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MicroRNA-21 accelerates hepatocyte proliferation *in vitro* via PI3K/Akt signaling by targeting PTEN

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ABSTRACT

MicroRNAs (miRNAs) are involved in controlling hepatocyte proliferation during liver regeneration. In this study, we established the miRNAs-expression patterns of primary hepatocytes *in vitro* under stimulation of epidermal growth factor (EGF), and found that microRNA-21 (miR-21) was appreciably up-regulated and peaked at 12 h. In addition, we further presented evidences indicating that miR-21 promotes primary hepatocyte proliferation through *in vitro* transfecting with miR-21 mimics or inhibitor. We further demonstrated that phosphatidylinositol 3′-OH kinase (PI3K)/Akt signaling was altered accordingly, it is, by targeting phosphatase and tensin homologue deleted on chromosome 10, PI3K/Akt signaling is activated by miR-21 to accelerate hepatocyte rapid S-phase entry and proliferation *in vitro*.

1. Introduction

Primary hepatocytes constitute the foremost hepatic cell population and are characterized by a unique stem-like capability for growth [1,2]. This property protects the host from liver malfunction after extreme or continual loss in liver organ size via swift regrowth and expansion. Within the regenerative course, an abundance of growth factors, cytokines and signaling pathways interplay at various levels [3]. Among the list of pathways, the phosphatidylinositol 3′-OH kinase (PI3K)/Akt signaling pathway plays a pivotal role in liver regeneration. Total or selective PI3K blockage substantially reduces proliferation after partial hepatectomy (PH) [4], while treating mice with activated Akt increased liver size by 3- to 4-fold [5].

MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules that regulate gene expression post-transcriptionally. Recently, various periodicals separately identified several de-regulated miRNAs during liver regeneration (LR) in patients or animal models [6–16]. One of the de-regulated miRNAs, miR-21, was appreciably up-regulated during LR phases [6–8,11,14,15]. Prior researchers have revealed that miR-21 was involved in tumor progression by activating Akt signaling [17,18]. It remains ambiguous whether miR-21-mediated PI3K/Akt activation explains primary hepatocyte proliferation.

In the present study, we sought to investigate the regulatory role of miR-21 in hepatocyte proliferation *in vitro*. Our results determined the miRNA profiles of primary hepatocytes that were cultured *in vitro* within the initial 12 h. Importantly, we determined a role of miR-21 and PI3K/Akt signaling in promoting hepatocyte proliferation by gain- or loss-of function strategies.

2. Materials and methods

2.1. Mice

Animal experiments were approved by the West China School of Medicine, Sichuan University, China Institutional Review Board. Male C57BL/6 mice aged 6–8 weeks were purchased from the Sichuan University Laboratory Animal Center.

2.2. Two-thirds PH

Studies including 2/3 PH were performed on 25 mice in accordance with a standardized protocol that was published previously [19]. Another 25 sham hepatectomy (SH) mice were used as controls. At time intervals of 2, 24, 48, 72 and 96 h after the operation, liver tissue was harvested in 5 duplicates.

2.3. Hepatocyte isolation, culture and transfection

Primary mouse hepatocytes were isolated by a two-step in situ collagenase perfusion technique derived from male C57BL/6 mice as in [20] and cultured as in (Bailly-Maitre et al., 2007), supplemented with epidermal growth factor (EGF, 30 ng/ml). For all hepatocyte transfection assays, 5×10^5 or 10,000 cells per well were seeded onto 6- or 96-well plates (Corning), respectively. After

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cell attachment for 6 h, hepatocytes were transfected with miR-21 mimics, inhibitor, or the negative control (RiboBio) using Lipofectamine™ 2000 (Invitrogen). The miR-21 mimic and its negative control, and the miR-21 inhibitor along with its negative control are referred to as miR-21 and miR-con or anti-miR-21 and anti-miR-con, respectively.

2.4. miRNA expression profiling

Mouse primary hepatocytes cultured *in vitro* were used to analyze miRNA expression changes. Cell samples were harvested at 0, 2, and 12 h after seeding into 6-well plates. A minimum of 3 duplicates at every time point were harvested and pooled for RNA extraction. miRNA expression profiling was performed at Exiqon. Differentially expressed miRNAs were identified through Fold Change filtering (Fold Change $\geqslant 2.0$). Hierarchical clustering was performed using MEV software (v4.6, TIGR).

2.5. qRT-PCR

Total RNA was extracted with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and miR-21 detection was carried out as described in [21]. Quantitative normalization was performed on U6 or β-actin, for miRNA or mRNA detection, respectively and the relative expression levels calculated as in [22].

2.6. Cell cycle analysis

Single-cell hepatocyte suspension were collected 24 h after treatment with miR-21 mimics, inhibitor or blank loading control. The sample preparation was performed using a Cell Cycle Detection Kit (KeyGen) and analyzed with a FACSCan flow cytometer (BD) according to the manufacturer's instructions. The data were analyzed using Mod Fit-LT v3.0 (Verity Software House Topsham).

2.7. Protein preparation and immunoblotting

Protein extracts were obtained using RIPA buffer [1% TritonX-100, 0.1% SDS,1% sodiumdeoxycholate,150 mM NaCl, 50 mM Tris–HCl (pH 7.2), 0.4 mM Na_3VO_4, 10 g/ml leupeptin, 4 g/ml pepstatin, 0.1 TIU/mlaprotinin]. Immunoblotting performed as in [21].

2.8. Statistical analysis

Statistical significance was estimated using a 2-tailed Student's *t*-test. A *P*-value less than 0.05 was considered to be significant.

3. Results

3.1. Genome-wide miRNA changes and qRT-PCR validation during hepatocyte proliferation

We analyzed genome-wide miRNA expression profiles at 0, 2, and 12 h in freshly isolated hepatocytes that had been seeded into 6-well plates under stimulation of EGF. Based on the miRNA expression levels, we grouped them into three sets and classified them as up-regulated (>2-fold), down-regulated (<0.5-fold) and unchanged (0.5- to 2-fold). Weighed against standard levels (0 h), there were 68 and 107 up-regulated and 191 and 189 down-regulated miRNAs at 2 and 12 h, respectively. To further depict the pattern of miRNA changes, unsupervised hierarchical miRNA clustering and the expression level change at the 3-fold threshold were selected to generate the heat map (Fig. 1A and B). Using a Venn diagram, only an integral miRNA subset demonstrated exactly the same expression patterns at 2 and 12 h after the hepatocytes were seeded; 40 miRNAs were up-regulated, 76 miRNAs were down-regulated and 228 miRNAs were unchanged (Fig. 1C). miR-21 was among the first cluster and was substantially up-regulated by 3-fold after 2 h and by 4-fold after 12 h compared with the 0 h time point (Fig. 1B). Thus, we focused our analyses on miR-21 in primary hepatocytes *in vitro*.

To validate miR-21 expression levels in hepatocytes *in vitro* we performed qRT-PCR assays at 0, 2, 6, 12, 24, and 48 h after cultivation. We determined that miR-21 expression substantially increased from 2 h (1.91 ± 0.14 vs. 0.26 ± 0.02, $P < 0.001$) to 48 h (2.43 ± 0.42 vs. 0.26 ± 0.02, $P < 0.001$), with a peak at 12 h (6.10 ± 1.38 vs. 0.26 ± 0.02, $P < 0.001$) (Fig. 2A). The miR-21 expression pattern as detected by qRT-PCR during the first 12 h was in good agreement with the microarray results.

3.2. miR-21 up-regulation during the early stages of LR

To determine the differentiated expression of miR-21 during LR, we collected liver tissue after 2/3 PH at 2, 24, 48, 72, and 96 h for qRT-PCR. We determined substantially altered expression following 2/3 PH compared with the SH control (Fig. 2B). miR-21 up-regulation was induced 2 h after 2/3 PH (2.51 ± 0.24 vs. 1.59 ± 0.16, $P = 0.005$). Its expression consistently rose although it began decreasing after 48 h (5.51 ± 0.19 vs. 2.25 ± 0.21, $P < 0.001$), which was immediately after most hepatocytes transitioned from the G1 to the S phase [23]. The miR-21 expression curve in regenerative liver parenchyma, which peaked 48 h after PH, was distinct from the results in hepatocytes *in vitro,* which peaked 12 h after cultivation.

3.3. Impacts of miR-21 transfection on hepatocyte proliferation in vitro

To determine the mechanism by which miR-21 regulated hepatocyte proliferation, we performed cell transfection assays. To assess the transfection efficiency, we detected miR-21 expression in hepatocytes after transfection with 40 nM miR-21 mimics, miR-21 inhibitors or miR-21 negative controls (Fig. 3A). In miR-21 mimic-treated hepatocytes, miR-21 expression levels were significantly up-regulated 6 and 12 h after transfection compared with the control (16.79 ± 1.37 vs. 8.40 ± 0.69, $P = 0.007$; 40.84 ± 7.65 vs. 16.49 ± 2.30, $P = 0.006$; respectively), while normalized after 24 h (3.66 ± 0.38 vs. 2.91 ± 0.31, $P = 0.06$). In miR-21 inhibitor-treated hepatocytes, miR-21 expression levels were significantly down-regulated at 6, 12, and 24 h compared with the control (0.0031 ± 0.002 vs. 8.40 ± 0.69, $P < 0.001$; 0.0089 ± 0.002 vs. 16.49 ± 2.30, $P < 0.001$; 0.0048 ± 0.003 vs. 2.91 ± 0.31, $P < 0.001$, respectively). These results indicated that miR-21 mimics or inhibitors have been efficiently transfected into hepatocytes, resulted in over-expression or deletion of miR-21 in hepatocytes.

Next, we performed cell cycle analysis by flow cytometry to determine the role of miR-21 on hepatocyte proliferation 24 h after infection (Fig. 3C–E). We determined that the proportion of S-phase hepatocytes after miR-21 mimic or miR-21 inhibitor treatment was appreciably enhanced or diminished, respectively, compared with negative controls (19.90 ± 3.36 vs. 10.34 ± 2.10, $P = 0.01$; 5.16 ± 1.22 vs. 10.34 ± 2.10, $P = 0.02$, respectively). These results indicated that miR-21 mimics promoted hepatocyte rapid S-phase entry and cell cycle progression, while miR-21 inhibitors impeded S-phase entry and cell cycle arrest.

3.4. miR-21 suppresses PTEN and promotes hepatocyte proliferation via PI3K/AKT signaling

PI3K/Akt signaling is a well-defined pathway that is directly associated with cell growth, proliferation and survival. As an up-

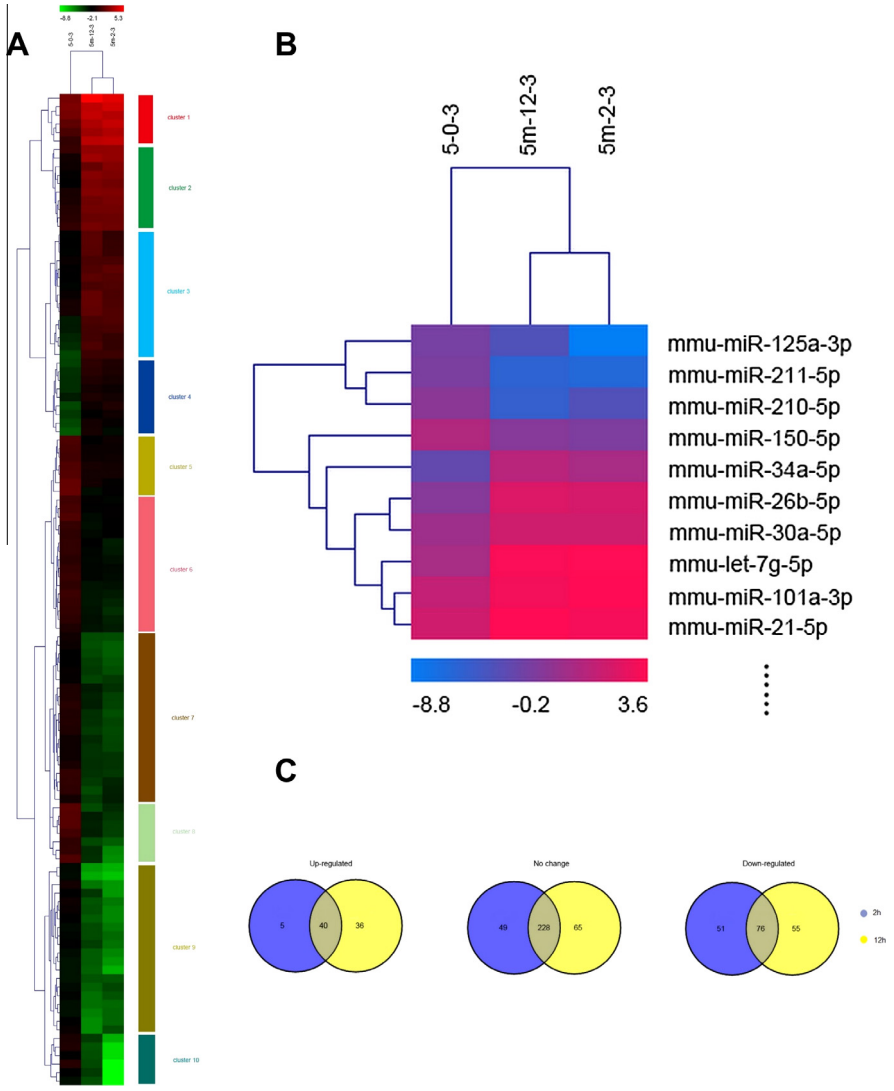

Fig. 1. Heatmaps of microRNA (miRNA) profiles and de-regulated miRNAs during the first 12 h of primary hepatocyte cultivation *in vitro* after EGF stimulation. (A) Unsupervised hierarchical cluster analysis diagram based on 3100 probe sets at 2 h or 12 h after seeding hepatocytes into 6-well plates compared with freshly isolated hepatocytes. The miRNA clustering tree is demonstrated on the left and the sample clustering tree is at the top. The rows represent individual probe sets. The columns represent the experimental samples: 0, 2 and 12 h. Red bars indicate the relative expression level of the above mean. Green bars indicate expression lower than the mean. (B) Heatmap of the miRNAs that were significantly de-regulated during hepatocyte proliferation *in vitro*. The de-regulated miRNAs were filtered at a 3-fold threshold (..... represents other unlisted miRNAs). (C) Up-regulation (left), no-change (middle) and down-regulation (right) of miRNAs in hepatocytes *in vitro* were represented by Venn diagrams based on 3-fold threshold expression compared with freshly isolated hepatocytes (0 h). Blue circles represent miRNAs that were detected at 2 h, yellow circles represent those that were detected at 12 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

stream kinase, PI3K activity could be negatively regulated by PTEN, a miR-21 target gene for which the expression could be post-transcriptionally inhibited in cancer cells [17,24–26]. However, whether PTEN is involved in miR-21 regulation in hepatocyte proliferation remains to be elucidated. To elucidate this, we first measured PTEN expression in cultured hepatocytes. We determined that PTEN expression was inhibited in hepatocytes from 6 h after cultivation (Fig. 1C). miR-21 expression trends changed in a mirror opposite pattern (Fig. 3C). Next, we used RT-PCR to determine the effects of miR-21 on hepatocyte *Pten* mRNA expression after transfection. We determined that hepatocyte PTEN expression was inversely regulated by miR-21; miR-21 over-expression significantly reduced *Pten* mRNA expression, and reduced miR-21 expression significantly increased *Pten* mRNA expression (Fig. 3B).

To determine whether PTEN is post-transcriptionally inhibited by miR-21, we further measured PTEN protein levels in hepatocytes by immunoblotting. We determined markedly reduced PTEN protein levels in hepatocytes that were transfected with miR-21 mimics as well as markedly enhanced PTEN levels in cells that were treated with miR-21 inhibitors (Fig. 4A). These findings affirm our speculation that PTEN is a direct target of miR-21 in cultured primary hepatocytes.

Given the negative regulation of PI3K/Akt signaling by PTEN, we investigated whether PTEN inhibition was responsible for the effect of miR-21 on PI3K/Akt signaling. We used immunoblotting to determine Akt protein levels and Ser473 phosphorylation (p-Akt). As expected, Akt levels and activity were markedly elevated in miR-21 mimic-treated hepatocytes but were markedly decreased after miR-21 inhibitor treatment (Fig. 4A and B). Certainly, PI3K/Akt signaling activation is essential for the early phase of LR, which ultimately triggers the subsequent phosphorylation of downstream targets, induces cyclin E and inactivates CDK inhibitor proteins p21 and p27, thereby promoting cell growth and survival [4,5,27]. Additionally, while contemplating our findings above that

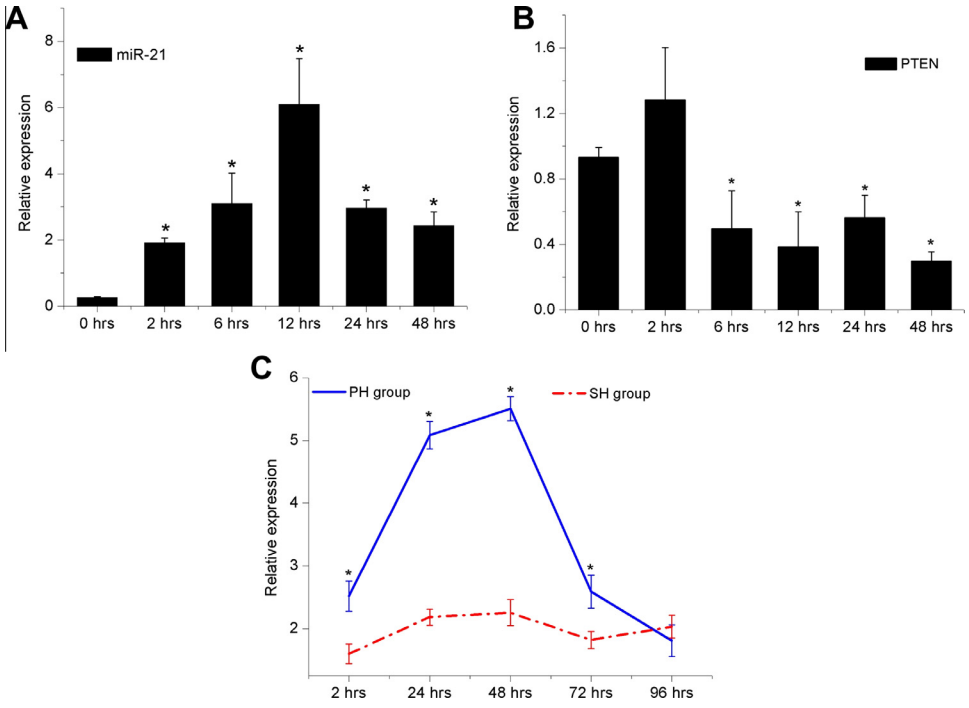

Fig. 2. miR-21 up-regulation in primary hepatocyte proliferation *in vitro and in vivo* during LR, and its inverse correlation with the target gene PTEN. (A and B) miR-21 expression levels were examined by SYBR green RT-PCR at the indicated time points after primary hepatocytes were seeded in 6-well plates (A) or partial hepatectomy in mice (B) during LR. (C) PTEN expression levels were examined by Taqman RT-PCR at the indicated time points after seeding primary hepatocytes in 6-well plates. (*$P < 0.05$ vs. 0 h).

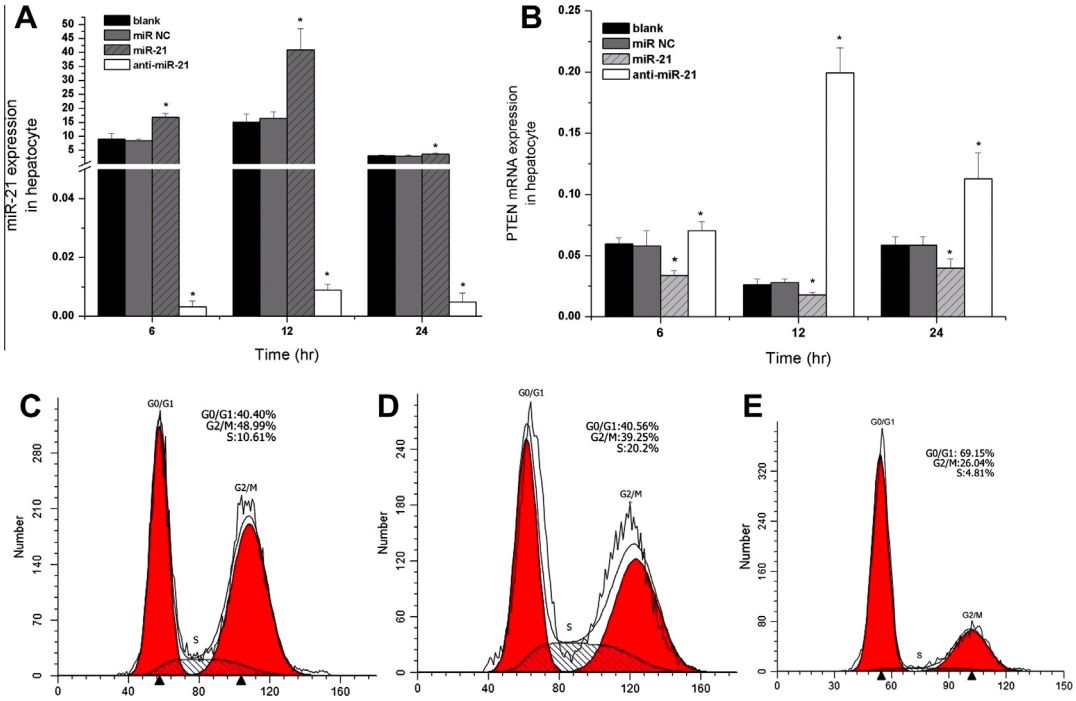

Fig. 3. miR-21-regulated primary hepatocyte cell cycle progression *in vitro* after transfection. (A) Primary hepatocytes that were treated with miR-21 mimics (miR-21), inhibitor (anti-miR-21) or negative controls (miR NC) were seeded in 6-well plates. miR-21 expression levels were examined at the indicated time points by SYBR green RT-PCR. (B) *Pten* mRNA expression levels were examined at the indicated time points by Taqman RT-PCR. (C–E) Cell cycle analyses were performed by flow cytometry. Cell cycle distribution data were presented as a percentage. (*$P < 0.05$ vs. miR NC group).

demonstrated an altered S-phase distribution of hepatocytes after miR-21 transfection, we investigated whether over- or low-miR-21 expression affected cyclin E, p21 and p27 translation (Fig. 4C). We determined that miR-21 over-expression in hepatocytes increased cyclin E1 and decreased p21 and p27 protein levels. Although we failed to notice increased p27 protein levels in miR-21 inhibitor-treated hepatocytes, cyclin E expression was decreased and p21 expression was significantly increased.

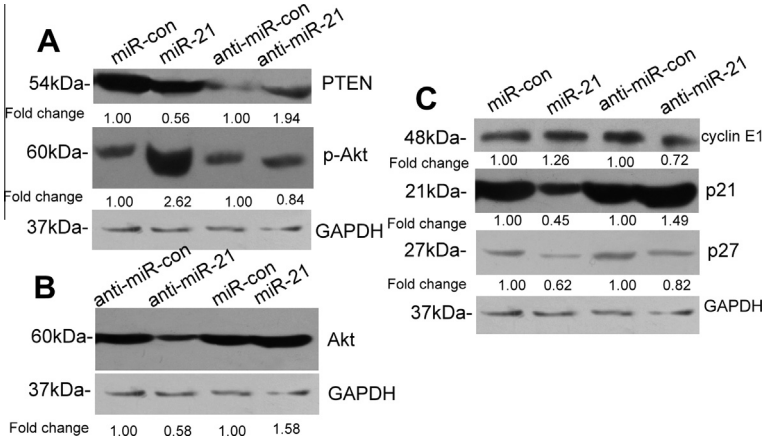

Fig. 4. miR-21 modulates hepatocyte proliferation-associated PI3K/Akt signaling by targeting PTEN, which facilitates cyclin E1 expression and represses p21 and p27 expression. (A and B) Immunoblotting revealed that miR-21 mimic (miR-21) transfection into primary hepatocytes decreased PTEN levels, increased Akt levels and activation by enhancing its Ser473 phosphorylation (p-Akt), while miR-21 inhibitor (anti-miR-21) transfection increased PTEN expression, and decreased Akt expression and activation by inhibiting Ser473 dephosphorylation. Negative control siRNA-transfected cells (miR-con, anti-miR-con) were used as controls. (C) Immunoblotting demonstrated that miR-21 transfection into hepatocytes increased cyclin E1 and decreased p21 and p27 levels, while anti-miR-21 transfection decreased cyclin E1 and increased p21 levels.

4. Discussion

Several previous studies have characterized genome wide miRNA expression patterns in the regenerative liver parenchyma during LR [6,7,9,11,14,15]. However, very little is understood about the role of miRNAs in primary hepatocytes *in vitro*. In the present study, we investigated genome wide miRNA expression levels in the first 12 h after cultivating purified primary hepatocytes. There initially were 259 and 296 de-regulated miRNAs in the purified primary hepatocytes 2 and 12 h after cultivation, respectively, compared with the baseline levels (0 h). The key finding was that miR-21 was up-regulated by 3- to 4-fold during cultivation of hepatocytes *in vitro*, which was the equivalent expression pattern as the regenerative liver parenchyma in the early phase of LR [6–8,11,14,15,28].

Recently, miRNA and LR research has been gradually changing from identifying miRNA characteristics to identifying functional miRNAs and their regulated mechanism such as miR-21 [28], miR-23b [16] and miR-221 [29], which promote hepatocyte proliferation, whereas miR-26a [30], miR-33 [31], miR-34a [9], miR-127 [32] inhibit hepatocyte proliferation.

The expression curve of miR-21 in regenerative liver parenchyma, which peaked 48 h after PH, was similar to that obtained from prior scientific studies [11,15,28] but was distinct from the results in cultured hepatocytes *in vitro,* which peaked 12 h after cultivation. This might be attributed to the data that was obtained from the different material; the former was from regenerative tissue homogenates, which consists of several cell types, while the latter was from hepatocytes alone [3]. Because miRNA expression patterns *in vivo* are a consequence of the integral regulation of complicated signaling pathways and eventually interaction with other non-parenchymal cells in the regenerative liver parenchyma [3], we focused entirely on purified primary hepatocytes to filter out unexpected confounders.

Lately, the analysis of miRNAs and LR is progressively transforming from pinpointing miRNA characteristics to identifying functional miRNAs and their regulatory mechanisms including miR-21 [28], miR-23b [16], and miR-221 [29], which promote hepatocyte proliferation, and miR-26a [30], miR-33 [31], miR-34a [9], and miR-127 [32], which inhibit hepatocyte proliferation.

miR-21 is among the first recognized mammalian miRNAs, is overexpressed in most tumor types, and is characterized by high proliferative property [17,33,34]. Former research demonstrates that miR-21 is also up-regulated in the early phase of LR after PH, which regulates hepatocyte proliferation by targeting critical genes such as Peli1 [11], Btg2 [15], Rhob [28], among others. Our present study mainly investigated the relationship between miR-21 and PI3K/Akt signaling. This survival pathway is essential for activating proliferation in normal LR after PH [35]. Our data demonstrated that miR-21 overexpression in primary hepatocytes increased Akt expression levels and activation (p-Akt); inversely, low miR-21 expression decreased Akt expression and activation.

Our finding that miR-21 activated PI3K/Akt signaling in primary hepatocytes encouraged us to investigate whether PTEN, a miR-21 target that inhibits Akt phosphorylation [24], was involved in this process. We found that PTEN expression was inhibited in normal primary hepatocytes from 6 h after cultivation, which was opposite of miR-21 expression. After transfection with miR-21 mimic or inhibitor, we determined that *Pten* mRNA and protein levels were up- or down-regulated, respectively. Our findings suggested that miR-21 promoted PI3K/Akt activation in a PTEN-dependent manner in primary hepatocytes *in vitro*. Nevertheless, an extremely recent publication by Ng et al. suggested that PTEN accumulation did not inhibit Akt1 activation in normal LR [28]. They first described that miR-21 knockdown impaired cyclin D1 translation by derepressing Rhob, which inactivates Akt1/mTORC1 signaling. However, it remains to be investigated whether miR-21 modulation *in vivo* could also appreciably accelerate primary hepatocyte proliferation *in vitro* in a similar pattern. miR-21 promotes cell proliferation via PTEN-dependent PI3K/Akt activation in cancer cells [17,25,26]. miR-21 has numerous targeted genes, which coupled with our results, suggests that miR-21 is a central regulator of Akt signaling in hepatocyte proliferation.

Our results further revealed that miR-21 controlled the G1/S phase transition of the cell cycle in primary hepatocytes *in vitro*. In miR-21 mimic-treated hepatocytes, the proportion of hepatocytes in S phase was markedly enhanced compared with controls. Cyclin E1 was increased and cyclin-dependent kinase inhibitors (p21, p27) were decreased in these cells. In contrast, in miR-21 inhibitor-treated hepatocytes, the percentage of cells in S phase was markedly decreased. Cyclin E1 and p21 protein levels changed inversely compared with miR-21 mimic-treated cells. Within the cell cycle regulatory machinery, cyclin E1 is essential for controlling cell cycle progression at several stages. The protein levels peak near the G1/S checkpoint and are reduced following S-phase entry. Cyclin E1 overexpression facilitates rapid cellular S-phase entry by

specifically activating cyclin-dependent kinase Cdk2 as well as promoting DNA synthesis and proliferation [36,37]. Moreover, cyclin E-Cdk2 also inactivates p21 and p27 by phosphorylation during G1 and S phases and accelerating cell cycle progression [38]. Thus, our findings established that miR-21 controlled cell cycle S-phase progression in primary hepatocytes *in vitro*.

In conclusion, we offer data detailing which de-regulated miRNAs regulate hepatocyte proliferation after cultivation with EGF stimulation. By targeting PTEN, PI3K/Akt signaling is activated by miR-21 to accelerate rapid S-phase entry by hepatocytes and proliferation *in vitro*.

Conflict of interest declaration

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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