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MiR-34a affects hepatocyte proliferation during hepatocyte regeneration through regulating Notch/HIF-1α signaling pathway

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Abstract. – OBJECTIVE: To explore the influences of micro ribonucleic acid (miR)-34a on liver function and hepatocyte proliferation during hepatocyte regeneration in rats and its mechanism.

MATERIALS AND METHODS: A total of 80 Sprague-Dawley rats were randomly divided into 4 groups: Sham-2 d group (2 days after hepatectomy), Sham-10 d group (10 days after hepatectomy), miR-34a siRNA-2d group (miR-34a knockdown + 2 days after hepatectomy) and miR-34a siRNA-10 d group (miR-34a knockdown + 10 days after hepatectomy), with 20 rats in each group. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were detected at 2 d and 10 d after the operation. The rat liver was harvested for calculating the liver/body weight ratio. In addition, the deoxyribonucleic acid (DNA) content in rat hepatocytes was detected *via* Feulgen staining. The pathological changes in rat liver were detected *via* hematoxylin-eosin (H&E) staining. Moreover, the hepatocyte apoptosis in each group was detected *via* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Expression levels of proliferating cell nuclear antigen (PCNA), Notch1 intracellular domain (NICD), and hypoxia-inducible factor-1α (HIF-1α) in liver tissues of each group were detected *via* immunohistochemistry and Western blotting.

RESULTS: No significant differences in the liver/body weight ratio, serum levels of ALT, AST, LDH, pathological structure of the liver, hepatocyte apoptosis level, and PCNA expression in hepatocytes were found between miR-34a siRNA-2 d group and Sham-2 d group. However, the expression levels of NICD and HIF-1α in the liver significantly increased in miR-34a siRNA-2 d group compared with those in Sham-2 d group ($p<0.05$). On the contrary, compared with those in Sham-10 d group, the liver function and hepatocyte regeneration level significantly increased in miR-34a siRNA-10 d group. Increased liver/body weight ratio, remarkable decline in serum levels of ALT, AST, and LDH, significant alleviation of pathological injury of liver tissues, decreased the apoptosis level and upregulated PCNA protein were observed in miR-34a siRNA-10 d group than those of Sham-10 d group. The Notch/HIF-1α signaling pathway was also significantly activated.

CONCLUSIONS: MiR-34a knockdown can significantly enhance the liver function and hepatocyte regeneration ability in rats at 10 d after hepatectomy through activating the Notch/HIF-1α signaling pathway.

Key Words

Hepatectomy, Hepatocyte proliferation, MiR-34a, Notch/HIF-1α.

Introduction

Partial hepatectomy (PH) is the preferred therapeutic method for malignant hepatic tumors[1]. Hepatocyte regeneration is the liver repair process after the partial loss of liver caused by the operation, trauma, poisoning, infection, necrosis or liver transplantation[2]. The liver of mammalians has strong regeneration ability, and it can still repair itself even if most of it (up to 2/3) is resected[3]. Adult hepatocytes are usually in a resting phase, namely the mitotic G0 phase, and cell cycle of them (about 95% in young rats and about 75% in elderly rats) is progressed for cell proliferation and division in the case of PH or liver injury[4]. In the mouse experiment, the liver mass begins to increase at 48 h after hepatectomy or 3 d after deoxyribonucleic acid (DNA) synthesis in liver tissues, and it returns to the normal level at 5-7 d[5]. Clarifying the molecular mechanism of hepatocyte regeneration is of great significance in the treatment of liver cancer and liver transplantation in the future.

Micro-ribonucleic acids (miRNAs) are a group of single-stranded non-coding RNAs[6] existing in

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eukaryotes, with regulatory functions and 20-24 nt in length. MiRNAs, through the targeted binding to specific genes, can regulate the expression of a variety of genes. They play important roles in physiological activities, such as cell proliferation, differentiation, and apoptosis. MiR-34a has been proved to be an important tumor-suppressor gene in various tumors[7,8]. For example, miR-34a can inhibit the proliferation and invasion of breast cancer cells through the targeted inhibition on Wnt1 gene in the Wnt/β-catenin signaling pathway[9]. In addition, miR-34a can directly act on the Snail gene, thereby inhibiting the endothelial-mesenchymal transition, invasion, and differentiation of ovarian cancer cells[10]. However, the role of miR-34a in hepatocyte regeneration after hepatectomy and its mechanism have not been reported yet.

In this study, the rat model with miR-34a knockdown was established *via* the intravenous injection of miR-34a small interfering RNA (siRNA) into the cecum. Rat liver was partially resected *via* an operation. At 2 d and 10 d after the operation, the liver function, pathological changes in the liver, hepatocyte apoptosis, and proliferation were detected, and the potential mechanism was explored.

Materials and Methods

Animal Grouping and Establishment of MiR-34a Knockdown Model

A total of 80 Sprague-Dawley rats weighing (284.02±9.66) g aged 12-14 weeks old were divided into 4 groups: Sham-2 d group (2 days after hepatectomy), Sham-10 d group (10 days after hepatectomy), miR-34a siRNA-2d group (miR-34a knockdown + 2 days after hepatectomy), and miR-34a siRNA-10 d group (miR-34a knockdown + 10 days after hepatectomy), with 20 rats in each group. No significant differences in the basic data, such as age and body weight of rats, were found among the 4 groups. MiR-34a siRNA was intravenously injected into the cecum of rats in miR-34a siRNA-2 d group and miR-34a siRNA-10 d group at 2 d and 10 d before the operation.

Hepatectomy

The operation process was as follows: 1) Rats were anesthetized and fixed (2% pentobarbital, 45 mg/kg). 2) After the hair on the abdomen was shaved off, the abdominal cavity was opened to fully expose the liver. 3) The middle and left lobes of the liver were cut off and ligated with the silk thread. 4) After the residual blood in the abdominal cavity and liver surface was cleared, the remaining liver was placed back to the abdominal cavity. 5) The abdominal cavity was continuously sutured and disinfected. 6) After the operation, rats were placed under the light and resuscitated, and they had free access to food and water. All animals' operations were approved by the Animal Ethics Committee of Luoyang Central Hospital.

Detection of Protein Expression Via Western Blotting

The rat liver in each group was fully ground in the lysis buffer, followed by ultrasonic lysis. The lysis buffer was centrifuged for collecting the supernatant into the Eppendorf (EP) tube. The protein concentration was detected *via* ultraviolet spectrometry, and the protein samples were quantified to the same concentration. The protein was sub-packaged and placed in the refrigerator at -80°C. After the total protein was extracted, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The proteins loaded on the gel were transferred onto a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and incubated with the primary antibody at 4°C overnight. At the other day, the membrane was incubated with the goat anti-rabbit secondary antibody in the dark for 1 h. The protein band was scanned and quantified using the Odyssey scanner, and the protein level to be detected was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Immunofluorescence Detection of Hypoxia-Inducible Factor-1α (HIF-1α) Expression in Tissues

The liver tissue sections were baked in an oven at 60°C for 30 min, deparaffinized with xylene (5 min × 3 times), and dehydrated with 100% ethanol, 95% ethanol and 70% ethanol for 3 times, respectively. The endogenous peroxidase activity was inhibited with 3% hydrogen peroxide methanol. The tissues were sealed with goat serum for 1 h, incubated with the anti-HIF-1α antibody [diluted at 1:200 with phosphate-buffered saline (PBS)] at 4°C overnight, washed with PBS on a shaker for 4 times, and incubated with the fluorescein isothiocyanate (FITC) secondary antibody at 37°C for 1 h. The nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, Waltham, MA, USA). After color development, 6 samples were randomly selected in

each group, and 5 fields of view were randomly selected in each sample, followed by photography under a microscope (200×).

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining

The liver tissue sections were baked in an oven at 60°C for 30 min, deparaffinized with xylene (5 min × 3 times), and dehydrated with 100% ethanol, 95% ethanol and 70% ethanol for 3 times, respectively. Then, the sections were incubated with protein kinase K for 0.5 h, washed with PBS, reacted with the terminal deoxynucleotidyl transferase (TdT) and luciferase-labeled dUTP at 37°C for 1 h. Sections were subsequently incubated with the horseradish peroxidase (HRP)-labeled specific antibody at 37°C for 1 h. The nucleus was stained with DAPI, followed by photography and counting under the fluorescence microscope.

Detection of Serum Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Lactate Dehydrogenase (LDH) Levels Using a Full-Automatic Biochemical Analyzer

After collection of rat liver, the anti-coagulated blood was collected for detecting serum levels of ALT, AST, and LDH in each group using a full-automatic biochemical analyzer (Beckman Coulter, AU5800, Miami, FL, USA).

Feulgen Staining

The paraffin on the sections was dissolved with xylene and alcohol in gradient concentrations. Sections were incubated with 5% trichloroacetic acid at 90°C for 15 min, incubated with HCl for 8-15 min in the water bath or incubator. Subsequently, sections were incubated with Schiff reagent for 60-90 min, and incubated with sulphurous acid water I, II, and III for 5 min, respectively. Finally, sections were washed with running water for 5-15 min, counterstained with 5% fast green aqueous solution for 1 min and sealed.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for analysis of all data. Measurement data were expressed as mean ± standard deviation, and *t*-test was used for the data comparison between two groups. $p<0.05$ suggested that the difference was statistically significant.

Results

Liver/Body Weight Ratio of Rats in Each Group at 2 d and 10 d

As shown in Figure 1, the liver/body weight ratio of rats in each group at different time points was first detected. It was found that the liver/body weight ratio had no significant difference between Sham-2 d group and miR-34a siRNA-2 d group ($p>0.05$). However, it was significantly higher in miR-34a siRNA-10 d group than that in Sham-10 d group ($p<0.05$), indicating that knockdown of miR-34a can promote hepatocyte regeneration in rats.

Liver Function of Rats in Each Group at 2 d and 10 d

As shown in Figure 2, serum levels of ALT, AST, and LDH in each group were detected using the full-automatic biochemical analyzer. It was found that the liver function in miR-34a siRNA group was superior to that in Sham group at 10 d ($p<0.05$), and it had no significant difference between the two groups at 2 d ($p>0.05$).

Histological Staining of Liver of Rats in Each Group

Pathological changes in rat liver harvested from each group were detected *via* hematoxylin-eosin (H&E) staining. At 2 d in Sham group and miR-34a siRNA group, the hepatocytes were arranged disorderly, even in a reticular or honeycomb shape. There were lipid droplets filling in cells, and the nucleus of most cells was squeezed

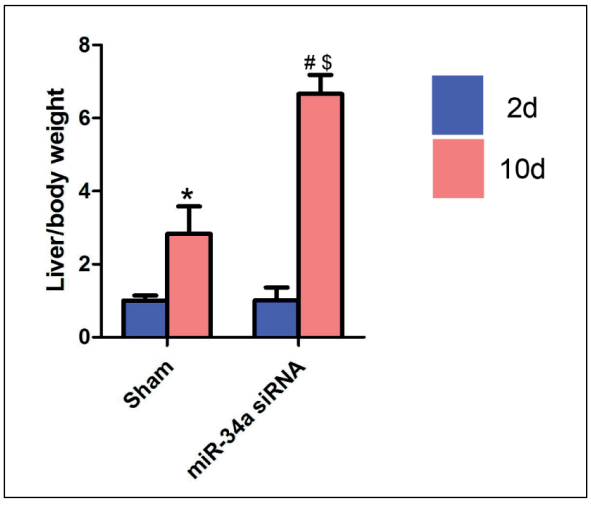

Figure 1. Liver/body weight ratio of rats in each group at 2 d and 10 d. Sham: hepatectomy group, miR-34a siRNA: miR-34a knockdown + hepatectomy group. *$p<0.05$ *vs.* Sham-2 d group, #$p<0.05$ *vs.* miR-34a siRNA group, $$p<0.05$ *vs.* Sham-10 d group.

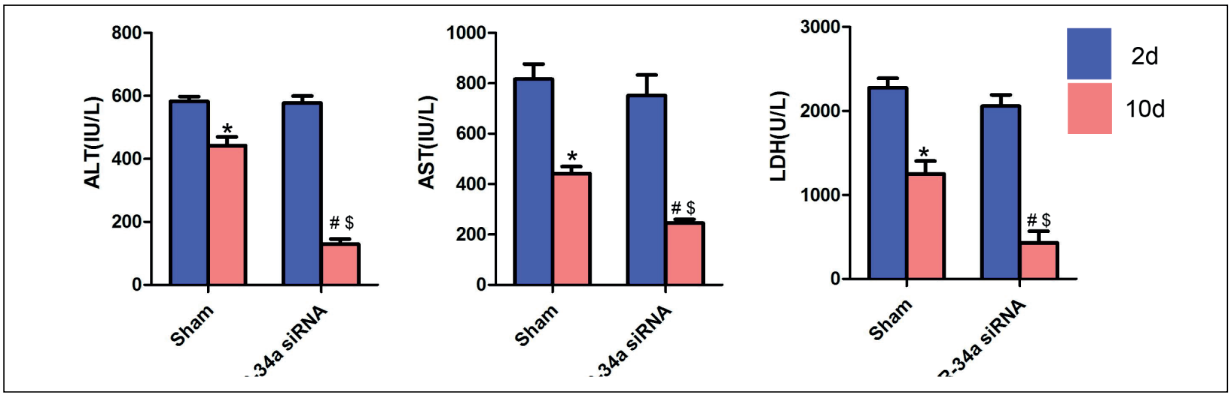

Figure 2. Liver function of rats in each group at 2 d and 10 d (ALT, AST and LDH). Sham: hepatectomy group, miR-34a siRNA: miR-34a knockdown + hepatectomy group. *p<0.05 *vs.* Sham-2 d group, #p<0.05 *vs.* miR-34a siRNA group, $$p$<0.05 *vs.* Sham-10 d group.

to one side. At 2 d in both groups, there were evident water-like and balloon-like changes in hepatocytes. At 10 d, the pathological injury of liver tissues in Sham group and miR-34a siRNA group was significantly alleviated, which was more pronounced in miR-34a siRNA group (Figure 3).

Influence of MiR-34a Knockdown on DNA Content in Liver Tissues of Rats

DNA content in liver tissues of rats was quantified *via* Feulgen staining. It is revealed that the DNA content in Sham group and miR-34a siRNA group was higher at 10 d than that at 2 d, and the increased DNA content in liver tissues was much more pronounced in miR-34a siRNA group than that in Sham group (p<0.05) (Figure 4).

TUNEL Staining of Rat Liver Tissues in Each Group

TUNEL staining was performed to evaluate the apoptosis of liver tissues. The results showed that the number of apoptotic hepatocytes increased significantly in Sham group from 2 d to 10 d (p<0.05). It also increased in miR-34a siRNA group from 2 d to 10 d, but the increasing degree was significantly lower than that in Sham group (p<0.05) (Figure 5).

Immunohistochemical Staining of Proliferating Cell Nuclear Antigen (PCNA) in Liver Tissues of Rats in Each Group

PCNA is an important index for cell proliferation. Here, we detected the expression level of PCNA in rat liver tissues *via* immunohistochemical staining. As shown in Figure 6, the expression level of PCNA in liver tissues increased significantly in miR-34a siRNA group at 10 d, which was 7.45 times higher than that at 2 d. However, PCNA expression was only 2.11 times higher at 10 d than that at 2 d in Sham group (p<0.05).

Immunohistochemical Staining of HIF-1α in Liver Tissues of Rats in Each Group

Considering the important role of Notch/HIF-1α signaling pathway in hepatocyte regeneration, HIF-1α expression in rat liver tissues was further

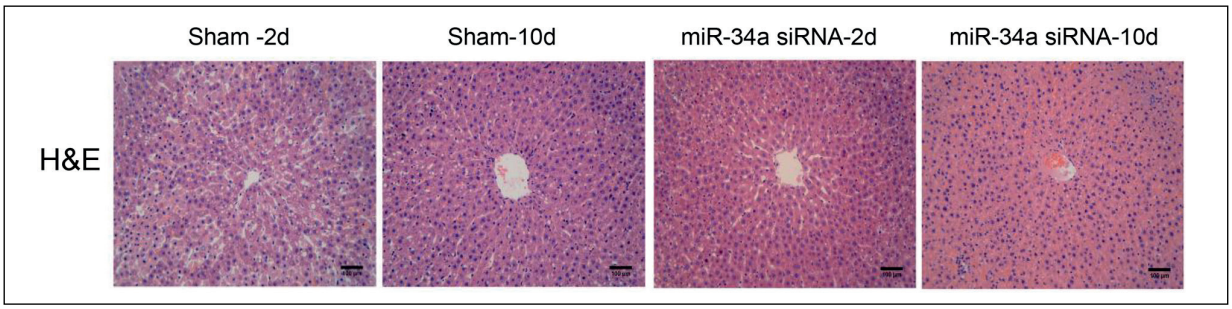

Figure 3. Histological staining of the liver of rats in each group (magnification x 20). Sham: hepatectomy group, miR-34a siRNA: miR-34a knockdown + hepatectomy group.

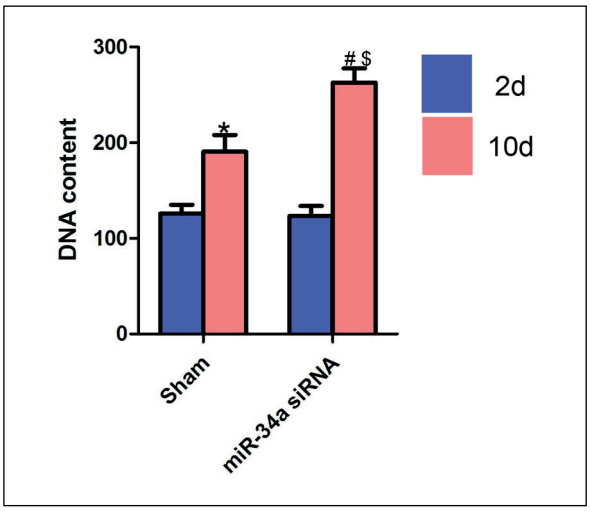

Figure 4. DNA content in liver tissues of rats in each group. Sham: hepatectomy group, miR-34a siRNA: miR-34a knockdown + hepatectomy group. *p<0.05 *vs.* Sham-2 d group, #p<0.05 *vs.* miR-34a siRNA group, $$p$<0.05 *vs.* Sham-10 d group, with a statistical difference.

detected *via* immunohistochemical staining. The results manifested no statistically significant difference in the HIF-1α expression level between Sham group and miR-34a siRNA group at 2 d (p<0.05). At 10 d, the increasing degree of HIF-1α expression level in miR-34a siRNA group was significantly higher than that in Sham group (p<0.05) (Figure 7).

Influence of MiR-34a Knockdown on Notch/HIF-1α Signaling Pathway in Liver Tissues of Rats

Furthermore, the protein expressions of Notch1 intracellular domain (NICD) and HIF-1α in rat liver tissues were detected *via* Western blotting. It was found that the Notch/HIF-1α signaling pathway in miR-34a siRNA group was significantly activated compared with that in Sham group (p<0.05). The protein expressions of NICD and HIF-1α in Sham group and miR-34a siRNA group significantly increased at 10 d compared with those at 2 d, but the increasing degree in miR-34a siRNA group was higher than that in Sham group (p<0.05).

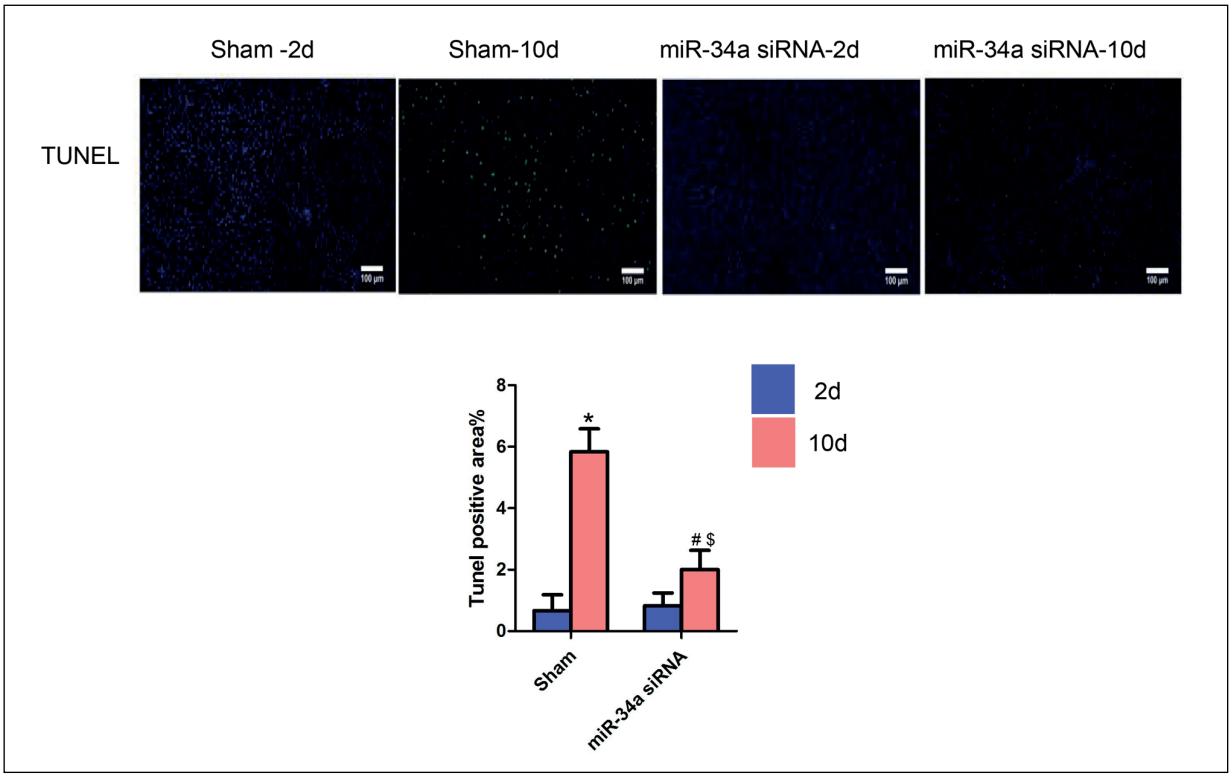

Figure 5. TUNEL staining of liver tissues of rats in each group (magnification x 20). Sham: hepatectomy group, miR-34a siRNA: miR-34a knockdown + hepatectomy group. *p<0.05 *vs.* Sham-2 d group, #p<0.05 *vs.* miR-34a siRNA group, $$p$<0.05 *vs.* Sham-10 d group, with a statistical difference.

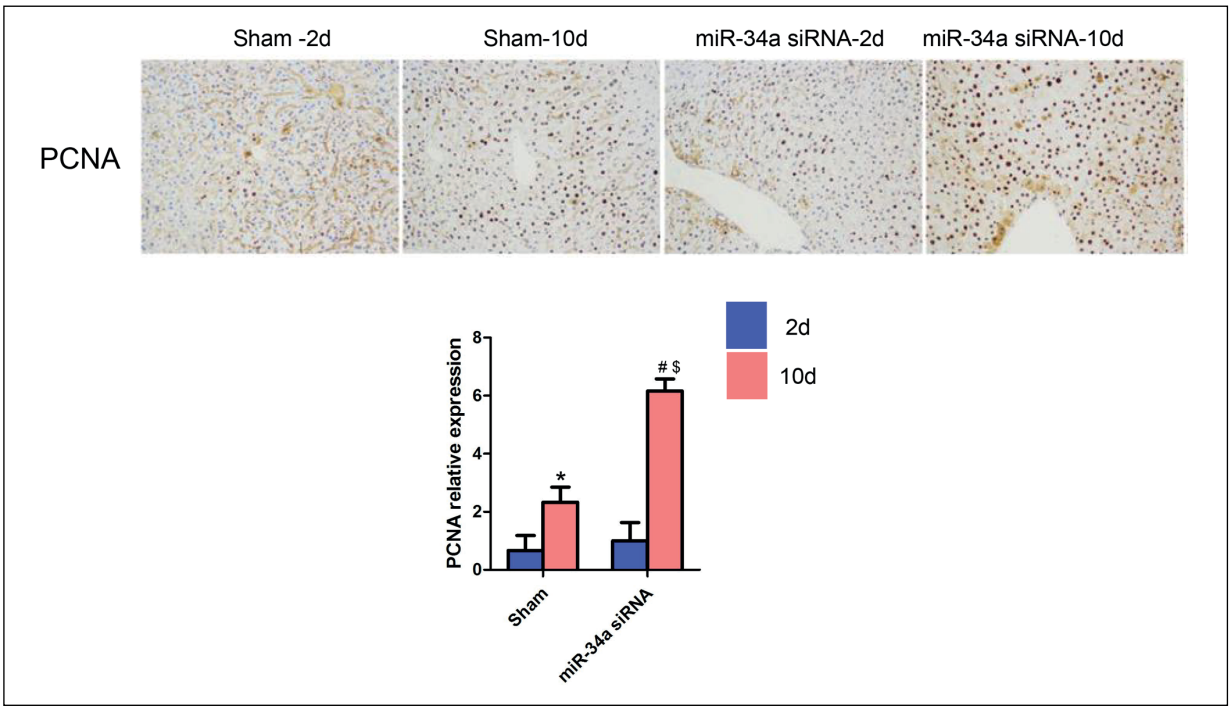

Figure 6. Immunohistochemical staining of PCNA in liver tissues of rats in each group (magnification x 40). Sham: hepatectomy group, miR-34a siRNA: miR-34a knockdown + hepatectomy group. *p<0.05 *vs.* Sham-2 d group, $^{\#}p$<0.05 *vs.* miR-34a siRNA group, $^{\$}p$<0.05 *vs.* Sham-10 d group, with a statistical difference.

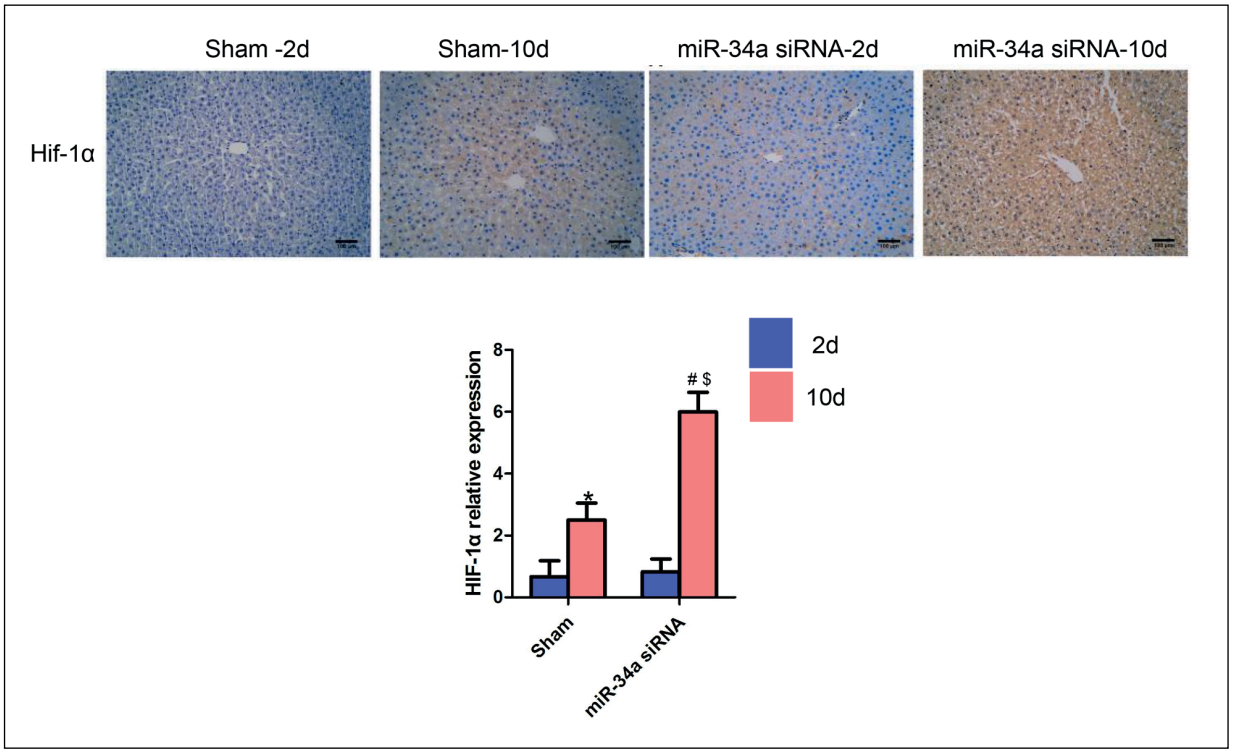

Figure 7. Immunohistochemical staining of HIF-1α in liver tissues of rats in each group (magnification x 20). Sham: hepatectomy group, miR-34a siRNA: miR-34a knockdown + hepatectomy group. *p<0.05 *vs.* Sham-2 d group, $^{\#}p$<0.05 *vs.* miR-34a siRNA group, $^{\$}p$<0.05 *vs.* Sham-10 d group, with a statistical difference.

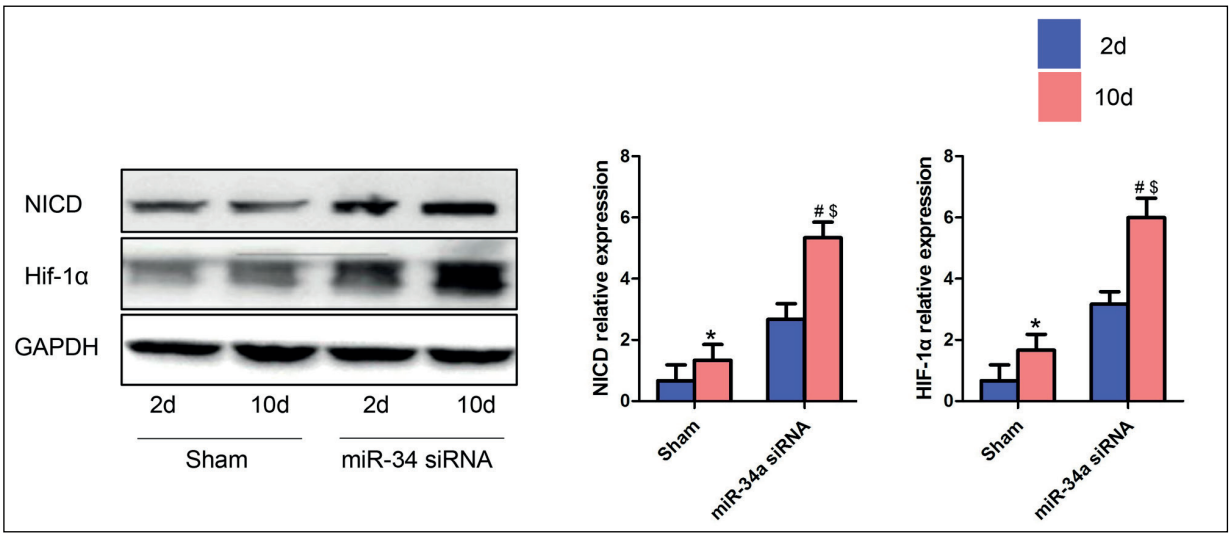

Figure 8. Influence of miR-34a knockdown on Notch/HIF-1α signaling pathway in liver tissues of rats. Sham: hepatectomy group, miR-34a siRNA: miR-34a knockdown + hepatectomy group. *p<0.05 *vs.* Sham-2 d group, $^{\#}p$<0.05 *vs.* miR-34a siRNA group, $^{\$}p$<0.05 *vs.* Sham-10 d group, with a statistical difference.

Discussion

Hepatocyte regeneration has an important significance in liver and gallbladder surgeries (such as liver tumor resection, liver transplantation, and split liver transplantation), and the hepatocyte regeneration ability is a key factor determining the success of the above surgeries[11]. The poor hepatocyte regeneration will lead to the poor prognosis of patients, such as small-for-size syndrome[12]. Hepatocyte regeneration is a complex physiological process involving multiple genes, proteins, and cells, in which the proliferation ability of hepatic stellate cells and hepatocytes can directly affect the hepatocyte regeneration ability[13]. When hepatocyte regeneration starts, hepatocytes and hepatic stellate cells in the G0 phase will quickly enter the rapid growth stage under the stimuli of various feedback signals[14]. Therefore, understanding the mechanism of hepatocyte regeneration is of great significance in clinical practice.

MiR-34a is a member of the miRNA family, which is highly conserved and located on human chromosome 1, and it can inhibit a variety of oncogenes. Therefore, miR-34a is considered as a tumor-suppressor gene[15]. Daige et al[16] have demonstrated that miR-34a, due to its ability to inhibit the growth of tumor cells, can serve as a novel targeted drug for liver cancer. The Notch signaling pathway is highly conserved and contains 4 Notch receptors (Notch1-4) and 5 Notch ligands (Delta-like-1/3/4 and Jagged1/2)[17]. Some Notch proteins, especially Notch1, have been proved to be highly expressed in the liver, and play crucial roles in hepatocyte regeneration[18]. Overexpression of Notch1 can inhibit the proliferation and cell cycle progression of liver cancer cells, thus suppressing the occurrence and development of hepatocellular carcinoma[19]. It has been proved that miR-34a can inhibit Notch1 expression in a targeted manner in various tumors, including malignant glioma, breast cancer, cervical cancer, and choriocarcinoma. In addition, miR-34a is transcribed and activated by p53, thereby regulating the biological behaviors of hepatocytes and liver cancer cells[20,21]. Therefore, expression change of p53 may directly affect the level of miR-34a, thus affecting the hepatocyte regeneration. Moreover, overexpression of miR-34a in liver tissues can significantly inhibit hepatocyte proliferation in rats in the late stage of hepatocyte regeneration by down-regulating INHBB and Met (important factors promoting proliferation of liver cancer cells) through targeting miR-34a[22]. After hepatectomy, the activation of Notch and Jagged signaling pathways can remarkably promote the proliferation and differentiation of hepatocytes, and protein expression of Hes1 in the downstream of Notch signaling pathway also significantly increased. In cervical cancer and choriocarcinoma, the high expression of miR-34a can significantly inhibit the Jagged1 and Notch1 signaling pathways, thus lowering the invasion ability of tumor cells[22]. The targeted inhibition of miR-34a on Notch1 has also

been evidenced to be able to significantly reduce the progression of prostate cancer[23]. In this study, the miR-34a knockdown model and the PH model were established. We detected liver function, liver/body weight ratio, histological changes in the liver, hepatocyte proliferation, and apoptosis level at 2 d and 10 d. The miR-34a knockdown could effectively inhibit liver dysfunction and pathological injury of the liver caused by hepatectomy, greatly inhibited the apoptosis of hepatocytes and hepatic stellate cells, and enhanced the proliferation ability of hepatocytes. Furthermore, Western blotting results showed that the effect of miR-34a on hepatocyte regeneration and liver function after hepatectomy was achieved by Notch/HIF-1α signaling pathway. However, there are still some limitations in this study: 1) *In vitro* experiments were lacked for verification, and 2) the direct target of miR-34a in this model was not verified.

Conclusions

We found that the miR-34a knockdown can improve the liver function and facilitate the hepatocyte regeneration in rats receiving hepatectomy through activating the Notch1/HIF-1α signaling pathway. Therefore, miR-34a siRNA is expected to become a targeted drug promoting the hepatocyte regeneration in the future.

Conflict of Interests

The authors declare that they have no conflict of interest.

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