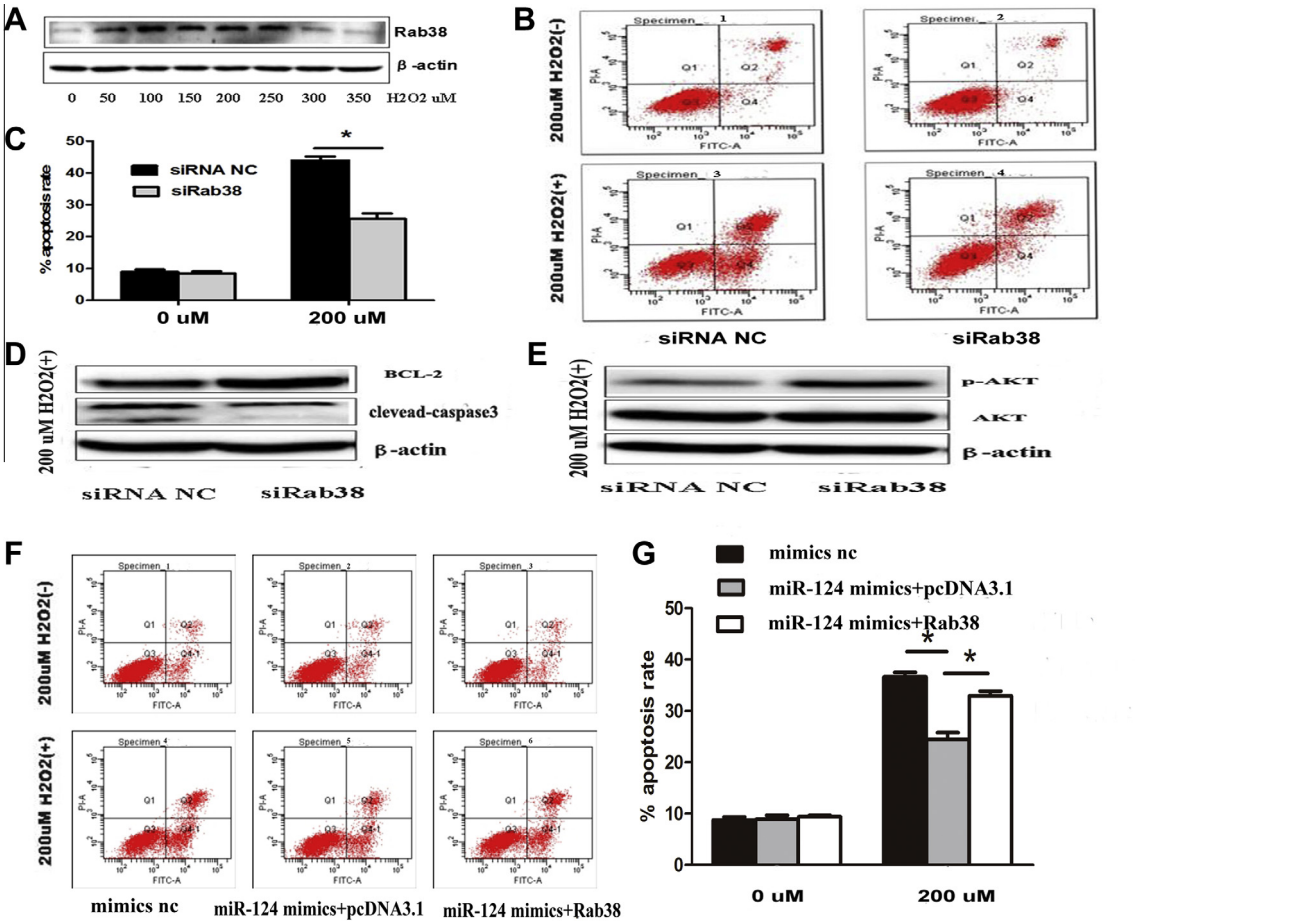

Fig. 4. MiR-124 protects hepatic L02 cells from H_2O_2-induced apoptosis by targeting Rab38. (A) The expression of the Rab38 protein under H_2O_2 stimulation was detected by Western blotting ($n = 3$). (B, C) The apoptosis rate of the cells exposed to siRab38, as detected by flow cytometry, was significantly decreased compared with that of the siRNA NC groups after treatment with 200 μM H_2O_2, while those left untreated showed no significant difference($n = 3$). (D) Apoptosis-associated proteins were detected by Western blotting ($n = 3$). (E) A Western blot showing that activation of the AKT pathway was increased in siRab38 groups compared with NC groups ($n = 3$). (F, G) The flow cytometry data showed that overexpression of Rab38 can attenuate the anti-apoptotic effect of miR-124 ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$.

diseases [16,17]. Therefore, we hypothesized that miR-124 is a key protective factor of hepatic IRI.

Hepatic IRI is characterized by the generation of reactive oxygen species (ROS), which include superoxide anions, hydrogen peroxide and hydroxyl radicals [18,19]. Therefore, in this study, we established an in vitro oxidative stress model induced by H_2O_2 and found that the rate of apoptosis of hepatic L02 cells induced by H_2O_2 was dose dependent, which was consistent with results from a previous study [20]. Furthermore, the over-expression of miR-124 remarkably protected hepatic L02 cells from H_2O_2-induced apoptosis, which indicated that miR-124 is a positive regulator of hepatic IRI.

The PI3K-AKT signaling pathway plays a pivotal role in oxidative stress, and the activation of AKT significantly protected cells from H_2O_2-induced cell apoptosis [21,22]. Previous studies confirmed that PI3K-AKT acts upstream of Bcl-2 family proteins and caspase-3 in oxidative stress that is induced by H_2O_2 [23,24]. In our study, we found that miR-124 increased the phosphorylation levels of AKT. Furthermore, the expression of Bcl-2 was increased and the activity of caspase-3 was decreased after treatment with miR-124. These results indicated that miR-124 protected hepatic L02 cells from H_2O_2-induced apoptosis through the AKT pathway, but the role of miR-124 in vivo need to be further confirmed.

The Targetscan and miRBase websites were used for locating the target gene and we found that miR-124 had 770 potential targets, including the Rab38 gene, which belongs to the Rab family of small GTPases. A previous study reported that Rab proteins and PI3 K are composed of a negative adjustment ring that is involved in the regulation of cell proliferation and apoptosis [25]. In this study, our data showed that Rab38 is a direct target gene of miR-124. More importantly, we further confirmed that the expression of the Rab38 protein was up-regulated under H_2O_2 stimulation and a Rab38 siRNA remarkably protected hepatic L02 cells from H_2O_2-induced apoptosis through the AKT pathway. Meanwhile, the rescue experiment showed that the protection of miR-124 against H_2O_2-induced apoptosis of hepatic L02 cells was, at least in part, attributed to its inhibition of Rab38.

In conclusion, our data reveal that miR-124 is down-regulated during hepatic IRI and protect human hepatic L02 cells from H_2O_2-induced injury through the AKT pathway by targeting Rab38. This finding may be used to develop novel therapeutic target for patients suffering from hepatic IRI.

Acknowledgments

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