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MicroRNA-181a-5p suppresses cell proliferation by targeting Egr1 and inhibiting Egr1/TGF-β/Smad pathway in hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is the leading cause of cancer mortality worldwide. Early growth response factor 1 (Egr1) plays a crucial role in cancer progression. However, its precise role in HCC has not been clear. Here, we identified the aggravating role of Egr1 in cell proliferation of HCC firstly. The expression of Egr1 was significantly increased in HCC tissues. Functionally, overexpression of Egr1 enhanced, whereas silenced Egr1 expression attenuated HCC cells proliferation in vitro. Mechanistically, up-regulated Egr1 induced cell proliferation through activating Transforming growth factor (TGF)-β1/Smad signaling pathway concomitantly with upregulation of p-Smad2 and p-Smad3. Secondly, miR-181a-5p was down-regulated in clinical HCC specimens and its expression was inversely correlated with Egr1 expression. Functionally, overexpression of miR-181a-5p inhibited, whereas decreased expression of miR-181a-5p promoted HCC cells proliferation in vitro. Furthermore, we demonstrated that miR-181a-5p overexpression directly suppressed Egr1, resulting in a down-regulated TGF-β1/Smad pathway. Besides, the silenced Egr1 expression could rescue the enhanced cell proliferation induced by miR-181a-5p inhibitor. Thus, we concluded that miR-181a-5p is a negative regulator of Egr1 that can suppress tumor proliferation in HCC through targeting Egr1/TGF-β1/Smad pathway, which may be a potential therapeutic approach of HCC.

1. Introduction

Hepatocellular carcinoma (HCC), accounting for 70% - 90% of the primary liver cancers, is the sixth most common malignancy worldwide, and ranked as the second deadliest human cancers (Torre et al., 2015; Finn, 2012). Despite of the therapeutic advances such as surgical resection, liver transplantation, and adjuvant treatment, most patients with HCC present with advanced stage disease and are not eligible for these therapies. The five-year survival for patients with HCC generally did not improve in the past few decades (Coleman, 2014). Uncontrolled cell proliferation and metastasis contribute to the major obstacles to the clinical therapy and are responsible for the high mortality (Allemani et al., 2015;Reeves and Aisen, 2015). Hence it is important to discover the molecular pathways underlying tumor progression in order to identify biomarkers for early detection and develop new therapeutic strategies of HCC.

Early growth response factor 1 (Egr1) as a zinc finger transcription factor, is rapidly induced in response to a variety of extracellular and environmental signals such as growth factors, cytokines, vascular injury, and hypoxia (Kaufmann and Thiel, 2002; Rössler and Thiel, 2009;Lohoff et al., 2010). Aberrant expression of Egr1 is related to many human diseases such as atherosclerosis, inflammation, ischemic injury and cancer (Bhattacharyya et al., 2013). Besides, increasing studies found that the function of Egr1 in cancer is complex. Egr1 expression has been shown to be variable among tumor cells of different tissue origin. Some studies proved that Egr1 is associated with tumor progression in some cancers such as gastric and prostate cancer (Kang et al., 2013; Choi et al., 2016). However, other studies found that Egr1 act as tumor suppressor in some cancers such as esophageal squamous cell carcinoma, non-small-cell lung cancer, and breast cancer (Gao et al., 2017; Sun et al., 2017; Parra and Ferreira, 2010). Moreover, abnormal Egr1 expression has been proved to be related to HCC. Some

Abbreviations: CCK-8, Cell counting kit-8; Egr1, early growth response factor 1; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; miR-181a-5p, microRNA-181a-5p; qRT-PCR, quantitative real-time polymerase chain reaction; siEgr1, small interfering RNA targeting Egr1; TGF-β1, transforming growth factor β1; UTR, untranslated region

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studies found that Egr1 can accelerate HCC progression (Ma et al., 2016; Lu et al., 2012). Conversely, other studies proved that Egr1 act as a tumor suppressor in HCC (Hao et al., 2002; Wang et al., 2016). The specific role of Egr1 in HCC remains unclear.

Transforming growth factor (TGF)-βsignal pathway can interfere with hepatocyte proliferation, but it also can promote HCC progression (Yoshida et al., 2018). TGF-β has been reported to act as both oncogene and suppressor in tumors (Bellam and Pasche, 2010). As disease progresses to malignancy, HCC selectively reduces the suppressive activity and increase the oncogenic activity of TGF-β (Matsuzaki, 2011). Furthermore, the Smad family plays a crucial role in the carcinogenesis of HCC (Yoshida et al., 2018). A previous study showed that transcription factor Egr1 can directly regulate the expression of TGF-β in prostate cancer (Baron et al., 2006). However, the relationship between Egr1 and TGF-β signal pathway in HCC has not been elucidated so far.

MicroRNAs (miRNAs) are a group of highly conserved and single-strand small non-coding RNAs that play important roles in physiological and pathological processes by changing the expression and translation of their target mRNA genes (He and Hannon, 2004). As the post-transcriptional regulator, miRNAs play important roles in tumor progression (Weis and Cheresh, 2011; Negrini et al., 2007; Peng and Croce, 2016). A miRNA can function as either an oncogene or a suppressor of tumor depending on its target gene (Zhang et al., 2007). Dysfunction of miRNAs is also associated with HCC tumorigenesis and progression. MiR-181a-5p belongs to miR-181 s family including miR-181a, b, c and d. This family has recently been recognized to play a central role in malignant transformation (Li et al., 2015; Taylor et al., 2013). Several studies have indicated that miR-181 s serve as tumor promoting genes, but the function of miR-181a-5p is tumor-type specific. MiR-181a-5p has been suggested as a tumor suppressor in glioma and non-small cell lung cancer (Shi et al., 2008; Cao et al., 2017). However, overexpression of miR-181a-5p was also reported to promote ovarian, breast, gastric, colorectal cancer and leukemia progression through different mechanisms (Parikh et al., 2014; Mi et al., 2017; Ji et al., 2014; Verduci et al., 2015). Aberrant expression of miR-181a-5p in HCC has been determined. Some studies addressed overexpression and tumorigenesis activity of miR-181a-5p in HCC (Brockhausen et al., 2015; Zou et al., 2015). Conversely, Korhan et al. (2014) reported that down-regulated miR-181a-5p served as a suppressor in HCC. Nevertheless, the role and the molecular mechanisms underlying miR-181a-5p-mediated in HCC are not entirely clear. Interestingly, Egr1 was identified to be a miR-181a-5p target gene in leukemia (Verduci et al., 2015).

In this study, we demonstrated that miR-181a-5p suppressed and Egr1 promoted the cell proliferation in HCC. Egr1 was identified as a direct target gene of miR-181a-5p in HCC cells. We further demonstrated that Egr1 accelerate HCC proliferation in vitro by up-regulating TGF-β1 to subsequently activate the TGF-β1/Smad pathway. Taken together, our findings illustrate that miR-181a-5p/Egr1/TGF-β1 pathway play a crucial role in HCC progression.

2. Materials and Methods

2.1. Patient samples and cell lines

Twenty pairs of fresh HCC and adjacent non-tumorous liver samples were obtained from the patients who received surgery at the Second Affiliated Hospital of Jinan University from 2015 to 2016. All fresh tissues were immediately frozen in liquid nitrogen and store at −80 °C or fixed in 10% formalin for paraffin embedding. All patients were histologically diagnosed. None of the patients received radiotherapy or chemotherapy before surgery. Sample obtaining and usage in the present study were approved by the Ethics Committee of the Second Affiliated Hospital of Jinan University. The clinical characteristics are presented in Table 1. Human HCC cell lines HepG2 and Hep3B were purchased from the Cell Bank of Type Culture Collection (Chinese Academy of Sciences, Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) (Gibco) and incubated at 37 °C in a humidified atmosphere containing 5% CO2. The mediums were changed every 48 h. All cells were grown to 60-70% confluence and serum-deprived for 24 h before performing experiment.

Table 1
Clinical characteristics of HCC patients (n = 20).

Characteristics	Number of cases
Age (years)	
≤ 65	14
> 65	6
Sex	
Male	17
Female	3
Type of hepatitis	
HBV	13
HCV	2
HBV + HCV	1
Tumor size (cm)	
≤ 3	11
3-5	6
> 5	3
Tumor number	
Solitary	18
Multiple	2
Liver cirrhosis	
with	15
without	5
TNM stage	
I	9
II	7
III	3
IV	1

2.2. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from cultured cells or tissues using Trizol reagent (Invitrogen) according to the manufacturer's protocols. For mRNA, cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen, MA, USA). For miRNA, reverse transcription was performed using miRcute miRNA cDNA first strand synthesis Kit (Tiangen, Beijing, China). RNA expression levels were measured using the above reverse transcription products with SYBR Green qPCR Kit (Takara, Dalian, China) and miRcute miRNA qPCR Detection Kit (Tiangen, Beijing, China) respectively on Roche LightCycler 480II system (Roche, Basle, Switzerland). Data were presented as relative quantification to β-actin or U6 based on calculation of $2^{-\Delta\Delta Ct}$ or as ΔCt. All experiments were performed in triplicate. The primers used are presented in Table 2.

Table 2
Sequences of primers for qRT-PCR in this study.

Genes	Sequences	
Egr1	Sense:	5'-CACCTGACCGCAGAGTCTTT-3'
	Antisense:	5'-CTGACCAAGCTGAAGAGGGG-3'
TGF-β1	Sense:	5'-CTTTGTACAACAGCACCCGC-3'
	Antisense:	5'-CGGGTGACTTCTTTGGCGTA-3'
β-actin	Sense:	5'-GCGAGTACAACCTTCTTGCAG-3'
	Antisense:	5'-GCCTTGCACATGCCGGA-3'
miR-181a-5p	Sense:	5'-GCGTCCAACATTCAACGCTGTCGGTGAGT-3'
	Antisense:	universal reverse primer (Tiangen, Beijing, China)
U6	Sense:	5'-CTCGCTTCGGCAGCACA-3'
	Antisense:	universal reverse primer (Tiangen, Beijing, China)

2.3. Western blot analysis

RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Shanghai, China) freshly mixed with protease and phosphatase inhibitors were used to isolate proteins from tissues or cells. BCA assay (Dingguo, Beijing, China) was performed according to the manufacturer's protocols to determine the protein concentration. Identical quantities of proteins were separated on sodium dodecylsulfate-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene difluoride membranes (Merck Millipore, MA, USA). Membranes were blocked with 5% nonfat in 0.1% tris-buffered saline with Tween-20. The membranes were incubated with primary antibodies specific for Egr1 (1:500, Santa Cruz Biotechnology), TGF-β1 (1:500, Santa Cruz Biotechnology), phospho-SMAD2 (1:1000, Sigma), SMAD2 (1:1000, Sigma), phospho-SMAD3 (1:1000, Sigma), SMAD3 (1:1000, Sigma) and β-actin (1:200, Abcam) at 4℃ overnight, the blots were incubated with peroxidase-conjugated secondary antibodies at room temperature for 1 h. Immune complexes were visualized using the Odyssey infrared imaging system (LI-COR, Lincoln, NE) and quantified by ImageJ software.

2.4. Overexpression or suppression of miR-181a-5p

Synthetic miR-181a-5p mimics and inhibitor, and the appropriate negative controls were purchased from Ribobio (Guangzhou, China). HCC cells were cultured at approximately 50-60% confluence in 12-well plates and transfected with RNA oligoribonucleotides using Lipofectamine™ 3000 reagent (Invitrogen, USA) following the manufacturer's instructions at a final concentration of 50-100 nM. The transfected cells were harvested for the following experiments after 48 h.

2.5. Overexpression or suppression of Egr1

The pENTER-Egr1 plasmid and pENTER as the empty vector were purchased from Vigene Biosciences (Shandong, China). Small interfering RNA targeting Egr1 (siEgr1) was synthesized by Ribobio (Guangzhou, China), and a scrambled siRNA (Ribobio) was used as the negative control. Cells were cultured at approximately 50-60% confluence in 12-well plates and transfected with 2 μg plasmid or 2.5 μl siRNA. All transfections were performed using Lipofectamine™ 3000 reagent (Invitrogen, USA) following the manufacturer's instructions. Cells were harvested 48 h after transfection.

2.6. Luciferase activity assay

PCR was performed to amplify the full-length 3'UTR of Egr1 using the following primers: (forward) 5'-GGCGGCTCGAGGGACAATTGAAATTTGCTAAAG-3' and (reverse) 5'-AATGCGGCCGCTCCCCTAACCCAGGAAAC-3'. The mutant 3'UTR fragment containing a substitution of six nucleotides (GAAUGU to CTTACA) within the core miR-181a-5p binding site was synthesized using the following primers: (forward) 5'-AGGAGTTGCTTACATGTAGTTACCTACTGAGT-3' and (reverse) 5'-TAACTACATGTAAGCAACTCCTGAATATATTT-3'. The amplified Egr1 3'UTR fragment and mutant fragment were inserted into the pmiR-RB-REPORTTM vector and control vector (Ribobio, Guangzhou, China) respectively, using the XhoI and NotI sites. Furthermore, HepG2 and Hep3B cells were plated into 96-well clusters and co-transfected with 100 ng constructs with 50 nM miR-181a-5p mimics or miRNA negative control or with 100 nM miR-181a-5p inhibitor or miRNA inhibitor negative control, respectively. At 48 h after transfection, the Dual-luciferase reporter system (Promega, Madison, WI, USA) was used to determine the luciferase activity. The data were expressed as the quotient of Renilla/Firefly luciferase activities. The experiments were conducted in triplicate.

2.7. Cell counting kit-8 assay

The proliferation of HCC cells were measured by CCK-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's protocols. The absorbance was measured at 450 nm. The proliferation curves were plotted using the absorbance at each time point.

2.8. Statistical analysis

All values were obtained from three independent experiments and were presented as mean ± SD. Statistical analysis were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Quantitative data between groups were compared using the Student *t*-test. Spearman's correlation coefficient was used to calculate the correlations between two groups. The statistical significant level was set at $P < 0.05$.

3. Results

3.1. The expressions of Egr1 and TGF-β1 are elevated in HCC tissues

To determine the role of Egr1 and TGF-β1 in HCC, we first analyzed the Egr1 and TGF-β1 protein expressions in clinical specimens from the human protein atlas (http://www.proteinatlas.org). We found that Egr1 and TGF-β1 had the positive strong expression in HCCs and negative weak expression in normal liver tissues (Fig. 1A). Furthermore, we detected the expressions of Egr1 and TGF-β1 in 20 paired HCC and tumor-adjacent tissues using qRT-PCR and western blot analysis. The mRNA and protein levels of Egr1 and TGF-β1 were dramatically increased in HCC tissues (Fig. 1B, **C, D and E**). These findings indicated that aberrant expressions of Egr1 and TGF-β1 may have a potential role in HCC progression.

3.2. Egr1 promotes TGF-β1-induced proliferation in HCC cells

To determine whether Egr1 promote the HCC cells proliferation through TGF-β1 pathway, we treated HepG2 and Hep3B cells with pENTER-Egr1 plasmid and small interfering RNA targeting Egr1 (siEgr1). It was shown that up-regulated Egr1 significantly increased the expression of TGF-β1 by qRT-PCR and western blot analysis (Fig. 2A, **B and C**). The cell growth curves measured by CCK-8 assays demonstrated that overexpression of Egr1 enhanced cell proliferation in HepG2 and Hep3B cells (Fig. 2D**).** In contrast, silenced Egr1 expression resulted in a decreased expression of TGF-β1 (Fig. 2E, **F and G**). After treating with siEgr1, the proliferative ability of HepG2 and Hep3B cells were significantly reduced (Fig. 2H**).** These results suggested that Egr1 enhances the proliferative ability of HCC cells through promoting TGF-β1 signaling pathway.

3.3. Elevated Egr1 expression activate TGF-β1/Smad signaling pathway in HCC cells

To analyze the mechanism underlying Egr1/TGF-β1-mediate proliferation of HCC cells, we performed western blot analysis to measure several crucial molecules involved in TGF-β1/Smad signaling pathway. We found that overexpression of Egr1 caused a significant increase in the phosphorylation levels of Smad2 and Smad3 in both HepG2 and Hep3B cells (Fig. 3A, **B).** By contrast, inhibition of Egr1 expression obviously reduced the levels of phosphor-Smad2 (p-Smad2) and phosphor-Smad3 (p-Smad3) in both HepG2 and Hep3B cells (Fig. 3C, **D).**

3.4. MiR-181a-5p directly targets Egr1 3'UTR

We used public available algorithms (PicTar, http://pictar.mdc-belin.de/; TargetScan, www.targetscan.org/; and miRanda, www.microrna.org) to predict which miRNAs could target Egr1 3'UTR.

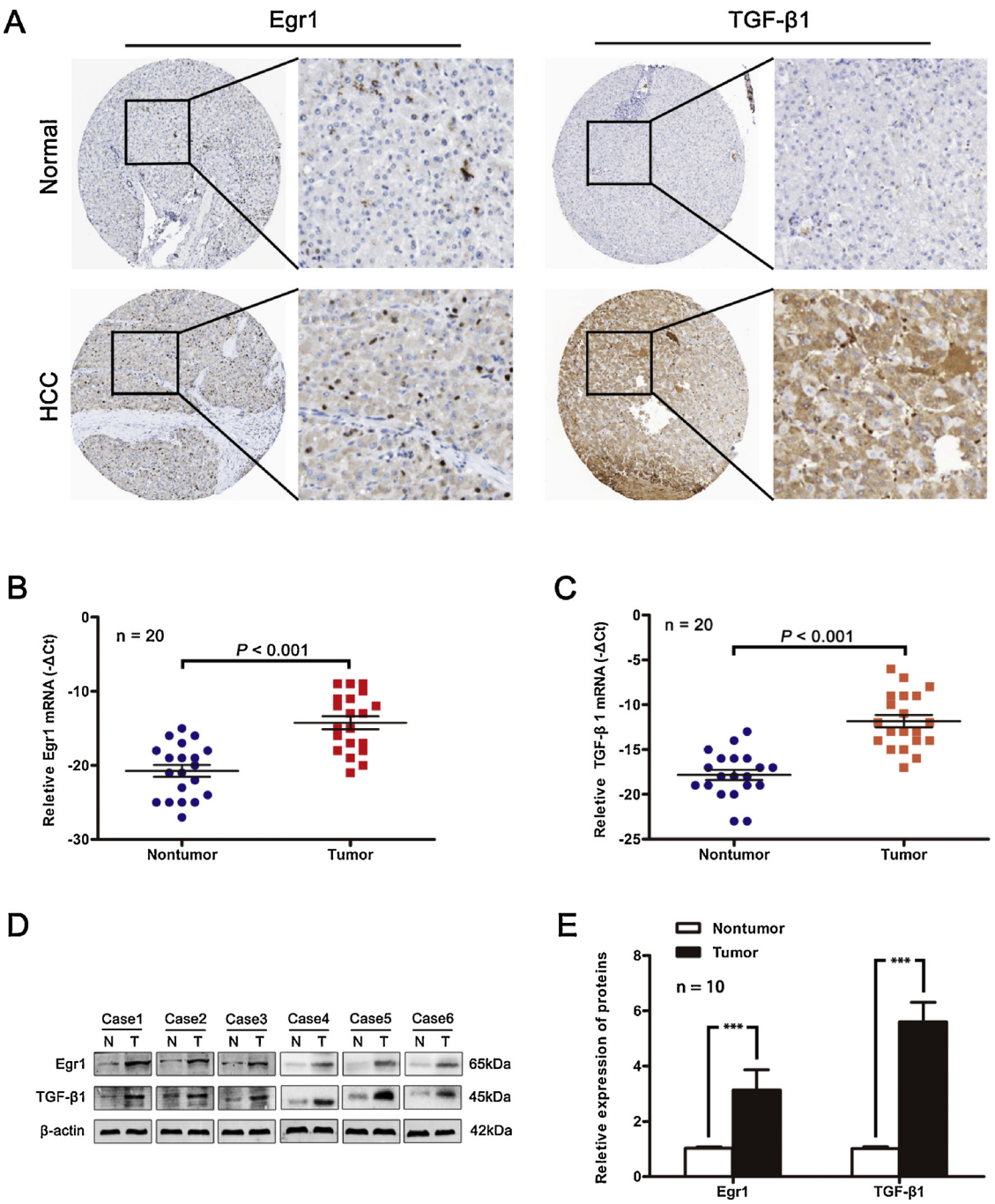

Fig. 1. Elevated expressions of Egr1 and TGF-β1 in human HCC tissues. (A) Immunohistochemical staining of Egr1 and TGF-β1 in normal liver tissues and HCC specimens. Images were taken from the Human Protein Atlas (http://www.proteinatlas.org) online database. **(B, C)** The mRNA levels of Egr1 and TGF-β1 in 20 paired fresh HCC specimens were detected by qRT-PCR. **(D)** The protein expressions of Egr1 and TGF-β1 in above 20 pairs of sample tissues were evaluated by western blot. **(E)** The relative protein densities were measured and are shown. The data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. N: nontumor; T: tumor.

MiR-181a-5p and other 8 miRNAs were identified to have potential target sites in 3'UTR of Egr1 mRNA. Furthermore, qRT-PCR analysis was performed to determine the expressions of these miRNAs in above 20 paired HCC specimens. Among these, miR-181a-5p was proved to down-regulate in HCC tissues (Fig. 4A). One conserved target of miR-181a-5p in the 3'UTR of Egr1 mRNA was predicted by bioinformatics prediction tools and then we constructed dual-luciferase reporters containing normal or mutant binding site for miR-181a-5p (Fig. 4B). In the meanwhile, a significant inverse correlation between miR-181a-5p expression and Egr1 mRNA expression in 20 paired HCC tissues was confirmed with Pearson correlation analysis, R = 0.435, $P = 0.0016$ (Fig. 4C). To further confirm Egr1 mRNA as a direct target of miR-181a-5p, the 3'UTR of Egr1 mRNA and it's mutant type containing the putative miR-181a-5p binding sites were cloned downstream of the luciferase open reading frame. These reporter constructs were co-transfected into either HepG2 cells with miR-181a-5p mimics or Hep3B cells with miR-181a-5p inhibitor. The results demonstrated that overexpression of miR-181a-5p in HepG2 cells dramatically decrease luciferase activity of wild-type (wt) construct of Egr1 3'UTR but not the mutant (Fig. 4D). In contrast, silenced miR-181a-5p expression in Hep3B cells remarkably increased the luciferase activity of wt construct of Egr1 3'UTR but not the mutant (Fig. 4E). Taken together, these data

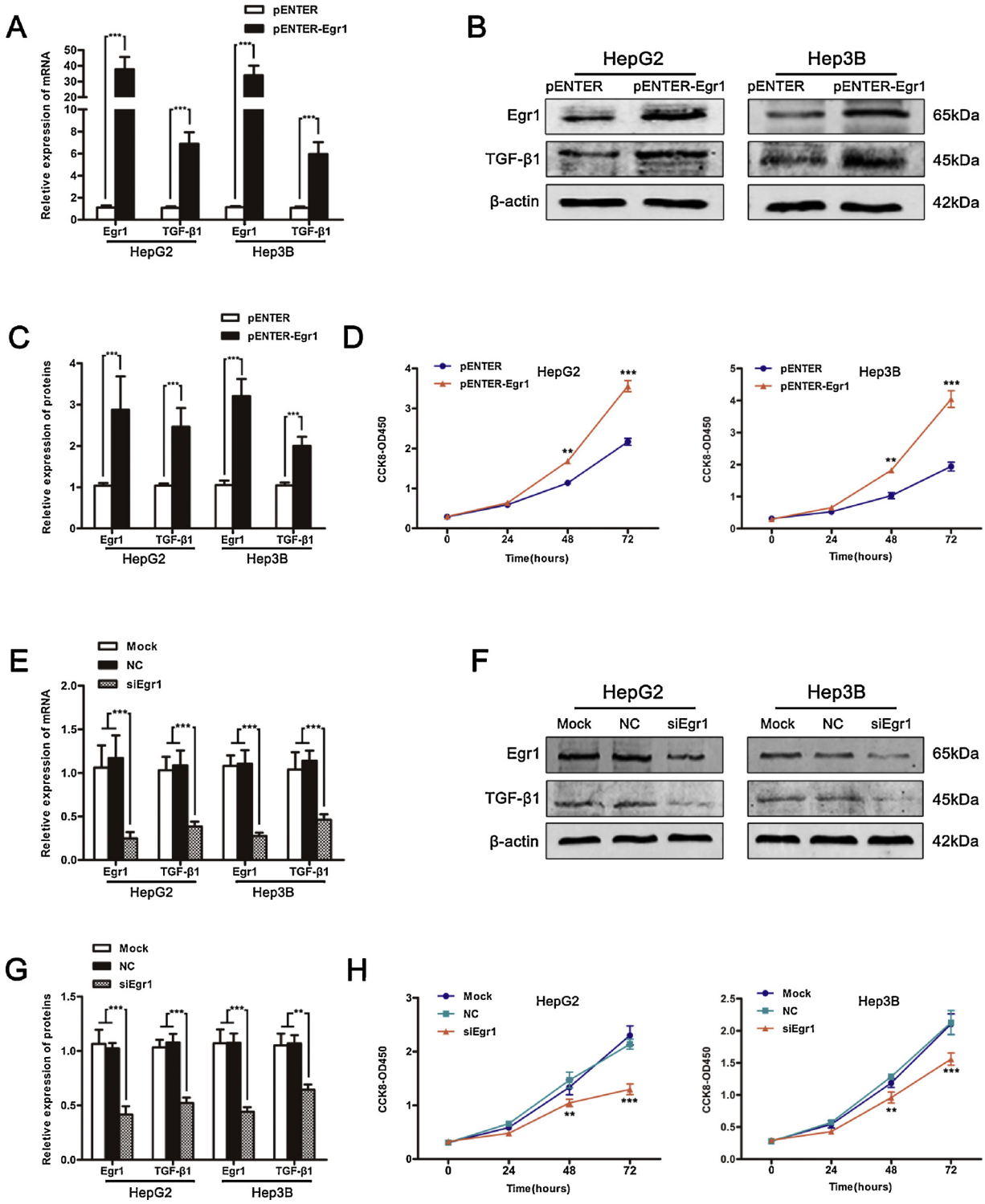

Fig. 2. Ectopic expression of Egr1 promotes TGF-β1-induced HCC cell proliferation in vitro. (A-C) HepG2 and Hep3B cells were transfected with pENTER or pENTER-Egr1 plasmids, and the expressions of Egr1 and TGF-β1 were determined by **(A)** qRT-PCR and **(B)** western blot with **(C)** quantitative analysis. **(D)** CCK-8 assays showed cell viability of HepG2 and Hep3B cells with overexpressed Egr1. **(E-G)** Small interfering RNA targeting Egr1 (siEgr1) decreased the expressions of Egr1 and TGF-β1 by **(E)** qRT-PCR and **(F)** western blot with **(G)** quantitative analysis. **(H)** Cell viability was assessed by CCK-8 assays in HepG2 and Hep3B cells with silenced Egr1 expression. The experiments were performed in triplicate; the data are expressed as mean ± SD. NC: negative control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

indicated that Egr1 could be a direct downstream target of miR-181a-5p in HCC cells.

3.5. MiR-181a-5p alleviates Egr1-induced cell proliferation in HCC

To further elucidate whether miR-181a-5p is involved in Egr1-mediated cell proliferation in HCC, we firstly transfected HepG2 and Hep3B cells with miR-181a-5p mimics and then decreased miR-181a-5p

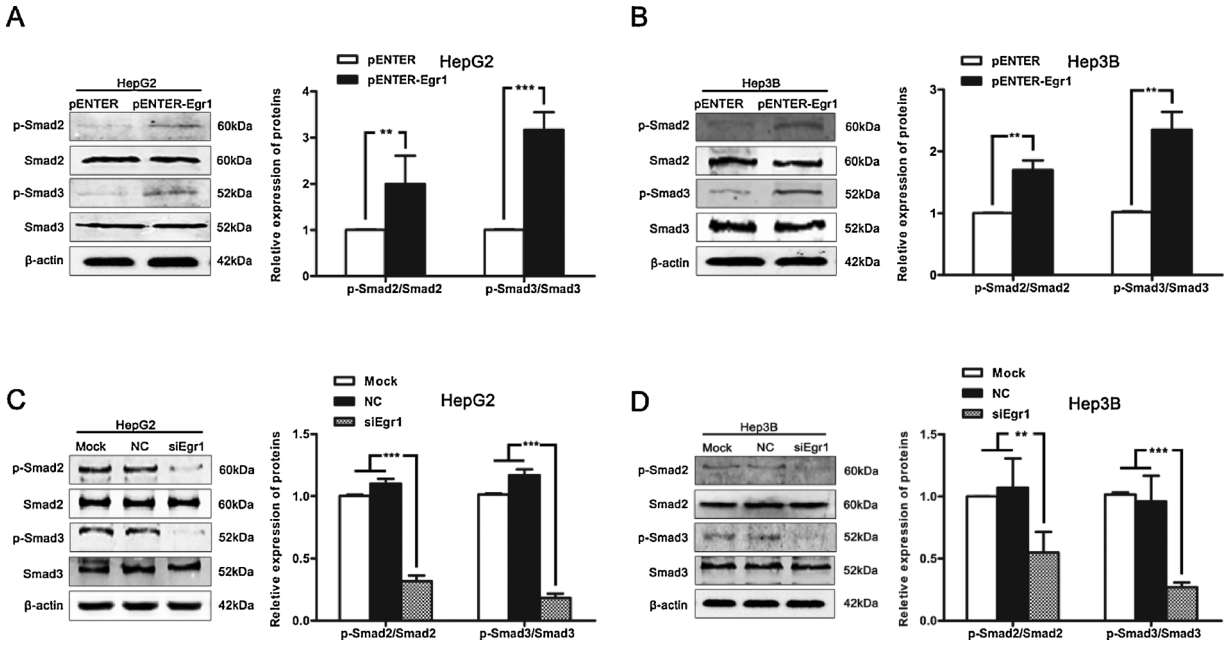

Fig. 3. Upregulation of Egr1 activates TGF-β/Smad signaling pathways. The levels of Smad2, Smad3, p-Smad2, p-Smad3 were detected by western blot analysis in HepG2 and Hep3B cells transfected with pENTER-Egr1 plasmids **(A, B)** or small interfering RNA targeting Egr1 (siEgr1) **(C, D)**. Results are presented as mean ± SD of three independent experiments. NC: negative control. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

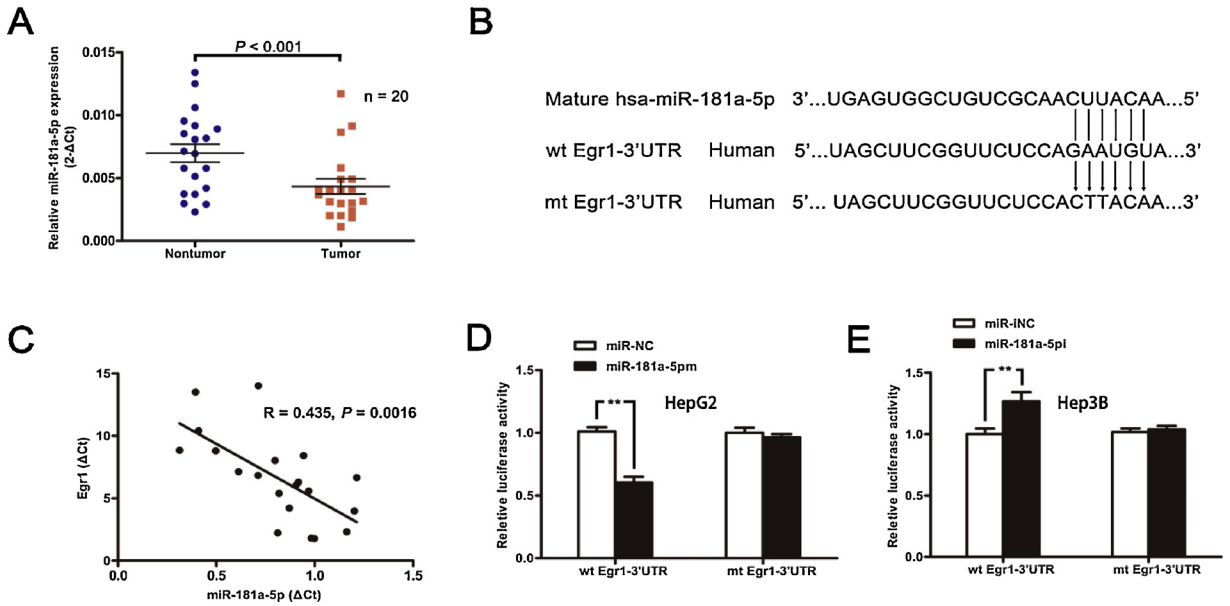

Fig. 4. Egr1 is a direct downstream target of miR-181a-5p. (A) The relative expression of miR-181a-5p was measured by qRT-PCR in 20 paired fresh HCC specimens. **(B)** miR-181a-5p and its putative binding sequence in the 3'UTR of Egr1. The mutant Egr1 3'UTR binding site was generated in the complementary site for the seed region of miR-181a-5p. **(C)** The correlation between Egr1 mRNA and miR-181a-5p was determined in 20 paired fresh HCC samples. The ΔCt values were subjected to Pearson correlation analysis.
(D) Overexpression of miR-181a-5p significantly decreased the Luciferase activity of HepG2 cells carried wild type 3'-UTR of Egr1. **(E)** Inhibition of miR-181a-5p significantly increased the Luciferase activity of Hep3B cells carried wild type 3'-UTR of Egr1. Results are presented as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. wt: wild type; mt: mutant type; miR-iNC: miRNA inhibitor negative control; miR-181a-5pi: miR-181a-5p inhibitor; miR-NC: miRNA negative control; miR-181a-5pm: miR-181a-5p mimics.

expression in HepG2 and Hep3B cells through transfecting inhibitor. It was shown that the elevated expression of miR-181a-5p significantly down-regulated the mRNA and protein levels of Egr1 and TGF-β1 in both HepG2 and Hep3B cells (Fig. 5**A, B, C and D)**. Next, we investigated the effect of miR-181a-5p on the viability of HCC cells using CCK-8 assays. The results revealed that overexpression of miR-181a-5p remarkably suppressed the viability of HepG2 and Hep3B cells (Fig. 5**E, F)**. Conversely, both HepG2 and Hep3B cells that were transfected with inhibitor showed noticeably increased expressions of Egr1 and TGF-β1 (Fig. 5**G, H, I and J)**. The CCK-8 assays demonstrated that suppression of miR-181a-5p resulted in stimulated cell growth in HepG2 and Hep3B cells (Fig. 5**K, L)**. These results revealed that miR-181a-5p attenuates Egr1-mediated cells proliferation in HCC.

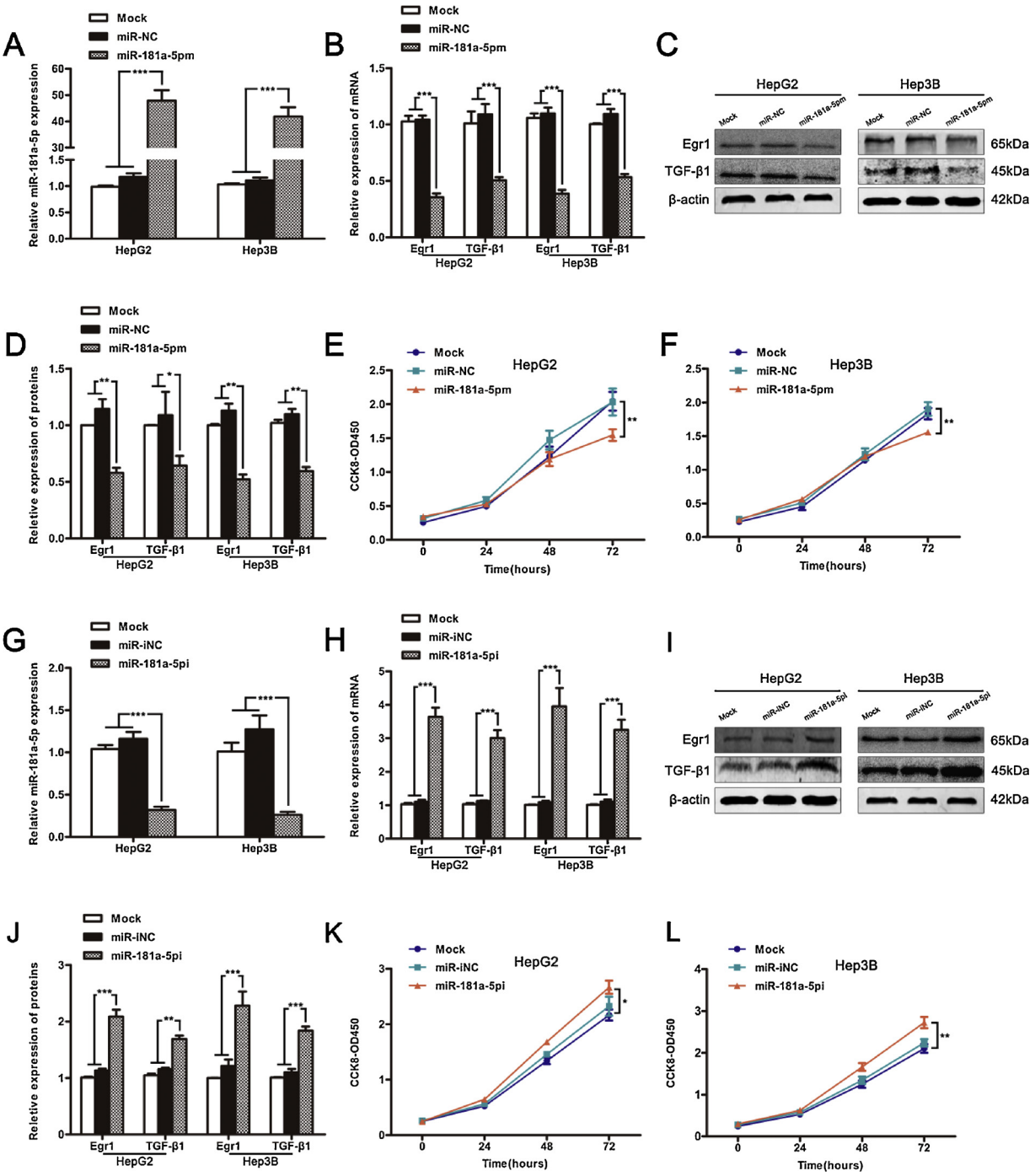

Fig. 5. MiR-181a-5p prevents Egr1-induced proliferation in HCC cells. (A) The relative expression of miR-181a-5p was determined by qRT-PCR analysis in HepG2 and Hep3B cells transfected with miR-181a-5p mimics. **(B-D)** Overexpression of miR-181a-5p decreased the mRNA and protein expressions of Egr1 and TGF-β1 in HepG2 and Hep3B cells by **(B)** qRT-PCR and **(C)** western blot with **(D)** quantitative analysis. **(E, F)** Cell viability was detected daily by CCK-8 assay for 3 days in HepG2 and Hep3B cells with overexpression of miR-181a-5p. **(G)** MiR-181a-5p mRNA expression was measured by qRT-PCR analysis in HepG2 and Hep3B cells transfected with miR-181a-5p inhibitor. **(H-J)** Inhibition of miR-181a-5p increased the mRNA and protein levels of Egr1 and TGF-β1 by **(H)** qRT-PCR and **(I)** western blot with **(J)** quantitative analysis. **(K, L)** Cell viability was assessed by CCK-8 assays daily for 3 days in HepG2 and Hep3B cells that were transfected with miR-181a-5p inhibitor. Data shown are the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. miR-NC: miRNA negative control; miR-181a-5pm: miR-181a-5p mimics; miR-iNC: miRNA inhibitor negative control; miR-181a-5pi: miR-181a-5p inhibitor.

3.6. Requirement of Egr1 for the antagonism effect of miR-181a-5p on cell proliferation in HCC

To determine whether the effect of miR-181a-5p on proliferation of HCC cells depends on Egr1, we treated HepG2 and Hep3B cells with siEgr1 first and then with miR-181a-5p inhibitor. QRT-PCR and western analysis were performed to detected the expressions of Egr1 and TGF-β1. CCK-8 assays were used to measure the cell viability of transfected cells. The results showed that the decreased expression of Egr1 and TGF-β1 were restored when Egr1-silenced cells were treated with miR-181a-5p inhibitor (Fig. 6A**, B and C)**. In the meanwhile, the suppressed cell viability induced by siEgr1 was rescued by miR-181a-5p inhibitor in HepG2 and Hep3B cells (Fig. 6D**)**. Overall, these data suggested that miR-181a-5p attenuates proliferation by directly targeting Egr1 in HCC

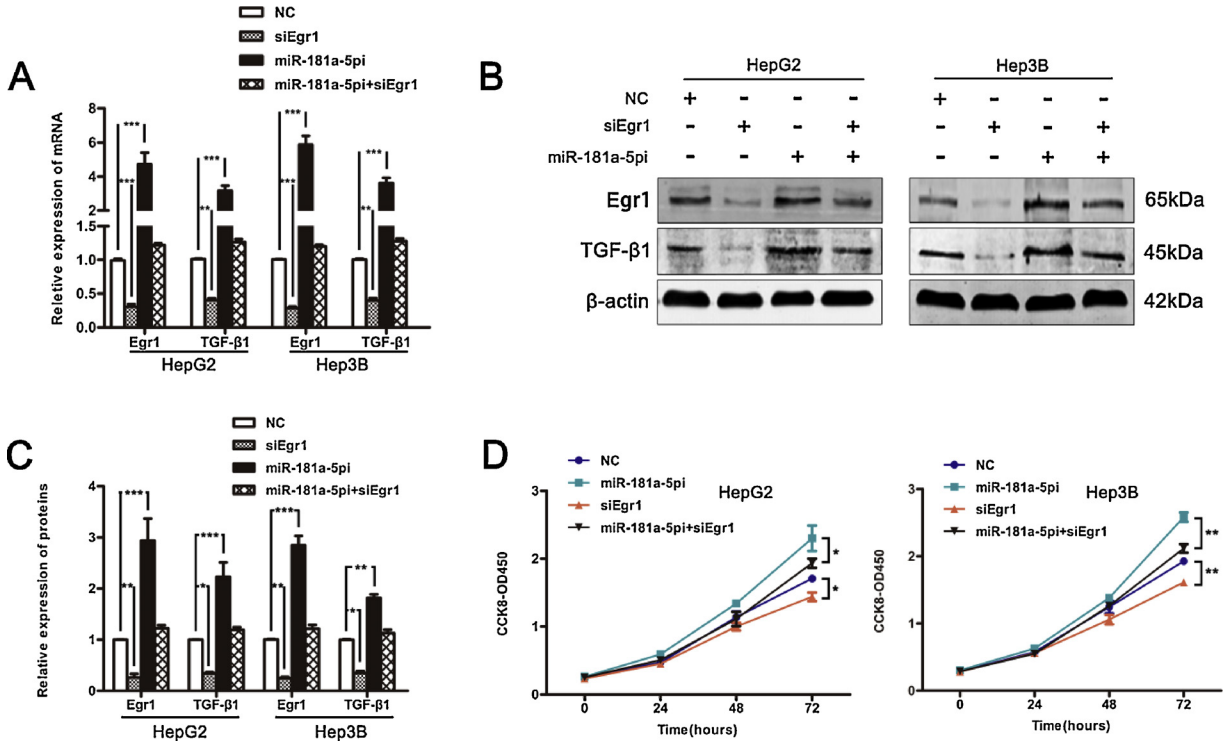

Fig. 6. MiR-181a-5p antagonism effect on the proliferation of HCC cells through Egr1. (A) MiR-181a-5p inhibitor restored the silencing effect of siEgr1 on the expressions of Egr1 and TGF-β1 by qRT-PCR analysis and **(B)** western blot with **(C)** quantitative analysis in HepG2 and Hep3B cells. **(D)** Proliferation of HepG2 and Hep3B cells was measured by CCK-8 assays after treatment of siEgr1, miR-181a-5p inhibitor, or siEgr1 with miR-181a-5p. The experiments were performed in triplicate. The results are presented as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. NC: negative control; miR-181a-5pi: miR-181a-5p inhibitor.

cells.

4. Discussion

HCC is one of the most common cancers worldwide with high prevalence and mortality. Efforts to discover the mechanisms of the unlimited growth of HCC cells have been increasing made over the last decades. Several biomarkers have been found and applied to the managements of HCC (Chai et al., 2016; Song et al., 2015). Here, we demonstrated the significant role of miR-181a-5p/Egr1/TGF-β1 pathway in HCC. We elucidated that Egr1 promotes TGF-β1/Smad pathway induced HCC progression through enhance tumor cells proliferation. Moreover, we identified miR-181a-5p, as a direct upstream regulator of Egr1, antagonized HCC progression through suppressing the expression of Egr1.

Aberrant expression of Egr1 has been reported in human cancers. Multiple studies have shown that Egr1 possess two opposing roles, i.e. that of a tumor suppressor or that of an oncogene (Kang et al., 2013; Choi et al., 2016; Sun et al., 2017; Parra and Ferreira, 2010). The role of Egr1 in HCC is a controversial topic. Several studies reported that Egr1 expression was down-regulated and served as a suppressor in HCC (Hao et al., 2002; Wang et al., 2016). Conversely, other studies addressed overexpression and promoting activity of Egr1 in HCC (Ma et al., 2016; Lu et al., 2012; Tian et al., 2014). In present study, we identified up-regulated Egr1 in HCC tissues. First, we analyzed Egr1 expression in clinical specimens from the human protein atlas and found that the expression of Egr1 was increased in liver cancer tissues compared with the normal counterparts. Then we proved overexpression of Egr1 in HCC tissues through q-RT PCR and western blot analysis. Furthermore, we investigated the biological functions of Egr1 by gain-of-function and loss-of-function experiments. We provided evidence that Egr1 promotes HCC cells proliferation in vitro. These results showed that Egr1 plays a crucial role in promoting HCC, in line with previous researches identifying the aggravating effect of Egr1 in HCC.

The TGF-β family signaling pathways have been demonstrated to regulate a variety of cellular processes, including proliferation, differentiation, migration or cell death, which is essential for the homeostasis of tissues and organs (Bissell et al., 2001). TGF-β plays a dual role in the control of growth and death in immature/proliferating hepatocytes and in liver tumor cells. It induces cell cycle arrest and apoptosis at early stages. However, it activates proliferative and

anti-apoptotic signals through transactivation of other pro-mitogenic pathways, such as PDGF or EGF at later stages (Caja et al., 2007, 2011; Sancho et al., 2009). Furthermore, Baron et al., (2006) found that Egr1 can up-regulated TGF-β1 in prostate cancer. Here, we identified up-regulated TGF-β1 in HCC tissues. Moreover, we observed high levels of p-Smad2, p-Smad3 in HepG2 and Hep3B cells overexpressing Egr1, as well as the decreased levels of p-Smad2, p-Smad3 in HepG2 and Hep3B cells with silenced Egr1 than control group. Our data suggested that TGF-β/Smad signaling pathway can be activated by Egr1 and may be partly responsible for Egr1-induced cell proliferation in HCC.

Increasing evidences have suggested the important role of specific miRNAs in the progression of human cancer. MiR-181a-5p and other 8 miRNAs (miR-19a-3p, miR-19b-3p, miR191-5p, miR-590-3p, miR-363-3p, miR-124-3p, miR-299-3p and miR-181b-5p) were identified to have potential target sites in 3'UTR of Egr1 mRNA by means of public available algorithms. We detected the expressions of these miRNAs in 20 paired HCC specimens by qRT-PCR analysis. Firstly, we identified that there was no obvious expression of miR-19b-3p, miR-191-5p and miR-181b-5p in HCC samples. Secondly, we found that the expressions of miR-19a-3p and miR-590-3p in HCC tissues were dramatically increased **(Fig. S1 A)** and then rejected these 2 miRNAs. Thirdly, we found that the expressions of miR-124-3p, miR-299-3p and miR-363-3p in 20 paired HCC samples were down-regulated but were not correlative with the expression of Egr1 mRNA **(Fig. S1B, C and D).** Lastly, only miR-181a-5p was selected because that the expression of which was decreased and correlative with Egr1 mRNA expression in 20 paired HCC samples.

MiR-181a-5p has already been identified to be a contributing factor to cancer (Li et al., 2015; Parikh et al., 2014). The role of miR-181a-5p in cancer development is complicated. In this study, we identified the decreased expression of miR-181a-5p in HCC tissues. Furthermore, in order to elucidate the role of miR-181a-5p in HCC, we overexpressed and silenced miR-181a-5p gene level to observe the biological effect in HCC cells. We found that miR-181a-5p plays a crucial role in attenuating cell proliferation in HCC.

A previous study demonstrated that Egr1 is a target gene of miR-181-5p in leukemia (Verduci et al., 2015). Thus, we wonder whether there is a relationship between miR-181a-5p and Egr1 in HCC. Here, we found that the expression of miR-181a-5p was negatively correlated with Egr1 in clinical HCC specimens. Furthermore, quantitative PCR and western blot analysis showed that miR-181a-5p could decrease the expression of Egr1 in both mRNA and protein levels. In addition, decreased expression of miR-181a-5p could increase Egr1 expression. Dual-luciferase activity experiment demonstrated that miR-181a-5p was a translational repressor of Egr1 by directly targeting 3'UTR. We also found that silenced Egr1 expression could reverse the promoting effect of miR-181a-5p inhibitor on HCC cells proliferation in vitro.

In summary, we demonstrated in this study that transcription factor Egr1 can promote HCC progression through enhancing tumor cells proliferation by activating TGF-β1/Smad signaling pathway. In addition, we revealed that miR-181a-5p can prevent Egr1/TGF-β1 pathway induced HCC cells proliferation through targeting Egr1 in vitro. Although we demonstrated that miR-181a-5p may act as a protective factor in the progression of HCC through suppressing Egr1 expression, the molecules mechanisms that directly regulate downstream of miR-181a-5p remain unclear. In the meanwhile, it remains unknown that whether miR-181a-5p and Egr1 have relationship with HCC prognosis. Hence it is necessary for us to conduct further studies to illuminate above question.

5. Author contributions

Jian-gang Bi and Ping Xu conducted most of the experiments, analyzed the results, and wrote the first draft of the manuscript. Qi Li and Jin-feng Zheng assisted in cell culture experiments. Cai-xian Liao and Shi-yun Bao designed the study and edit the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2018.11.011.

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