Int J Clin Exp Med 2019;12(2):1570-1576
www.ijcem.com /ISSN:1940-5901/IJCEM0076693

Original Article

MiR-124 inhibits the proliferation of human hepatic L02 cells by targeting *SGK1*

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Received March 25, 2018; Accepted October 20, 2018; Epub February 15, 2019; Published February 28, 2019

Abstract: Aim: This study was aimed to investigate the role of miR-124 in liver regeneration and its effect on hepatocyte proliferation. Material and methods: The liver regeneration model was established in rats, and the expression of miR-124 was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The proliferative capacity of hepatic L02 cells was examined by cell cycle analysis and the cell counting kit 8 (CCK8) assay, and SGK1 protein and mRNA levels were detected by western blot and qRT-PCR, respectively. A luciferase reporter assay was used to verify the expression of the target genes of miR-124. Results: Reduction in the miR-124 levels was observed in regenerative livers 4 h and 24 h after partial hepatectomy. MiR-124 inhibited the proliferative capacity of hepatic L02 cells. The activation of ERK signaling pathways was suppressed and the expression of *SGK1*, a direct target of miR-124 involved in the proliferation of hepatic L02 cells, was down-regulated by miR-124. Interestingly, perturbation in the expression of *SGK1* reduced the proliferation of hepatic L02 cells by inactivating ERK phosphorylation. Conclusion: Expression of miR-124 decreased significantly in the early proliferative phase of liver regeneration, and over-expression of miR-124 significantly inhibited the proliferation of hepatic L02 cells via targeting of *SGK1* and inactivation of ERK signaling.

Keywords: miR-124, liver regeneration, SGK1, L02 cells

Introduction

The liver has the intrinsic ability of regenerating itself by a process called liver regeneration (LR). In rats that have undergone 70% partial hepatectomy (PH), the remaining liver regenerates and recovers its original mass and function within 7-10 days. LR after PH can be divided into three distinct phases, namely, initiation, proliferation, and termination [1-3], which are characterized by the activation of inflammatory cytokines, growth factors, and signaling pathways that participate in LR [4]. Although LR has been studied extensively, the regulatory mechanism of LR is still not fully understood.

MicroRNAs (miRNAs) are a class of small regulatory RNAs that modulate a variety of biological processes, including cellular differentiation, metabolism, proliferation, and apoptosis. Recently, certain studies confirmed that miRNAs contribute to LR. Salehi showed that human liver regeneration is characterized by the coordinated expression of distinct microRNAs that govern cell cycle fate [5]; others demonstrated that miR-382 promotes liver regeneration in vivo by targeting *PTEN* [6], and miR-26a inhibited hepatocyte proliferation by targeting *CCND2* and *CCNE2* [7]. A recent study confirmed that 70% miRNAs are down-regulated in the early phases after PH, which indicated that miRNAs may play critical roles in the early phase of LR [8, 9]. A previous study reported that miR-124 was involved in various cellular functions [10-18]. Another study demonstrated that miR-124 exhibited pro-apoptotic capacity in human liver cancer [19]. Furthermore, we showed that miR-124 is down-regulated in liver ischemia reperfusion injury, and that it protects the human hepatic L02 cells from hydrogen peroxide-induced apoptosis [20]. Taken together, it appears that miR-124 may play a significant role in liver pathophysiology; however, the direct effect of miR-124 on hepatocyte proliferation remains to be investigated.

In the present study, we detected a change in miR-124 expression in a rat model of LR. We further studied the mechanism by which miR-124 affects proliferation of human hepatocytes.

Material and methods

Establishment of a rat model of 70% partial hepatectomy

Male Sprague-Dawley (SD) rats (180-200 g) were purchased from the animal center of the Sun Yat-sen University (Guangzhou, China). All animal experiments were performed in accordance with the institutional animal care instructions. The study protocol was approved by the Animal Ethics Review Committee, Sun Yat-sen University. The SD rats were randomly divided into PH and sham hepatectomy (SH) groups. The rats were anesthetized with chloral hydrate (3 mL/kg, intra-peritoneal route) and 70% partial hepatectomy was performed as described by Higgins [21]. The rats underwent abdominal surgery without liver resection for the sham hepatectomy. At the indicated time points (4 h and 24 h) after hepatectomy, the animals were sacrificed and the regenerated liver tissues were collected.

Cell culture and transfection

Human hepatic L02 cells (purchased from the Shanghai Cell Band of the Chinese Academy of Sciences) were grown in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum (FBS), 1% streptomycin (100 g/mL) and 1% penicillin (100 IU/mL) at 37°C and in an atmosphere of 5% carbon dioxide. The miR-124 mimics, *SGK1* siRNA, and the negative control (NC) were obtained from RiboBio (Guangzhou, China). Cells were transfected using a Lipofectamine RNAiMax kit (Invitrogen) according to the manufacturer's protocol and harvested after 48 h.

Reverse transcription and quantitative real-time PCR

Total RNA was isolated from prepared liver samples or transfected cells by Trizol (Life Technologies) according to manufacturer's instruction. Reverse transcription (RT) of liver samples was performed with 100 ng total RNA, multiplex RT human primer pools, and the TaqMan microRNA reverse transcriptase kit (Life Technologies). Quantitative real time polymerase chain reaction (qRT-PCR) was performed with TaqMan universal PCR master mix, no AmpErase UNG (Life Technologies) using the LightCycler 480 system. The cDNA of the transfected cells was synthesized following the manufacturer's protocol (Takara). qRT-PCR was performed using a standard SYBR green PCR kit, and PCR-specific amplification steps were applied in the LightCycler 480 system real-time PCR machine. The relative expression of the target genes (*miR-124*, *U6*, *IL6R*, SGK1, and *GADPH*) was calculated using the $2^{-\Delta\Delta}$Ct method [22].

Western blot analysis

Transfected cells were lysed in lysis buffer (KeyGen, Nanjing, China) and equal amounts of protein were separated discontinuously by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, MA, USA), following which the membrane was incubated with primary antibodies against serum glucocorticoid-regulated kinase (SGK1) (Abcam), extracellular signal-regulated kinase (ERK), p-ERK (Cell Signaling Technology), β-actin (Santa Cruz) overnight at 4°C. The membranes were washed thrice with Tris-buffered saline-Tween 20 (TBST) and incubated with a secondary antibody conjugated to horse radish peroxidase for 90 min at room temperature. After washing for 15 min, the membranes were treated with the enhanced chemiluminescence kit and recorded on Kodak films.

Cell counting kit 8 (CCK8) cell proliferation assay

Hepatic L02 cells were transfected with miR-124 mimics and corresponding NCs in 96-well plates at a density of 2,000 cells per well. At the indicated time points (0 h, 24 h, 48 h, 72 h, and 96 h) after transfection, 10 μL CCK8 was added to the cells and the proliferative ability of the cells was measured at 490 nm using an enzyme linked immunosorbent assay (ELISA) reader according to the manufacturer's instructions.

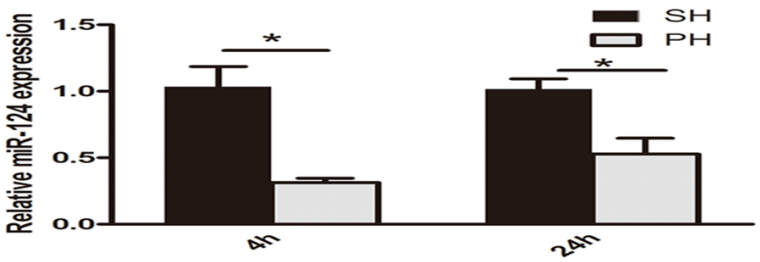

Figure 1. MiR-124 is down-regulated during the early phase of LR. The expression of miR-124 during LR was measured by qRT-PCR analysis. miR-124 levels were standardized to that of u6. *: P<0.05, **: P<0.01, compared with SH control at each time point.

Cell cycle analysis by flow cytometry

Hepatic L02 cells were cultured in 6-well plates and then transfected with miR-124 mimics and NC. After 48 h, the cells were collected, fixed with 70% ethanol overnight at 4°C and then washed with ice-cold phosphate buffered saline (PBS). The cell pellets were re-suspended in RNase A. Finally, the cells were stained with propidium iodide (PI) and analyzed by using a flow cytometer.

Luciferase reporter assay

The 3'UTR of *SGK1*, containing the *SGK1*-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 the SGK1-UTR-pLUC/Mu-SGK1-UTR-pLUC reporter plasmid and the miR-124 mimics/NC using Fugene HD transfection reagent (Roche) according to the manufacturer's instructions. After 48 h, luciferase activity was measured using the dual luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical analysis

A paired Student's *t*-test was used and $P<0.05$ was considered statistically significant.

Results

MiR-124 is down-regulated during the early phase of LR

We examined the miR-124 levels during the early stage of LR by qRT-PCR and found that miR-124 was down-regulated by at least 2-fold in the regenerated livers after PH compared to that in the SH control group 4 h and 24 h after operation (**Figure 1**).

MiR-124 inhibits hepatic L02 cell proliferation

Hepatic L02 cells were transfected with miR-124 mimics or NC to alter miR-124 expression. As shown in **Figure 2A-C**, the percentages of cells in the G1 phase in miR-124 mimic and NC groups were 72.05% ± 3.20% and 58.75% ± 1.81%, respectively, and the percentages of S-phase cells were 27.75% ± 2.14% and 35.16% ± 2.58%, respectively. In addition, CCK8 cell proliferation analysis showed that miR-124 markedly reduced hepatic L02 cell growth at 48 h, 72 h, and 96 h compared to that in the NC group (**Figure 2D**).

ERK activation was suppressed in hepatic L02 cells transfected with the miR-124 mimic

Western blotting showed that the activation of the ERK pathway was reduced in the miR-124 mimic group compared to that of the NC group (**Figure 3**).

SGK1 is a target of miR-124

The expression of *SGK1*, a candidate target gene, was significantly reduced after treatment with miR-124 mimics compared to that of the NC group at both the mRNA and protein levels (**Figure 4A-C**). To further validate whether *SGK1* is a direct target of miR-124, we fused the 3'UTR region of *SGK1* to a luciferase reporter construct. As shown in **Figure 4D**, miR-124 suppressed the luciferase activity of the construct harboring the 3'UTR of *SGK1*, but not that of the construct containing a mutant binding site (SGK1 3'UTR-MUT), compared to the NC group.

Targeting of SGK1 by RNA interference inhibited the proliferation of hepatic L02 cells via the ERK pathway

To delineate the function of *SGK1* in liver cell proliferation, hepatic L02 cells were transfected with *SGK1* siRNA or control siRNA. The *SGK1* knockdown efficiency was verified by real-time PCR and western blotting (**Figure 5A**, **5C**). Cells treated with the *SGK1* siRNA or control siRNA

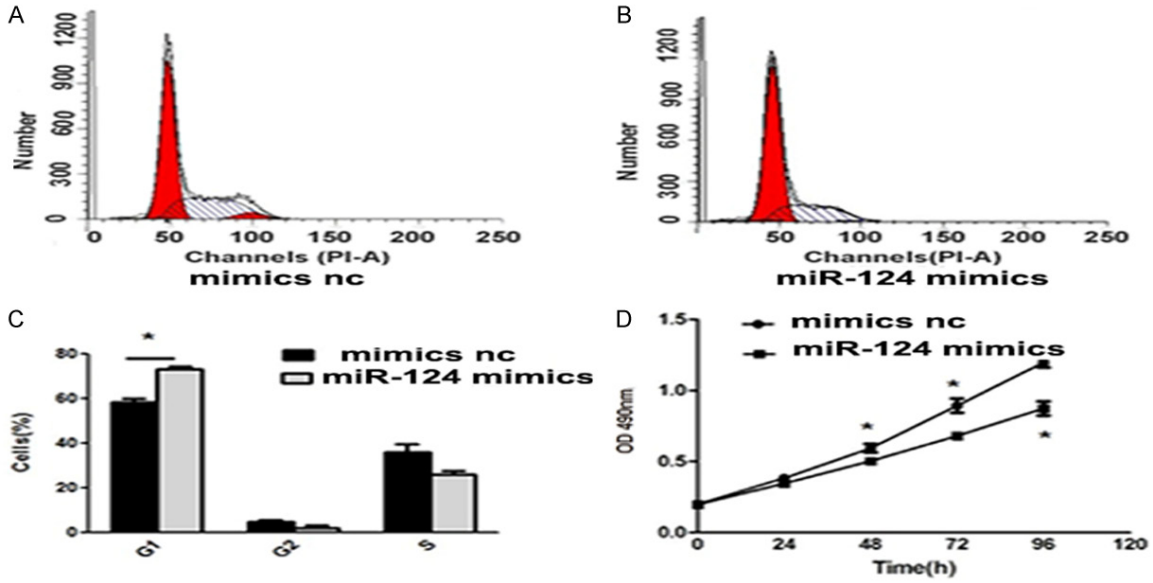

Figure 2. MiR-124 inhibits hepatic L02 cell proliferation. Hepatic L02 Cells treated with miR-124 mimics and mimics nc was analyzed by flow cytometry for cell cycle distribution analysis (A-C). After transfected with miR-124 mimics and mimics nc, the proliferation of the treated cells was measured by CCK8 assay at 490 nm (D). *: P<0.05.

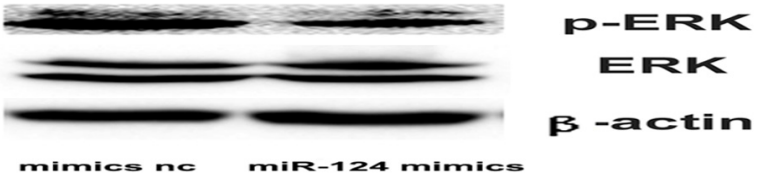

Figure 3. MiR-124 suppressed the activation of ERK by western-blot analysis compared with control group.

were re-seeded in 96-well plates and the CCK8 cell proliferation assay was performed. As shown in **Figure 5B**, the *SGK1* siRNA strongly repressed hepatic L02 cell proliferation. We further confirmed that *SGK1* down-regulation repressed hepatocyte proliferation via the ERK pathway (**Figure 5C**).

Discussion

During liver regeneration after partial hepatectomy, normally quiescent hepatocytes undergo one or two rounds of replication to restore the liver mass by a process of compensatory hyperplasia. A large number of genes are involved in liver regeneration, especially during the first few hours after PH (the "priming phase"). During this period, quiescent hepatocytes enter the cell cycle and the peak of DNA replication occurs at 24 h after PH [23]. Evidence shows that several miRNAs are involved in LR [24, 25]. However, little is known about the mechanisms utilized by these miRNAs for regulating their target gene expression during hepatocyte proliferation of LR. In the present study, we determined that miR-124 was down-regulated 4 h and 24 h after PH. Recent studies showed that miR-124 is a potential target for therapeutic intervention in hepatocellular carcinoma (HCC) and hepatic ischemia reperfusion injury, which indicated that miR-124 may play an important role in LR.

To evaluate the role of miR-124 in human hepatocytes, the hepatic L02 cells were transfected with miR-124 mimics, following which miR-124 increased the percentage of cells in the G1 phase, whereas the percentage of cells in the G2 phase decreased. These data indicated that miR-124 suppressed the proliferation of hepatic L02 cells by inducing G1-phase cell-cycle arrest, which was consistent with the results of a previous study showing that miR-124 suppressed HepG2 cell proliferation by inducing cell cycle arrest in the G1 phase [26].

We analyzed the early hepatic regenerative signaling pathways to further elucidate the mechanism by which miR-124 suppressed the prolif-

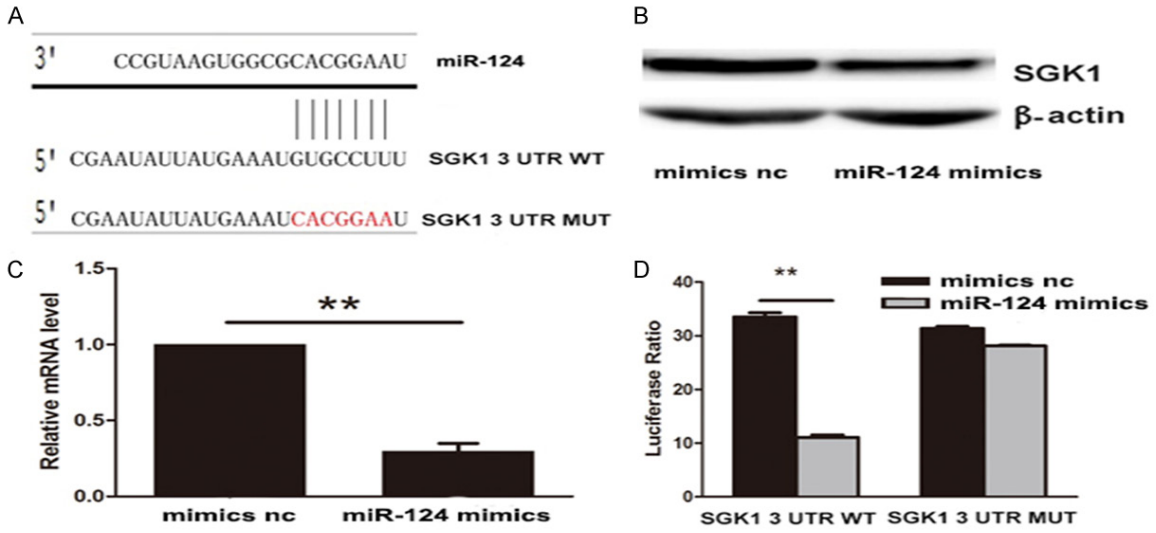

Figure 4. SGK1 is a target gene of miR-124. A. miR-124-binding sequences in the 3'-UTR of SGK1 and mutated sites in 3'-UTR of SGK1. B, C. The expression of SGK1 was significantly down-regulated at the protein and mRNA levels based on Western blotting and qRT-PCR, respectively, after treatment with miR-124 mimics compared with the mimics nc. D. MiR-124 suppressed the luciferase activities of constructs containing the 3'-UTR segment of SGK1. **: P<0.01.

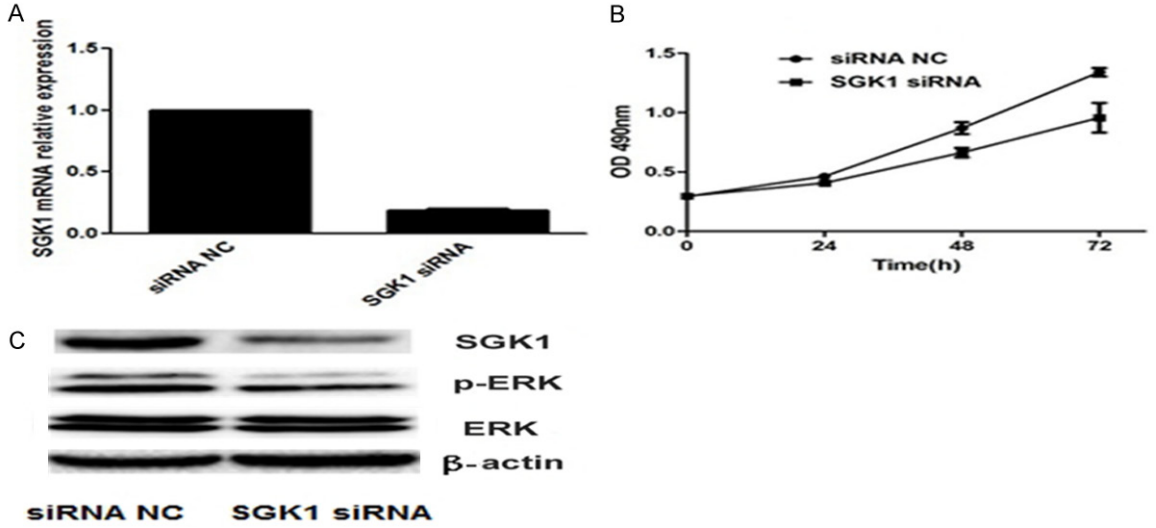

Figure 5. The down-regulated expression of SGK1 repressed hepatocyte proliferation through ERK pathway. A, C. Hepatic L02 cells were transfected with SGK1 siRNA or a negative control (siRNA NC), the levels of mRNA and proteins were analyzed by qRT-PCR and Western blotting respectively. B. Down-regulation of SGK1 suppressed hepatocyte proliferation were detected at the indicated time by using CCK8 assay. C. The expression of phosphorylated ERK in hepatic L02 cells was suppressed after treatment with SGK1 siRNA by Western blotting.

eration of hepatocytes. Previous studies showed that the early hepatic regenerative reaction was characterized by the induction of ERK and c-Jun amino-terminal kinase (JNK) activity [27, 28]. Interestingly, we found that miR-124 suppressed ERK phosphorylation, which is a downstream player in growth factor signaling during the process of liver regeneration [29]. We used Targetscan and miRBase to delineate the targets of miR-124, and identified that the reduced hepatocyte proliferation in the presence of miR-124 was partially due to the post-transcriptional regulation of the *SGK1* mRNA by miR-124. *SGK1* belongs to a family of serine/

threonine kinases, which has been implicated in diverse cellular activities such as cellular proliferation [30-32], and is known to have a positive regulatory role on ERK function during the early phase of liver regeneration [33, 34]. Furthermore, several studies have reported the inhibitory effect of *SGK1* on proliferation of various cell types [35, 36]. Interestingly, here we showed that the siRNA-mediated knockdown of *SGK1* suppressed the proliferation of hepatic L02 cells by inhibiting the activation of ERK, which implies that *SGK1* contributes to the miR-124-mediated suppression of proliferation. Importantly, the luciferase reporter assay confirmed that *SGK1* was the direct target of miR-124 at the post-transcriptional level.

In conclusion, miR-124 is highly down-regulated in the early phase of LR after PH. Overexpression of miR-124 suppressed hepatocyte proliferation via the ERK pathway by targeting *SGK1*, which suggests that miR-124 might be an "initiation" signal during LR.

Acknowledgements

This work was supported by General Program of the National Natural Science Foundation of China (No. 81660115), Jiangxi Provincial Department of Education Youth Project (700784002) and Science and Technology Plan Project of Jiangxi (20161BBG70158).

Disclosure of conflict of interest

None.

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