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RESEARCH ARTICLE

miR-582-5p inhibits proliferation of hepatocellular carcinoma by targeting CDK1 and AKT3

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Abstract microRNAs play an important role in the progression of hepatocellular carcinoma (HCC). In this study, we found that miR-582-5p expression was downregulated in hepatoma tissues and HCC cell lines. Upregulation of miR-582-5p reduced colony number, inhibited cellular proliferation, and arrested cell cycle in G0/G1 phase. When miR-582-5p was inhibited, the colony number was increased and cellular proliferation and cell cycle were promoted. Further studies showed that miR-582-5p regulated the progression of HCC through directly inhibiting the expression of CDK1 and AKT3, and indirectly inhibiting the expression of cyclinD1.

Yi Zhang and Wei Huang contributed equally to this work.

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Introduction

microRNAs (miRNAs) are small non-coding RNA of 19–25 nt in length which play an important role in posttranscriptional gene regulation, microRNAs bind to the 3′ UTR of target mRNA in a sequence-specific manner and lead to translational inhibition or mRNA destabilization. They regulate cell differentiation, proliferation, apoptosis and migration [1, 3]. Since the discovery the first microRNA *Lin-4* in *Caenorhabditis elegans*, hundreds of microRNAs were found in animals. A growing number of evidences show that aberrant expression of microRNAs promote the progression of many kinds of tumors [5, 24, 31]. Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world [6], many microRNAs play critical role in HCC, and they can function as oncogenes or tumor suppressor genes [4, 8, 22]. For example, miR-29c is a tumor suppressor gene, which represses cancer cell growth and proliferation by inhibiting the expression of SIRT1 [2]. miR-101 suppresses the tumorigenesis in nude mice and promotes apoptosis by inhibiting the expression of Mcl-1 and Bcl-2 [20]. miR-17-5p promotes HCC cell migration and proliferation by activating mitogen-activated protein kinases (MAPK) pathway and increasing the phosphorylation of heat shock protein 27 (HSP27) [28]. miR-224 promotes HCC cell proliferation and migration by activating AKT signaling pathway [29]. With the development of RNA-seq and microRNA microarray, more and more new microRNAs will be found that they regulate the development of HCC.

miR-582-5p suppresses forkhead box O1 (FOXO1) to inhibit apoptosis of monocytes, and promotes immune

responses against tuberculosis [10]. In bladder cancer, miR-582-5p and -3p inhibit cancer cell proliferation and invasion by downregulating PGGT1B, LRRK2 and DIXDC1 [23]. From the results of microRNA microarray [19], the expression of miR-582-5p was lower in HCC tissues than normal liver tissues, but the role of miR-582-5p in HCC has not been determined.

In the present study, we found miR-582-5p expression was reduced in HCC patients and HCC cell lines. To examine the role of miR-582-5p, we utilized miR-582-5p mimic to upregulate miR-582-5p expression, and miR-582-5p inhibitor to downregulate miR-582-5p expression. Gain- and loss-of-function studies revealed that miR-582-5p inhibited HCC cell proliferation and arrested cell cycle in G0/G1 phase. Further study shown the critical proteins that promote G1/S transition were inhibited. Moreover, we found both cyclin-dependent kinase 1 (CDK1) and v-akt murine thymoma viral oncogene homolog 3 (AKT3) were direct targets of miR-582-5p. These results showed that miR-582-5p inhibits the proliferation of HCC cells by targeting CDK1 and AKT3.

Materials and methods

Patient specimens

Eight HCC specimens and matched tumor-adjacent tissues were obtained from the First Affiliated Hospital, Sun Yat-Sen University. Samples were snap frozen immediately and were stored at −80 °C. For the research proposes using these clinic samples, prior patient's consent and approval from the Institutional Research Ethics Committee of the First Affiliated Hospital, Sun Yat-Sen University were obtained.

Cell culture

HCC cell lines QGY-7703, Huh7, BEL-7402, HepG2 and Hep3B, and the immortalized normal liver epithelial cell THLE3 were obtained from ATCC. HCC cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (HyClone, Logan, UT) and 1 % penicillin/streptomycin. THLE3 cells were growth in bronchial epithelial growth medium (Clonetics Corporation, Walkersville, MD), supplemented with 5 ng/ml epithelial growth factor, 70 ng/ml phosphoethanolamine, and 10 % fetal bovine serum. These cells were maintained in a humidified atmosphere at 37 °C with 5 % CO_2.

RNA extraction and real-time quantitative PCR

Total miRNA from cultured cells and fresh surgical HCC tissues were extracted using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 5 ng of total RNA using the TaqMan miRNA reverse transcription kit (Applied Biosystems, Foster City, CA), and the expression of miR-582-5p were quantified using miRNA-specific TaqMan miRNA Assay Kit (Applied Biosystems). Real-time PCR was performed using the Applied Biosystems 7500 Sequence Detection system. U6 small nuclear RNA was used as an endogenous control for miR-582-5p normalization. The expression of miR-582-5p was defined on the threshold cycle (Ct); the relative expression were calculated as $2^{-[(\text{Ct of miR-582-5p})-(\text{Ct of U6})]}$.

Relative expression of different genes was detected using Roche SYBR Green PCR Kit in an Applied Biosystems 7500 Sequence Detection system. The primers were showed in Table 1; GAPDH was used as an endogenous control for normalization. The expression of each transcript was calculated as $2^{-[(\text{Ct of cyclinD1,p21})-(\text{Ct of GAPDH})]}$ according to the Ct.

RNA oligonucleotide and cell transfection

miR-582-5p mimic, miR-582-5p inhibitor, and their cognate control RNAs were chemically synthesized by Guangzhou RiboBio Co (Guangzhou, Guangdong, China). Cells were plated in six-well plates, and 20 nm oligonucleotides were transfected into indicated HCC cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction, when the density of cells was up to 60 %.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

Cells, seeded on 96-well plates, were stained at indicated time point with 100 ul sterile MTT dye (0.5 mg/ml, Sigma) for 4 h at 37 °C, followed by removal of the culture medium and addition of 150 ul of DMSO (Sigma). The absorbance was measured at 570 nm, and 655 nm as the reference wavelength. All experiments were performed in triplicates.

Table 1 Primer sets

Purpose of gene	Primer sequences
CyclinD1	(F) 5′-TCCTCTCCAAAATGCCAGAG-3′ (R) 5′-GGCGGATTGGAAATGAACTT-3′
p21	(F) 5′-CGATGCCAACCTCCTCAACGA-3′ (R) 5′-TCGCAGACCTCCAGCATCCA-3′.
GAPDH	(F) 5′GGTGGTCTCCTCTGACTTC3′ (R) 5′CTCTTCCTCTTGTGCTCTTG3′

F forward, *R* reverse

Colony formation assay

Cells were seeded on six-well plates at 0.5×10^3 cells per well and cultured for 10 days. Colonies were fixed with 10 % formaldehyde for 5 min and then stained with 1.0 % crystal violet for 30 s.

Cell cycle assay

Cells were harvested and washed in cold PBS followed by fixation in 80 % alcohol for overnight in 4 °C. After washing in cold PBS three times, cells were resuspended in PBS solution with 2 ug/ml of bovine pancreatic RNase A (Sigma-Aldrich), then incubated at 37 °C for 30 min, followed by incubation with 20 ug/ml of propidium iodide (Sigma-Aldrich) for 20 min at room temperature; 5×10^4 cells were analyzed on a FACSCalibur cytometer (Becton Dickinson, San Jose, CA).

Western blot

Western blot assay was performed according to standard methods as described previously [9], using anti-CDK1 (1:2000), anti-AKT3 (1:2000), anti-cyclinD1 (1:2000), anti-P21 (1:2000), anti-Rb (1:3000) and anti-phosphorylated Rb antibodies (1:2000) antibodies. these antibodies were purchased from Abcam. The membranes were stripped and reprobed with an anti-β-actin antibody (1:5000, Abcam, Cambridge, MA) as a loading control.

Luciferase assay

Cells (3.5×10^4) were seeded in triplicate in 24-well plates and allowed to settle for 24 h, the psiCHECK™-2 luciferase reporter plasmids with the wild 3′ UTR sequence of CDK1 and AKT3 were transfected into the cell lines using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Luciferase activities were measured after 48 h by Dual Luciferase Reporter Assay Kit (Promega) in a luminometer. Three independent experiments were performed, and the data are presented as the mean±SD.

Statistical analysis

Results are presented as the mean±standard deviation (STDEV) for at least three repeated individual experiments for each group using SPSS version 10.0 software (SPSS, Chicago, IL. USA). Statistical differences were determined by using student's *t* test for independent samples. A *p* value of less than 0.05 was considered statistically significant.

Results

miR-582-5p expression is downregulated in HCC tissues and cell lines

According to the microRNA expression profiles of HCC tissues and adjacent normal live tissues, we found that miR-582-5p expression was lower in HCC tissues than adjacent normal live tissues through bioinformatics analysis (Supplementary Figure 1). In order to confirm these results, we used real-time PCR to determine the expression of miR-582-5p in clinic tissues of HCC patients. The results showed that miR-582-5p was downregulated in eight HCC tissues compared with matched tumor-adjacent tissues (Fig. 1a). Then, we measured miR-582-5p expression in HCC cell lines, the results also showed that, compared to THLE3, miR-582-5p was significantly downregulated in indicated HCC cell lines, including QGY-7703, Huh7, BEL-7402, HepG2, and Hep3B (Fig. 1b). Taken together, these results suggest miR-582-5p is downregulated in HCC; it may be a tumor suppressor.

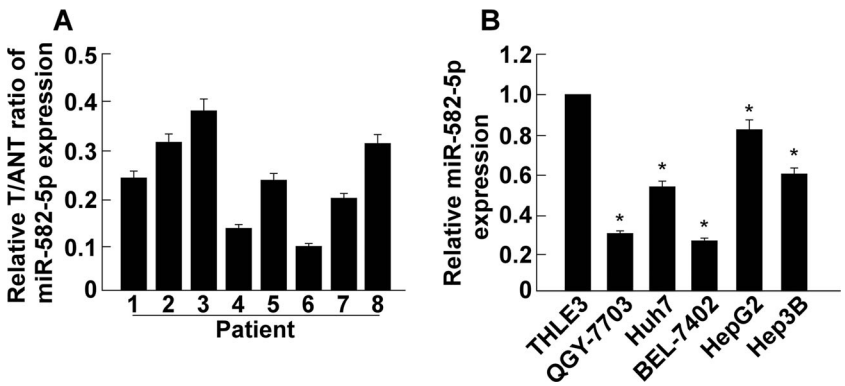

Fig. 1 miR-582-5p is downregulated in human HCC tissues and cell lines. **a** Real-time PCR analysis detected the miR-582-5p expression in eight paired primary HCC tissues (T) and their paired adjacent normal tissues (ANT). **b** Real-time PCR analysis detected the expression of miR-582-5p in THEL3-immortalized normal liver epithelial cells and HCC cell lines, including QGY-7703, Huh7, BEL-7402, HepG2, and Hep3B. **p*<0.05; *error bars* represent mean±STDEV

Ectopic expression miR-582-5p inhibits cell proliferation and induces cell cycle arrest

To investigate the biological role of miR-582-5p in the progression of HCC, we transfected has-miR-582-5p mimic into HCC cells, Huh-7 and Hep3B, to determine its effect on cellular proliferation (Fig. 2a). MTT assay found that ectopic expression of miR-582-5p reduced the proliferation rate in indicated HCC cells in contrast to negative control (NC) (Fig. 2b). Colony formation assays found that cell number also reduced significantly when miR-582-5p was overexpressed (Fig. 2c). Furthermore, we analyzed the relationship between miR-582-5p expression and cell cycle kinetic, and we found overexpression of miR-582-5p increased G0/G1 cell population from 60.09 to 69.17 % in Huh-7 cells, from 55.74 to 66.01 % in Hep3B cells, with a concomitant decrease in S cell population (Fig. 2d). These results indicated that miR-582-5p inhibits cell proliferation through regulation of G1/S transition.

Knockdown of miR-582-5p promotes cell proliferation and cell division

To further affirm that miR-582-5p regulates cell proliferation and cell cycle. We inhibited the expression of miR-582-5p by transfecting miR-582-5p inhibitor in indicated cells (Fig. 3a).

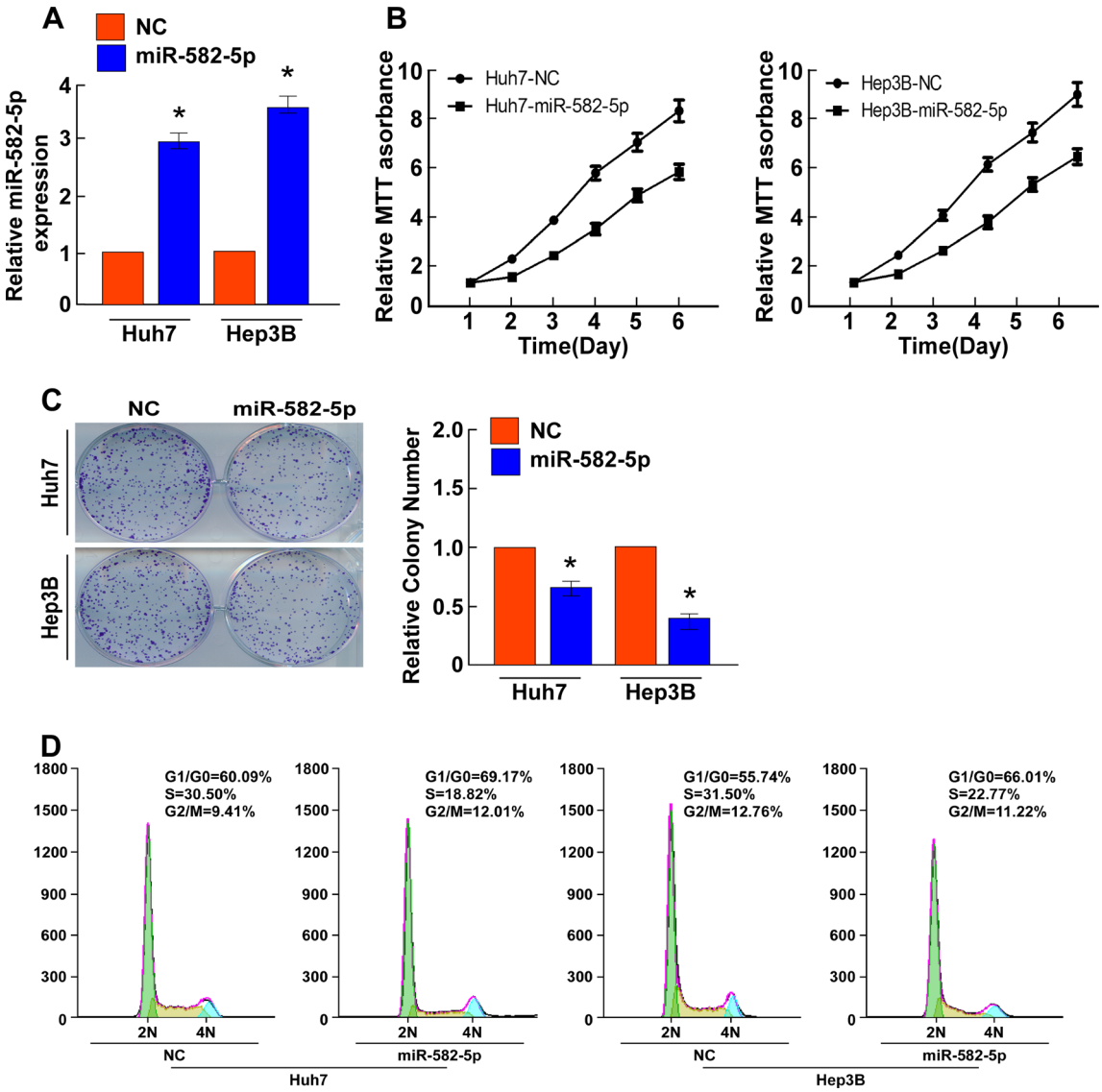

Fig. 2 Upregulation of miR-582-5p inhibits the proliferation of HCC cell lines and induces G1/G0 arrest. **a** The expression level of Huh-7 and Hep3B cells transfected with miR-582-5p mimic and negative control (NC). **b** The effects of ectopic expression of miR-582-5p on the proliferation of indicated HCC cells using MTT assays. **c** Representative micrographs (*left*) and quantification (*right*) of crystal violet stained cell colonies. **d** Cell cycle analysis of indicated HCC cell lines transfected with miR-582 mimic or NC. *$p<0.05$; *error bars* represent mean±STDEV

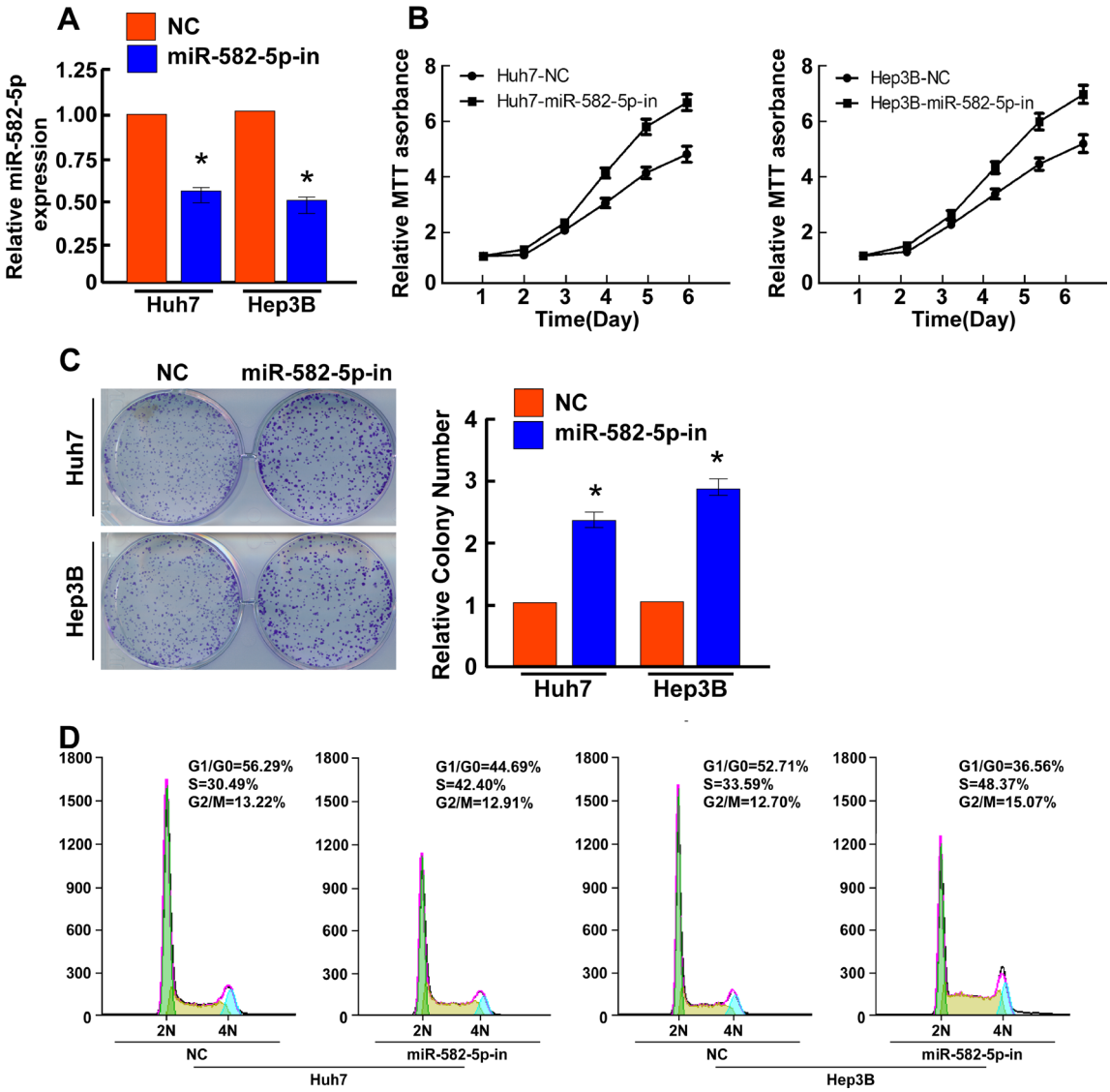

Fig. 3 Inhibition of miR-582-5p promotes the proliferation of HCC cell lines and accelerates S phase. **a** The expression level of Huh-7 and Hep3B cells transfected with miR-582-5p inhibitor and negative control (NC). **b** The effects of knockdown expression of miR-582-5p on the proliferation of indicated HCC cells using MTT assays. **c** Representative micrographs (*left*) and quantification (*right*) of crystal violet stained cell colonies. **d** Cell cycle analysis of indicated HCC cell lines transfected with miR-582 inhibitor or NC. *$p<0.05$; *error bars* represent mean±STDEV

MTT assay found inhibition of miR-582-5p increased the proliferation rate (Fig. 3b). Meanwhile, colony formation assay found that the cell number was significantly increased when miR-582-5p was inhibited (Fig. 3c). Cell cycle assay found that inhibition of miR-582-5p increased S cell population from 30.49 to 42.40 % in Huh-7 cells, from 33.59 to 48.37 % in Hep3B cells, with a concomitant increase in G0/G1 cell population (Fig. 3d). These results suggested that inhibition of miR-582-5p promotes HCC cells proliferation and accelerates cell division.

CDK1 and AKT3 are the targets of miR-582-5p

To identify candidate targets of miR-582-5p, we used TargetScan, PicTar, and miRBase to predict its target genes, and found AKT3 and CDK1 may be the target genes of miR-582-5p. miR-582-5p can bind to the 3′ UTR of AKT3 and CDK1 (Fig. 4a). Western blot analysis found that once miR-582-5p was ectopic expressed, the expression of CDK1 and AKT3 dramatically decreased. When the expression of miR-582-5p in indicated HCC cells was inhibited, the expression of CDK1 and AKT3 were upregulated (Fig. 4b). To determine whether miR-582-5p inhibits CDK1 and AKT3 by binding to the 3′ UTR of CDK1 and AKT3. We subcloned the 3′ UTR sequence which contains the binding site of miR-582-5p with CDK1 and AKT3 into the psiCHECK™-2 luciferase reporter vector, when miR-582-5p mimic were transfected into indicated HCC cells, the activity of luciferase was significantly inhibited. Inhibition of miR-582-5p significantly increased the

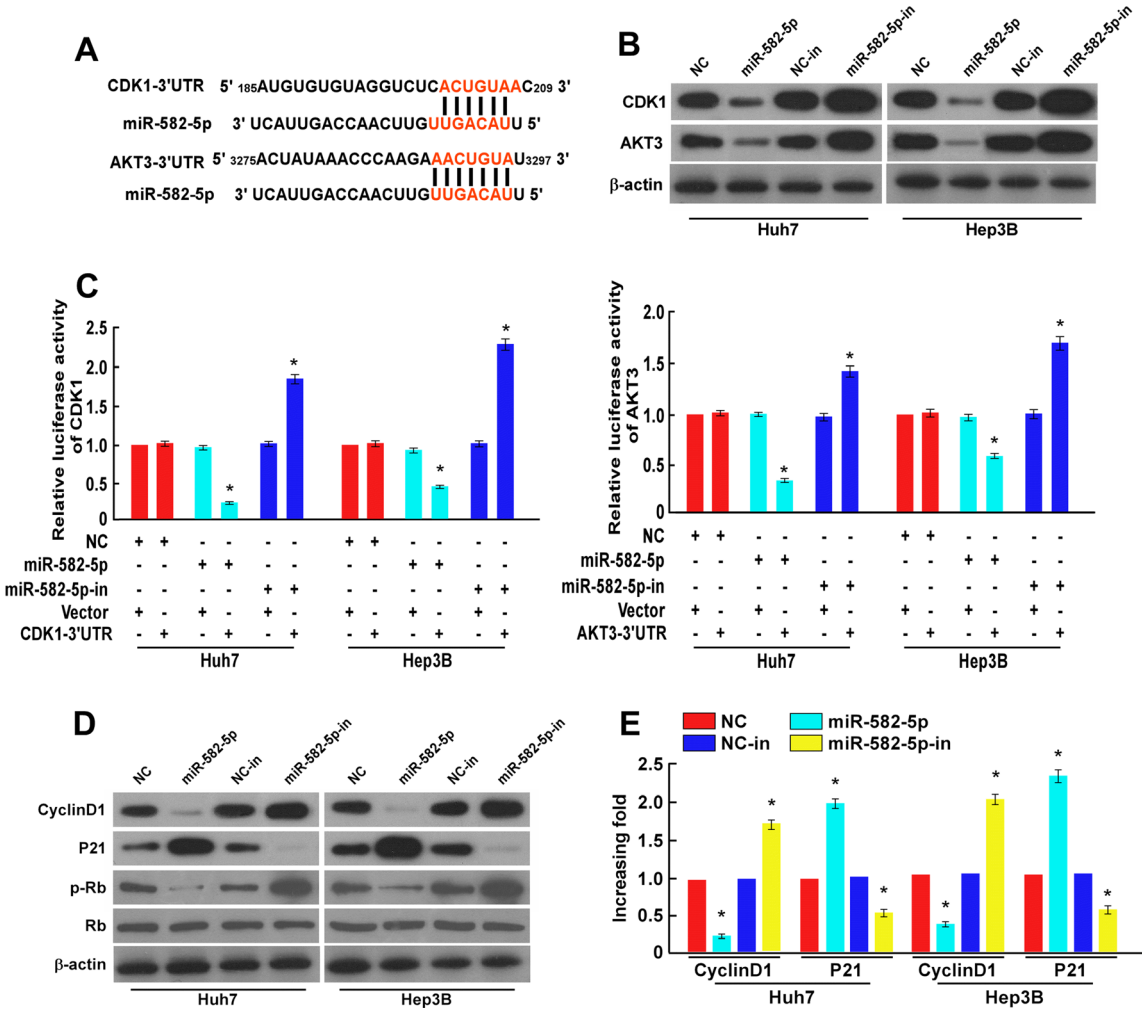

Fig. 4 miR-582-5p targets CDK1 and AKT3 by binding to its 3′ UTR and regulate the genes that regulate cell cycle progression. **a** Predicted miR-582-5p target sequences in 3′ UTR of CDK1 and AKT3. **b** Western blot analysis of the expression of CDK1 and AKT3 in indicated cells treated with miR-582-5p mimic and inhibitor. **c** Indicated HCC cells Huh-7 and Hep3B were contransfected with miR-582-5p mimic or miR-582-5p inhibitor and luciferase reporters containing the predicted miRNA target site in the 3′ UTR of CDK1 or AKT3. **d** Western blot analysis of the expression levels of cyclinD1, P21, p-Rb, and Rb in indicated HCC cells treated with miR-582-5p mimic and inhibitor. **e** Real-time PCR analysis of the expression levels of cyclinD1 and p21 in indicated HCC cells treated with miR-582-5p mimic and inhibitor

activity of luciferase in the indicated HCC cells (Fig. 4c). In order to further demonstrate CDK1 and AKT3 are the targets of miR-582-5p, we mutated the miR-582-5p-binding seed region of CDK1 and AKT3 3′-UTR, and found the activity of luciferase of CDK1 and AKT3 3′-UTR-mut did not change when miR-582-5p was overexpressed (Supplementary Figure 2). Collectively, these results showed CDK1 and AKT3 are direct targets of miR-582-5p.

Given that expression of miR-582-5p arrests cell cycle in G0/G1 phase, we asked whether miR-582-5p regulates cell cycle through cyclinD1, P21, and retinoblastoma protein (Rb). We used Western blot and real-time PCR to determine their expression. Overexpression of miR-582-5p downregulated the expression of cyclinD1 and the phosphorylation level of Rb, and the expression of P21 was increased, the expression of Rb was not changed. These results were in accord with previous studies [17]. Inhibition of miR-582-5p in the indicated cells, the expression of cyclinD1 and the phosphorylation level of Rb were upregulated, and the expression of P21 was downregulated (Fig. 4d, e). We also determined E2F1 expression, E2F1 activates genes which are essential to enter to and pass through S phase. Inhibition of miR-582-5p activated E2F1 expression, but overexpression of miR-582-5p suppressed E2F1 expression (Supplementary Figure 3). These results showed that miR-582-5p regulates HCC cell proliferation through proteins participating G1/S transition.

Discussion

In this study, we demonstrated that miR-582-5p was capable of modulating the HCC cell proliferation and cell cycle.

Further analysis found CDK1 and AKT3 were the direct targets of miR-582-5p. miR-582-5p arrested cell cycle in G0/G1 phase through the pathways regulated by CDK1 and AKT3.

CDK1, a serine/threonine kinase, is a key player in cell cycle progression. It's also significantly higher expression in HCC tissue and cells compared to non-tumor live tissues and normal liver cell lines. Inhibition of CDK1 suppresses cellular proliferation [11, 30]. CDK1 knockdown can arrest cell cycle in G2/M [26], but ectopic expression miR-582-5p causes cell cycle arrest in G0/G1, these suggest there may be some other genes or signaling pathways that are regulated by miR-582-5p. The analogy reporter also appeared. miR-302 inhibits the proliferation of endometrial cells and arrests cell cycle in G2/M phase. It directly targets cyclinD1, which is a key regulator of G0/G1 phase; these also suggest there may be some other targets of miR-30, these target genes may inhibits the expression of CDK1 and other gene regulation of progression of G2/M phase to influence cell cycle [27].

To confirm cell cycle arrests in G0/G1 phase, our analysis showed that overexpression of miR-582-5p led to inhibit the expression of cyclinD1 and the phosphorylation level of Rb, and increased the expression of cell cycle inhibitor P21. CyclinD1 is frequently overexpressed in some tumors, such as HCC and non-small cell lung cancer (NSCLC) [7, 18], cyclinD1 which belongs to D-type cyclins interacts with CDK4 or CDK6 to promote cell cycle G1 phase progression, and PI3K/AKT-GSK3β-cyclinD1 pathway regulates the expression and accumulation of cyclinD1. The serine/threonine kinase AKT is a primary PI3K effector. PI3K/AKT pathway can regulate diverse cellular process, including cell proliferation, survival, metabolism, growth and angiogenesis [12, 25]. More than 100 substrates of AKT have been identified in mammalian cells, including glycogen synthase kinase (GSK3), p21, p27 and forkhead box Os (FOXOs). Previous studies show that AKT regulates G1/S transition through inactivation of GSK3β, leading to reduction in cyclinD1 phosphorylation and increasing its protein stability [16, 21]. CyclinD1 also can as the sequestration of p21 by interacting with CDK4 or CDK6, leading to the indirect activation of CDK2, and promote restriction point progression during G1 phase. Knockdown of cyclinD1 causes P21 upregulation, and inhibits cell proliferation. Meanwhile, Rb is the substrate of cyclinD1-CDK4/CDK6 complex; this complex can phosphorylate Rb to promote G1/S transition [13, 14]. Rb plays an important role in the progression of G1 phase; it binds to E2F family proteins and inhibits their activity of transcription; so, Rb directly regulates the genes involving in cellular proliferation and DNA synthesis. Rounak Nassirpour and colleagues reported that the expression of AKT3 was upregulated in HCC cells to promote tumorigenesis; downregulation of AKT3 also inhibits HCC cell proliferation and migration, and induces apoptosis [15]. Hence, these suggest miR-582-5p arrests cell cycle in G0/G1 phase mainly through AKT pathway.

In summary, miR-582-5p is a tumor suppressor in HCC; it inhibits cell proliferation through directly inhibiting the expression of CDK1 and AKT3, and indirectly inhibiting cyclinD1. miR-582-5p may be employed as prognosis marker and therapeutic target for HCC. The effect of miR-582-5p on HCC growth in vivo and the mechanism is yet to be elucidated. The effect of anti-miR-582-5p in clinic will be determined further.

Conclusions

Collectively, we demonstrate that miR-582-5p was downregulated in HCC, and overexpression of miR-582-5p reduced cell proliferation and induced cell cycle arrest in G0/G1 phase through targeting CDK1 and AKT3. Our results also provided a novel target for HCC therapy.

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Conflicts of interest None.

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