MicroRNA-21 Contributes to Liver Regeneration by Targeting PTEN

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Data Collection B
Statistical Analysis C
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Manuscript Preparation E
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Background: Multiple microRNAs (miRNAs, miRs), including miR-21, have been documented to be critical regulators of liver regeneration, but the mechanism underlying their roles in hepatocyte proliferation and cell cycle progression is still far from understood.

Material/Methods: miR-21 levels were determined using qRT-PCRs in mouse livers at 48 h after 70% partial hepatectomy (PH-48 h). Cell proliferation was determined by use of a cell-counting kit-8 (CCK-8), EdU incorporation staining, and flow cytometry. Phosphatase and tensin homolog (PTEN) expressions were determined using qRT-PCR and Western blot analysis. PTEN siRNA was used to perform the rescue experiment.

Results: A marked upregulation of miR-21 was observed in mouse livers at 48 h after 70% partial hepatectomy (PH-48 h) compared to 0 h after PH (PH-0 h). Overexpression of miR-21 was associated with increased proliferation and a rapid G1-to-S phase transition of the cell cycle in BNL CL.2 normal liver cells *in vitro*. In addition, we showed that PTEN expression was inversely correlated with miR-21 in BNL CL.2 cells and demonstrated that PTEN expression is lower in mouse livers at PH-48 h. Moreover, the presence of PTEN siRNA significantly abolished the suppressive effect of miR-21 inhibitor on hepatocyte proliferation.

Conclusions: miR-21 overexpression contributes to liver regeneration and hepatocyte proliferation by targeting PTEN. Upregulation of miR-21 might be a useful therapeutic strategy to promote liver regeneration.

MeSH Keywords: Liver Regeneration • MicroRNAs • PTEN Phosphohydrolase

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Background

The liver has a central role in metabolic homeostasis and detoxification of xenobiotics, and possesses a remarkable restoring capacity following acute mass ablation, through proliferation of the remaining hepatocytes and/or differentiation of the liver stem cells [1–4]. Liver regeneration after partial hepatectomy (PH), predominantly associated with resident hepatocyte proliferation, is a very complex process that is attributed to the activation and interaction of multiple cytokines and growth factors regulating cell growth and proliferation [5–9]. Nevertheless, liver regeneration capacity is usually attenuated following liver transplantation or severe liver failure, highlighting the need to develop novel therapeutic strategies to prevent liver failure [10–12].

As posttranscriptional regulators, microRNAs (miRNAs or miRs) can reduce the stability and/or inhibit the translation of target messenger RNAs (mRNAs) by binding to their 3'-untranslated regions (3'-UTRs). It is estimated that these small noncoding miRNAs regulate about 70% of the human protein-coding genome, controlling cell differentiation, apoptosis, metabolism, and immune functions [13–16]. miRNAs, including miR-21, have been reported to be important regulators for liver regeneration after PH [17–21]. Several studies have documented the significant induction of miR-21 in the mouse liver during the proliferative phase of liver regeneration after PH by targeting Btg2, Pellino-1, and RhoB [22–24]. More recently, miR-21 has been shown to promote primary hepatocyte proliferation and induce a rapid S-phase entry via targeting phosphatase and tensin homolog (PTEN) *in vitro* [25]. In the current study, we established a murine model of 70% PH and found a remarkable upregulation of miR-21-5p (previous ID: miR-21) in mouse livers at 48 h after 70% PH (PH-48 h). We further investigated the effects and the underlying molecular mechanisms of miR-21 on the proliferation and cell cycle progress of hepatocytes using the BNL CL.2 normal mouse liver cell line.

Our data demonstrate that upregulation of miR-21 is involved in the proliferative phase of liver regeneration in a 70% PH animal model, as well as in the proliferation and the G1/S phase transition of BNL CL.2 cells *in vitro*. Moreover, an inverse correlation between miR-21 and PTEN expressions has been detected both *in vivo* and *in vitro*. Silencing PTEN abolishes the proliferation-suppressing effect of miR-21 inhibition on BNL CL.2 cells. Collectively, the present study indicates that miR-21 promotes liver regeneration and hepatocyte proliferation via targeting PTEN. Increasing miR-21 might be a potential strategy to enhance liver regenerative capacity.

Material and Methods

Animals and partial hepatectomy

Male C57BL/6 mice aged 8 weeks were purchased from the Shanghai Laboratory Animal Center (SLAC, Shanghai, China). The animals were allowed free access to food and tap water. Mice were anaesthetized with 1% pentobarbital sodium (i.p. 50 mg/kg) and subjected to 70% PH of the median and left lobes of the liver, as we have previously described [26]. Mice were then randomized to 2 groups: (1) mice sacrificed at 0 h after PH (PH-0 h, n=5) and (2) mice sacrificed at 48 h after PH (PH-48 h, n=5). At these 2 time points, mouse livers were harvested and stored at -80°C for further use. This study was approved by our local ethics committees and all animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by the National Institutes of Health (No. 85-23, revised 1996).

Cell culture

BNL CL.2 normal mouse liver cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in high- glucose Dulbecco's modified Eagle's medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Cell transfection

The miR-21 mimic, inhibitor, and their negative controls were purchased from RiboBio (China). BNL CL.2 cells were transfected with miR-21 mimic (50 nM), inhibitor (100 nM), or their negative controls for 48 h using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

Determination of cell size

A density of 1.0×10^5 cells per well were seeded into 24-well plates for 6 h. At 12 h after serum-deprivation, cells were transfected with miR-21 mimic (50 nM), inhibitor (100 nM), or their negative controls for 48 h. Then cells were labeled with non-immunological red fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil, Beyotime, China) according to the manufacturer's protocol. Cells were observed under an inverted fluorescence microscope (Leica, Germany) and digital images were acquired for determination of cell size with CellProfiler software.

Cell proliferation assay

A density of 2.0×10^4 cells per well were seeded into 96-well plates for 6 h. At 12 h after serum-deprivation, cells were

transfected with miR-21 mimic (50 nM), inhibitor (100 nM), or their negative controls for 48 h. A cell proliferation assay was then performed using the Cell Counting Kit-8 (CCK-8, Dojindo, Japan) according to the manufacturer's instructions. The absorbance was read at 450 nm using a spectrophotometer.

EdU incorporation assay

A density of 1.0×10^5 cells per well were seeded into 24-well plates for 6 h. After transfecting BNL CL.2 cells with miR-21 mimic (50 nM), inhibitor (100 nM), or their negative controls for 48 h, the incorporation of 5-ethynyl-2'-deoxyuridine (EdU) into actively proliferating cells was evaluated using a Cell-Light™ EdU Cell Proliferation Detection kit (RiboBio, China) according to the manufacturer's instructions. Cell immunostaining was observed with an inverted fluorescence microscope (Leica, Germany) and digital images were acquired and analyzed with Image J software. The results are presented as EdU-positive cells (%).

Cell cycle analysis

BNL CL.2 cells seeded at a density of 2×10^5 per well into 6-well plates were transfected with miR-21 mimic (50 nM), inhibitor (100 nM), or their negative controls. At 48 h after transfection, cells were detached using 0.25% trypsin (Gibco, USA), washed twice with PBS, and fixed in 70% ethanol at −20°C overnight. Cells were washed again with PBS and then incubated with propidium iodide (PI, 50 µg/mL) (Sigma, USA) and RNase for 30 min. Cellular DNA content was analyzed using a MoFlo XDP Cell Sorter (Beckman Coulter). The cell number in each phase of the cell cycle was determined using FlowJo software (Treestar Inc., USA).

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Germany). For mRNA analysis, cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA). A template equivalent of 300 ng of total RNA was subjected to 40 cycles of quantitative PCR with SYBR Premix Ex Taq™ (Tli RNaseH Plus, Takara, Japan) in the CFX96™ Real-Time PCR Detection System (Bio-Rad, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences (forward and reverse) were designed as follows: GAPDH, forward, 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse, 5'-TGTAGACCATGTAGTTGAGGTCA-3'; PTEN, forward, 5'-TGGATTCGACTTAGACTTGACCT-3' and reverse, 5'-GCGGTGTCATAATGTCTCTCAG-3'. For miRNA expression assay, the Bulge-Loop™ miRNA qPCR Primer Set (RiboBio, China) was used to detect miR-21 expression by qRT-PCR with the Takara SYBR Premix Ex Taq™ in CFX96™ Real-Time PCR Detection

System. 5S ribosomal RNA (5S rRNA) was used to normalize target miRNA expression. The relative expression level of target genes was defined as fold change using $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Liver tissues or cells were lysed using RIPA lysis buffer (Beyotime, China) containing 1% phenyl-methane-sulfonyl fluoride (PMSF). Equal amounts of 30 µg of total protein were subjected to electrophoreses on 10% SDS-Page gels, transferred to PVDF membranes (Bio-Rad), and incubated with primary antibody anti-PTEN (Abcam, 1: 1000 dilution). After incubation with corresponding HRP-conjugated secondary antibody, the protein bands were visualized using an enhanced chemiluminescence (ECL) system (Pierce Biotechnology Inc., Rockford, IL, USA) with a ChemiDoc XRS Plus luminescent image analyzer (Bio-Rad, USA). Densitometric analysis of protein bands was performed using Image Lab software (Bio-Rad, USA). The loading volume of each sample was normalized by β-actin (Abcam, 1:5000 dilution).

Target gene validation

PTEN, a cycle-related gene that functions as a negative control in cell proliferation, was chosen as a candidate target gene of miR-21 in BNL CL.2 cells [27,28]. First, miR-21 mimic (50 nM), inhibitor (100 nM), or their negative controls were transfected to BNL CL.2 cells. At 48 h after transfection, qRT-PCR and Western blot were performed to evaluate the mRNA and protein levels of PTEN. Second, as PTEN was found to be negatively regulated by miR-21, co-transfection of PTEN siRNA (75 nM, RiboBio, China) and miR-21 inhibitor (100 nM) was conducted to further determine whether PTEN is involved in the positive effect of miR-21 on the proliferation of BNL CL.2 cells.

Statistical analysis

The statistical analyses in this study were performed using SPSS software (version 19.0). Data are expressed as mean ±SEM. An independent-samples *t* test or one-way ANOVA with post-hoc test was conducted to evaluate the data. A P-value less than 0.05 was considered statistically significant.

Results

miR-21 is upregulated during the proliferative phase of liver regeneration

Because we previously documented that hepatocyte proliferation peaked at 48 h after PH [26], the expression level of miR-21 was then determined in the mouse liver at PH-48 h versus PH-0 h using qRT-PCR. We found that the miR-21 level
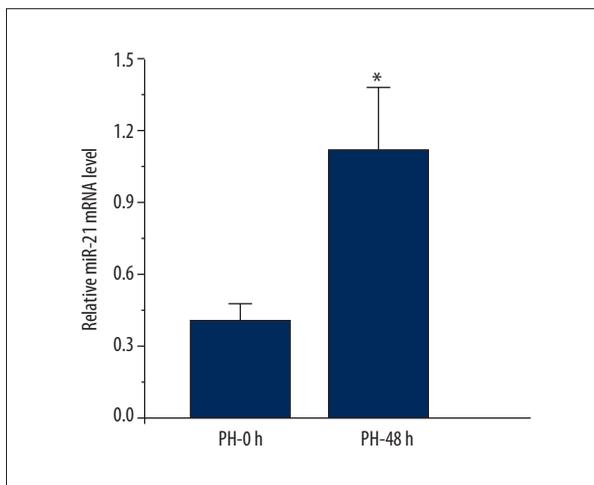
Figure 1. miR-21 is upregulated during the proliferative phase of liver regeneration. qRT-PCR analysis demonstrated a marked upregulation of miR-21 in mouse regenerating livers at 48 h after PH (PH-48 h, n=5) *versus* that at 0 h after PH (PH-0 h, n=5). * P<0.05.

was markedly upregulated at PH-48 h, verifying the induction of miR-21 during the proliferative phase of liver regeneration (Figure 1).

miR-21 accelerates hepatocyte proliferation without impact on the cell size *in vitro*

In order to investigate the effect of miR-21 on the proliferation of hepatocytes, BNL CL.2 normal liver cells were transfected with miR-21 mimic, inhibitor, or respective negative controls. We first showed that the miR-21 level was significantly increased by miR-21 mimic and was decreased by miR-21 inhibitor, confirming that the transfection took effect in BNL CL.2 cells (Figure 2A, 2B). Although cell size was not changed by miR-21 regulation (Figure 3), CCK-8 cell proliferation and EdU incorporation assay demonstrated that miR-21 mimic accelerated and miR-21 inhibitor reduced the proliferation of BNL CL.2 cells *in vitro* (Figure 4A, 4B). Flow cytometry showed that miR-21 mimic induced a G1-to-S phase transition of the cell cycle in BNL CL.2 cells *in vitro*, while miR-21 inhibitor led to G1 phase arrest (Figure 4C). These data indicate that miR-21 has a positive effect on the proliferation and cell cycle progression of BNL CL.2 normal liver cells.

PTEN expression is inversely correlated with miR-21 *in vivo* and *in vitro*

PTEN was first identified as a potent tumor suppressor gene that is inactivated in many types of human cancers [29]. To investigate whether PTEN is a target gene of miR-21 in BNL CL.2 cells, the PTEN expression level was determined in miR-21-overexpressing or -suppressing cells. Our data showed

that miR-21 negatively regulated PTEN expression at both the mRNA and protein levels (Figure 5A, 5B). We also observed decreased PTEN mRNA and protein expressions in mouse livers at PH-48 h compared to those at PH-0 h (Figure 5C, 5D). These data are consistent with *in vitro* transfection of BNL CL.2 cells with miR-21 mimic or inhibitor, indicating that PTEN expression is inversely correlated with miR-21 during the proliferative phase of liver regeneration *in vivo* and in BNL CL.2 normal liver cells *in vitro*.

PTEN is a target gene of miR-21 regarding its effect on the proliferation of hepatocytes

To further evaluate the contribution of PTEN as a target gene of miR-21 in the proliferation of hepatocytes, we assessed the impact of PTEN silencing by siRNA. First, PTEN siRNA (si-01 or si-02) was transfected to BNL CL.2 cells for 48 h, which induced a remarkable reduction in PTEN expression at mRNA level (Figure 6A). Then, co-transfection of miR-21 inhibitor and PTEN siRNA (si-01 or si-02) was conducted in BNL CL.2 cells, showing that the effect of miR-21 inhibitor in decreasing cell proliferation was reversed by the presence of PTEN-siRNA as represented by EdU staining (Figure 6B). These data confirm PTEN as a target gene of miR-21 regarding its effect on the proliferation of hepatocytes.

Discussion

Liver regeneration, especially after acute loss of liver tissue beyond a critical level, is a fundamental response of the liver to injury [30,31]. Partial hepatectomy (PH) is a common model used to investigate the cellular and molecular mechanisms, especially the proliferation of hepatocytes, during liver regeneration [32,33]. Although multiple microRNAs, including miR-21, have been found to be critical regulators of liver regeneration, the underlying mechanism regarding their roles in hepatocyte proliferation and cell cycle progression is still far from understood [5,18,34].

miR-21 has previously been proposed as a potential target for therapeutic intervention in hepatocellular carcinoma (HCC) and liver failure [35–38]. Studies have reported the induction of miR-21 in the liver during the early stages (from 2 to 48 h after PH) of liver regeneration, in which Btg2, Pellino-1, and RhoB have been identified as target genes of miR-21 [22–24]. In the present study we found that miR-21 was markedly upregulated in regenerating liver tissues at 48 h after PH, which is consistent with previous findings. Next, we continued to investigate the effects of miR-21 on the cell size, proliferation, and cell cycle progression of hepatocytes using BNL CL.2 normal liver cells *in vitro*. Our data showed that miR-21 overexpression increased the proliferation of BNL CL.2 cells *in vitro*
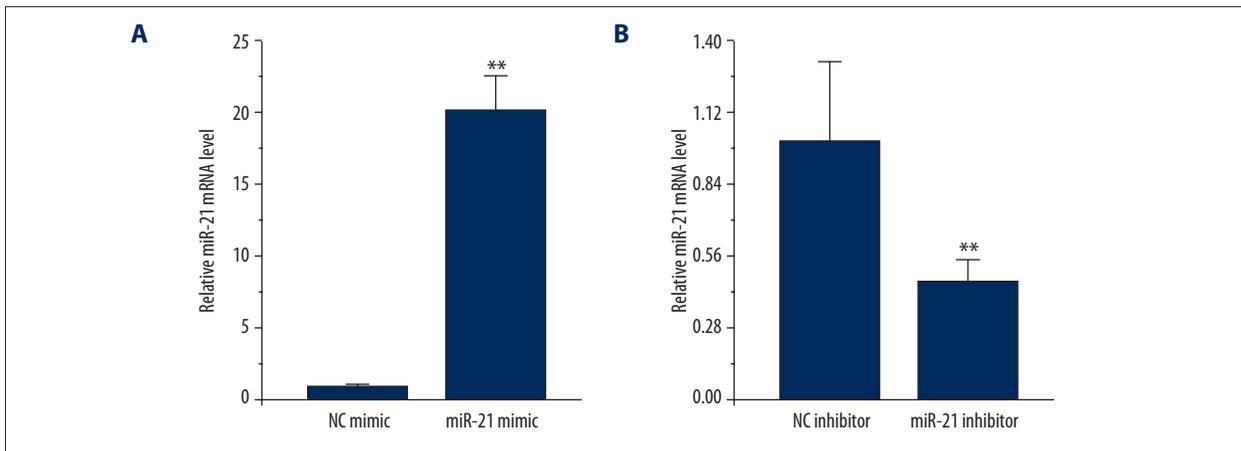
Figure 2. The efficiency of miR-21 mimic or inhibitor transfection in hepatocytes. qRT-PCR analysis confirmed that miR-21 mimic (**A**) increased and miR-21 inhibitor (**B**) reduced miR-21 level in BNL CL.2 cells (n=5). ** P<0.01.
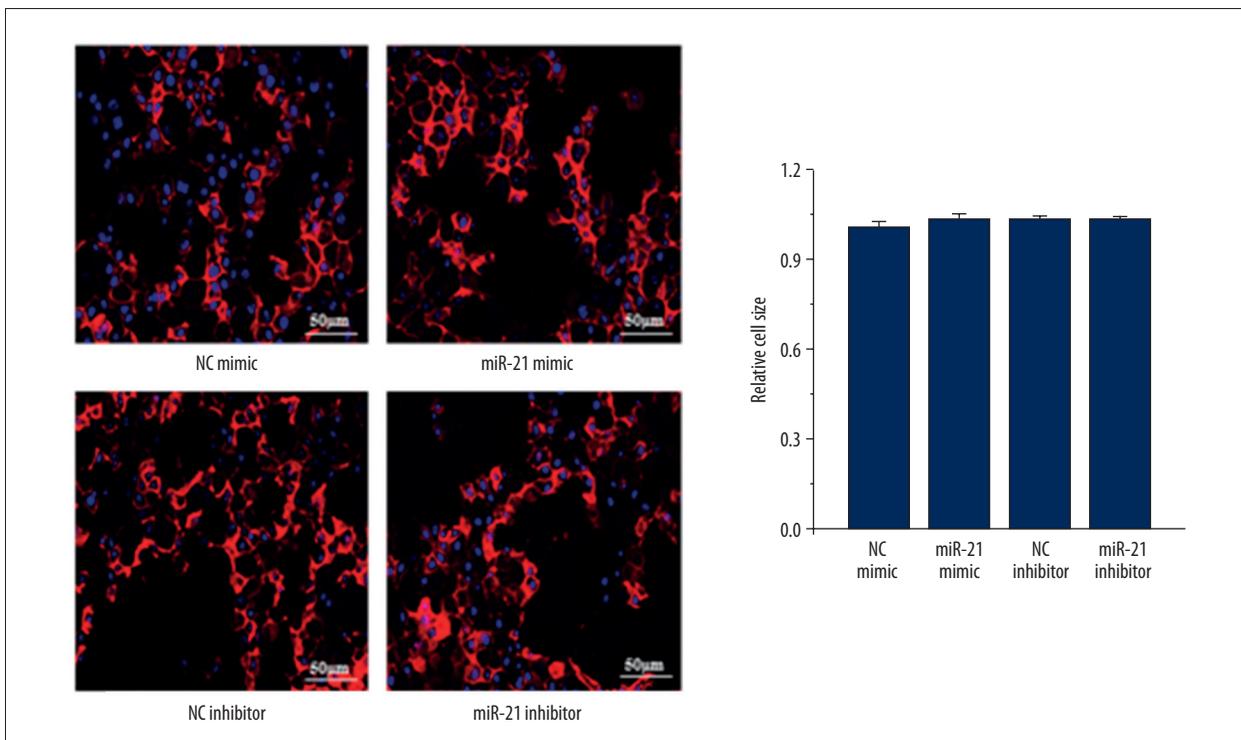

Figure 3. miR-21 regulation exhibits no effect on the cell size of hepatocytes. Dil (red) fluorescent staining showed that miR-21 mimic or inhibitor did not affect the size of BNL CL.2 cells *in vitro*. Nuclei were counterstained with DAPI (blue) (n=5). Scale bar=50 μm.

and induced a rapid G1-to-S phase progression of the cell cycle, although cell size was not altered. Given that liver regeneration after PH is predominantly mediated by the proliferation rather than the hypertrophy of hepatocytes, these data confirm miR-21 as an important promoter of hepatocyte proliferation during liver regeneration.

PTEN is a well-documented tumor-suppressor gene that inhibits cell growth and tumor development [39,40]. The loss of

PTEN expression leads to increased activity of Akt and mammalian target of rapamycin (mTOR) kinase signaling [41,42]. Recently, it has been shown that miR-21 overexpression increases the proliferation of primary hepatocytes *in vitro* via targeting PTEN [25]. To further investigate whether PTEN is a downstream target of miR-21 in BNL CL.2 normal liver cells, we conducted co-transfection of miR-21 inhibitor and PTEN siRNA and then analyzed the associated effect on hepatocyte proliferation. The results from the present study indicate that
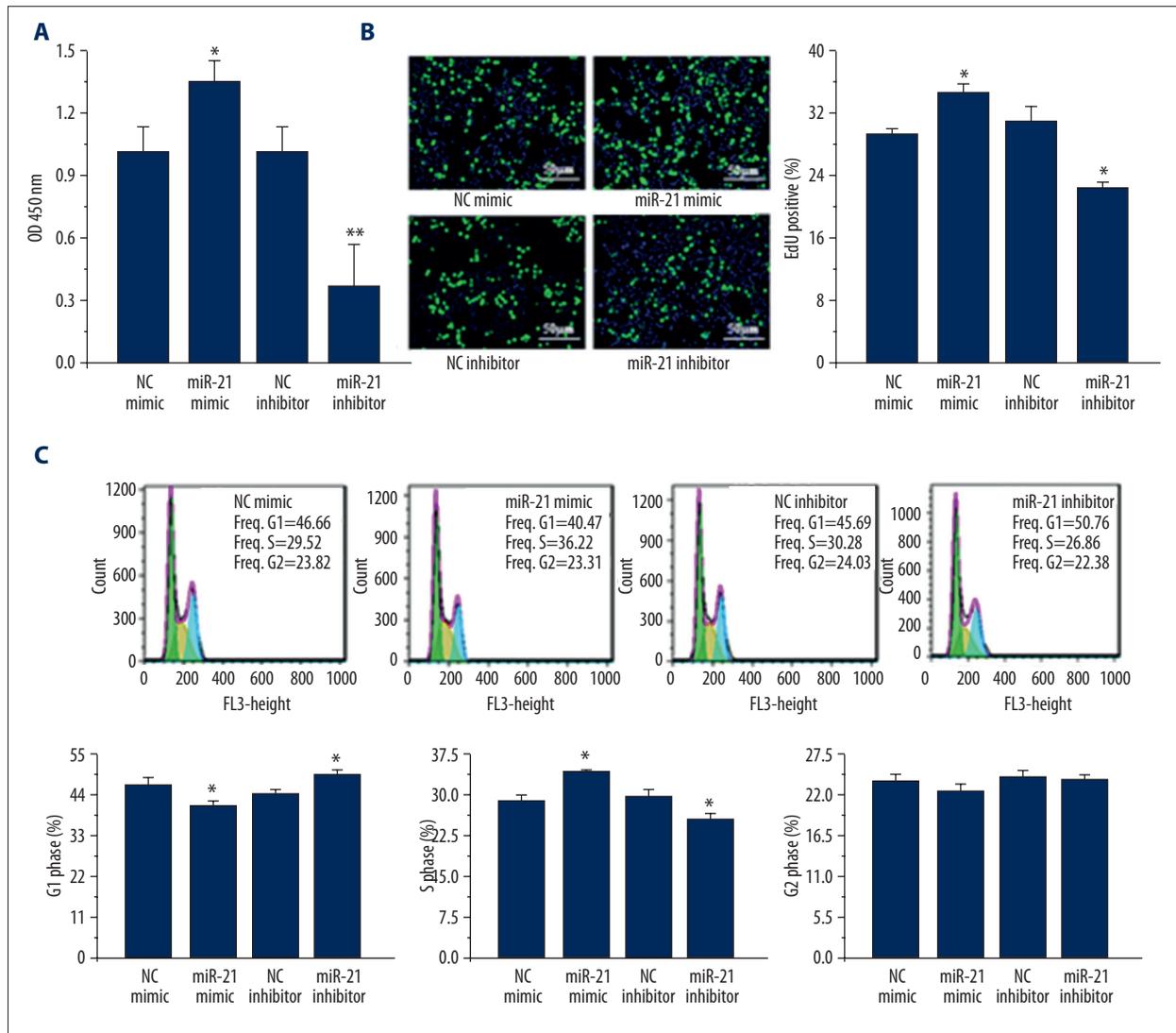
Figure 4. miR-21 accelerates the proliferation and G1/S phase transition of the cell cycle in hepatocytes. CCK-8 cell proliferation assay (**A**) and EdU (green) incorporation assay (**B**) demonstrated that miR-21 overexpression promoted and miR-21 inhibition reduced the proliferation of BNL CL.2 cells *in vitro*. Nuclei were counterstained with DAPI (blue) (n=10 and n=5, respectively). Scale bar=50 μm. (**C**) Flow cytometry showed that miR-21 mimic induced a G1/S phase transition of the cell cycle in BNL CL.2 cells, and miR-21 inhibitor resulted in G1 phase arrest (n=5). * P<0.05; ** P<0.01.

PTEN expression is inversely regulated by miR-21 in BNL CL.2 cells and that the PTEN mRNA and protein levels were significantly lower in mouse livers at PH-48 h *in vivo*. More importantly, we found that PTEN siRNA could significantly reverse the suppressive effect of miR-21 inhibitor on hepatocyte proliferation. Thus, these data verify that PTEN is a target gene of miR-21 in BNL CL.2 normal liver cells with downregulated PTEN expression, with miR-21 overexpression in the regenerating livers after PH.

The liver, which is the largest organ of the human body, has great potential for regeneration, and the underlying mechanism of liver regeneration has received much attention [43–45]. The

significant induction of miR-21 during liver regeneration has previously been reported in a murine model of partial hepatectomy (PH) [22–24]. Furthermore, miR-21 has recently been shown to promote primary hepatocyte proliferation via targeting PTEN *in vitro* [25]. However, the novelties of the present study are: (1) Because the proliferation-promoting effect of miR-21 has only been demonstrated in primary hepatocytes [25], here, we further clarify the role of miR-21 on the proliferation and cell cycle of normal liver cells by using the cell line BNL CL.2 *in vitro*; and (2) An inverse correlation of miR-21 and PTEN expressions has been shown in hepatocytes *in vitro*. To the best of our knowledge, we are the first to demonstrate that PTEN expression was lower than the induced expression of miR-21
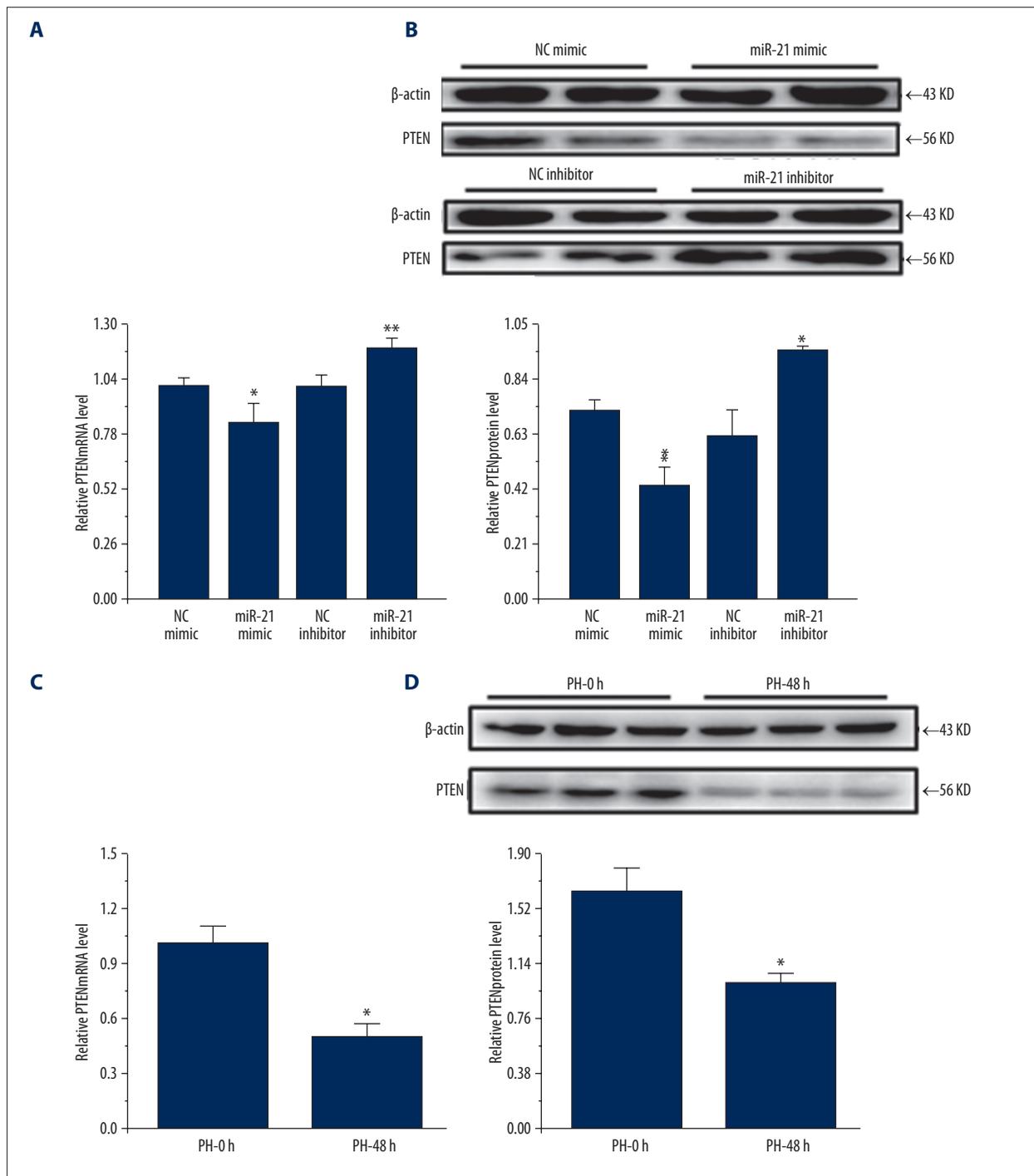
Figure 5. PTEN expression is inversely correlated with miR-21 both *in vitro* and *in vivo*. qRT-PCR (**A**) and Western blot (**B**) analysis showed that miR-21 negatively regulated PTEN expression at both mRNA and protein levels in BNL CL.2 cells *in vitro* (n=5 and n=3, respectively). A reduction of PTEN expression was also found at mRNA (**C**) and protein (**D**) levels in mouse livers at PH-48 h versus those at PH-0 h (n=5 and n=5, respectively). * P<0.05; ** P<0.01.

in mouse livers at PH-48 h, although the promoting effect of miR-21 on liver regeneration *in vivo* by targeting PTEN needs to be further investigated.

The upregulation of miR-21 during liver regeneration may be induced by multiple factors. It has been demonstrated that the induction of miR-21 is mediated by the IL-1 receptor and toll-like receptor-activated Pellino-1/NF-κB signaling, with Pellino-1
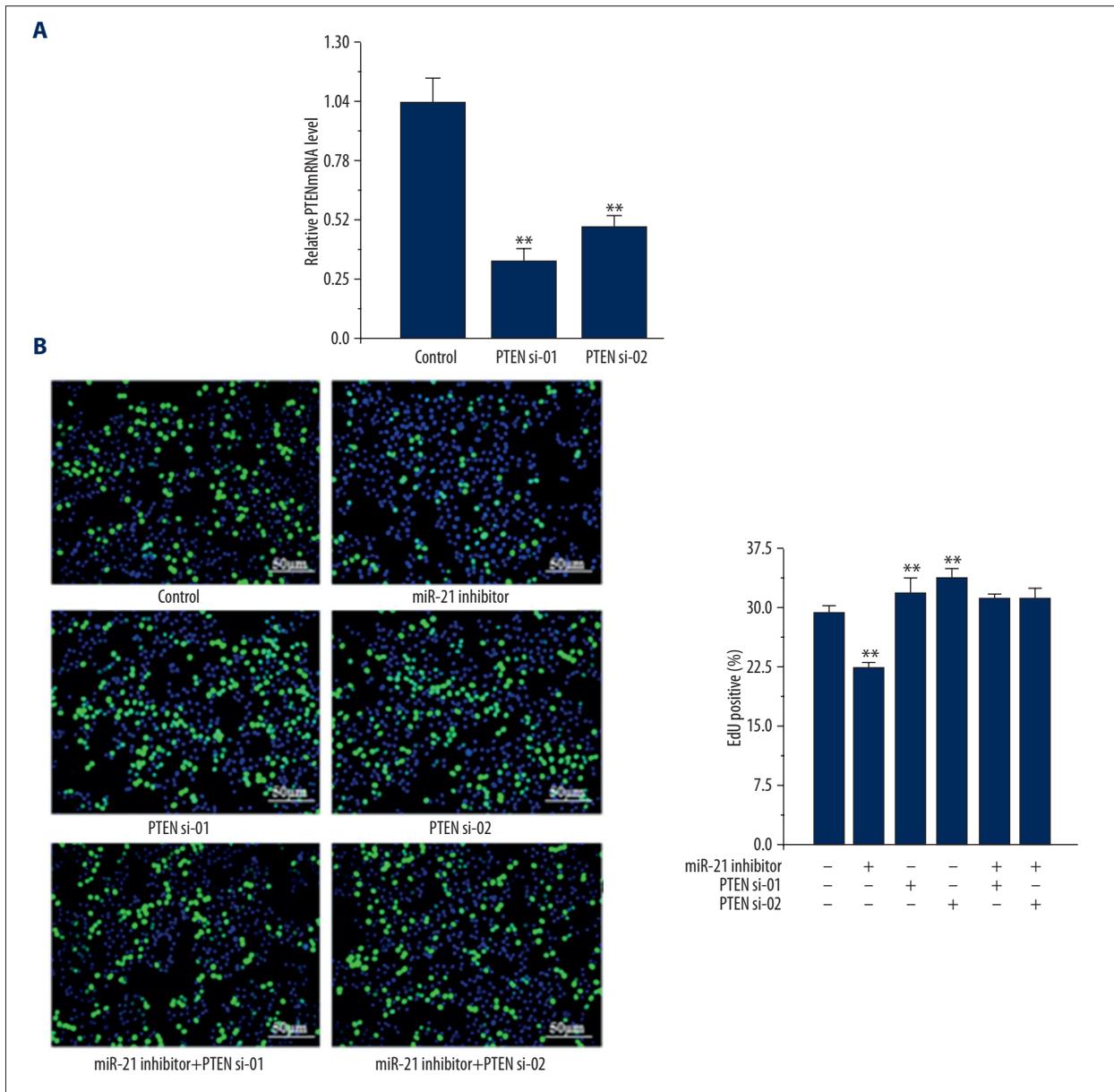
Figure 6. Silencing PTEN reverses the proliferation-suppressing effect of miR-21 inhibitor in hepatocytes. (**A**) qRT-PCR analysis verified that PTEN siRNA (si-01 or si-02) reduced PTEN expression at mRNA level in BNL CL.2 cells (n=5). (**B**) EdU (green) staining demonstrated that the presence of PTEN siRNA (si-01 or si-02) can abolish the reducing effect of miR-21 inhibitor on the proliferation of BNL CL.2 cells. Nuclei were counterstained with DAPI (blue) (n=5). Scale bar=50 μm. ** $P < 0.01$.

as a downstream effector of miR-21, performing feed-back regulation of NF-κB signaling by miR-21 [23]. Thus, it is important to study the upstream and downstream molecular mechanisms underlying the effects of miR-21 on hepatocyte proliferation and liver regeneration. Our data demonstrate upregulated miR-21 and downregulated PTEN expression in the proliferative phase of liver regeneration after PH *in vivo*. Further study is needed to confirm the functional relationship between miR-21 and PTEN by conducting gain- and loss-of-function studies *in vivo*.

Conclusions

Our findings indicate that miR-21 overexpression promotes liver regeneration and hepatocyte proliferation via targeting PTEN. Upregulation of miR-21 might be a useful therapeutic strategy to promote liver regeneration.

Competing Interests

The authors have declared that no competing interests exist.

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