Int J Clin Exp Pathol 2017;10(7):8031-8042
www.ijcep.com /ISSN:1936-2625/IJCEP0053818

Original Article

MicroRNA-29 family functions as a tumor suppressor by targeting RPS15A and regulating cell cycle in hepatocellular carcinoma

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Received March 26, 2017; Accepted June 13, 2017; Epub July 1, 2017; Published July 15, 2017

Abstract: Growing evidence shows that miRNA plays an important role in the development and progression of cancer. In this study, we found that the expression levels of miR-29 family were dramatically decreased in hepatocellular carcinoma (HCC) cell lines and clinical tissues. Then, we demonstrated that ectopic expression of miR-29 family could significantly suppress cell proliferation and induce apoptosis in HCC cells. Luciferase assay together with western blot assay confirmed that miR-29 family bound directly to the 3'-untranslated region (3'-UTR) of RPS15A and reduced the expression of RPS15A. In addition, the cell cycle related gene including cyclinA, cyclin D1 and p21 were also down-regulated when increased the expression of miR-29 family, which is similar as silencing RPS15A expression. Moreover, co-transfection of miR-29 mimics with 3'UTR-deleted RPS15A could rescue the expressions of cyclin A and cyclin D1 while down-regulate the p21 expression. In conclusion, miR-29 family functions as a novel tumor suppressor in HCC by regulate cell growth and cell cycle through binding to RPS15A 3'UTR. These findings may be utilized in developing novel therapeutic tools for HCC.

Keywords: Hepatocellular carcinoma, miR-29, RPS15A, cell cycle

Introduction

Primary liver cancer is the sixth most common cancer in the world and the third most common cause of cancer mortality. There is wide geographic variability in incidence with a majority of the cases occurring in developing countries. And hepatocellular carcinoma (HCC) accounts for approximately 85% of all primary liver cancers [1]. Thus, new anticancer agents and therapeutic strategies are required to improve the patients live quality and prolong their life.

The major factors for HCC include alcoholic liver disease, nonalcoholic fatty liver disease, cirrhosis, and especially hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. In China, there are more than 93 million HBsAg-positive subjects, of whom about 20 million have chronic HBV infection [2]. The hepatic B virus x protein (HBxAg) plays an important role in the development of HCC. Evidence showed that HBxAg promote hepatic cells proliferation partly through the upregulated expression of ribosomal protein s15a (RPS15A). In our previously study, we also found that down-regulation of RPS15A could inhibit the proliferation of human hepatic cancer cell in vitro [3]. However, detailed mechanisms of RPS15A in HCC development are still unclear.

MicroRNAs (miRNAs) function at the posttranscriptional level by negatively regulating translation of their target mRNAs by imperfect binding to their 3'UTRs. miRNAs have emerged as key regulators of diverse physiological and pathological processes, including cell proliferation, apoptosis, and cancer. Several recent researches showed miRNA played a vital role in HCC progression such as miR-101 [4], miR-224 [5, 6] promoted cell apoptosis, while miR-122a [7], miR-195 [8], miR-31 [9] suppressed HCC through regulate cell cycle, down regulated miR-152 [10] induced aberrant DNA methylation in HCC.

In addition, several research groups were showed miR-29 family inhibit HCC progression through different pathway. CM Wang [11] and HJ Bae [12] have showed miR-29c functions as

a tumor suppressor by targeting TNFAIP3 and SIRT1 respectively. Whereas, Xiong et al found miR-29 was HCC suppressor and promoted cell apoptosis by Bcl-2 and Mcl-1 through mitochondrial pathway [13]. However, miR-29 functions in inhibit HCC growth might have other pathway. In this study, we showed that miR-29 expression was obviously reduced in HCC tissues and cell lines. Ectopic expression of miR-29 could inhibit HCC cell lines growth and induce apoptosis. We also found that miR-29 could regulate the expression of cell cycle related protein through down-regulate RPS15A. Our study revealed that miR-29 family acts as a tumor suppressor by interacting with RPS15A mRNA.

Materials and methods

Clinical samples

We obtained 5 human hepatic para-carcinoma tissue samples and 11 hepatic cancer tissue samples from newly diagnosed HCC patients in The Affiliated Hospital of Nanjing University of Chinese Medicine. All patients gave informed consent and use of the samples for molecular studies.

Cell lines, cell culture and transfection

Hepatic cancer cell lines HepG2, and Hep3B were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Hepatic cancer cell lines Bel7404, SMMC-7721, the liver cell line LO2 and human embryonic kidney cell line 293T were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained in DMEM (Dulbecco's modified eagle medium), supplemented with 10% fetal bovine serum and 100 U/mL penicillin, 100 mg/mL streptomycin, cultured at 5% CO_2 and humidified 37°C chamber (Thermo Fisher). All cell culture related reagents were purchased from Gibco.

Harvest all the cells and seed 5×10^5 cells per well of six-well plate. After 18 h, mix 5 μL scramble, miRNA mimics or inhibitor (20 μM), 91 μL RNase-free water and 4 μl X-tremeGENE HP DNA transfection reagent (Roche, Indianapolis, IN, USA). After 20 min, pour the mix into a well of six-well plate and cultured in cell culture chamber. And the sequence of all the miRNA and siRNA were as follows: NC: 5'-TTCTCCGAACGTGTCACGT-3'; RPS15A siRNA: 5'-CATGGTTACATTGGCGAATT-3'; SiRNA was synthesized by Genepharma (Shanghai, China). miRNA mimics scramble: 5'-UUCUCCGAACGUGUCACGUTT-3'; miRNA inhibitor scramble: 5'-CAGUACUUUUGUGUAGUACAA-3'; miR29a, miR29b, and miR29c mimics, inhibitors were purchased from Genecopoeia (Guangzhou, China).

MTT proliferation assay

To detect cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed 1 day after transfection. All the cell lines were washed with phosphate-buffered saline (PBS) and digested by trypsin, seeded 2000 cells per well in 96-well plate. The plate was incubated for 1 to 5 days in 37°C and 5% CO_2 humidified chamber. 50 μL of MTT (10 mg/mL) were added and incubated for 2 h at 37°C, discarded the MTT and 100 μL dimethyl sulphoxide (DMSO, D8418, Sigma) was added to each well. The absorbance at 595 nm was measured using microplate reader (Bio Tek Instruments, Winooski, VT, USA).

Cell colony forming assay

Pour 1×10^3 cells per well in six-well plate on day after miRNA mimics or inhibitor transfection. The medium were changed every 3-days. After 14 days of culture at 37°C, discarded the medium, washed with PBS. Poured 4% paraformaldehyde to fix the cell for 10 min at room temperature, stained with crystal violet for 10 min at room temperature, washed with water and dried in baking oven at 55°C. Cell survival was counted according to the usual criterion of 50 cells or more per colony.

Flow cytometric analysis

Cell cycle and percentage of apoptotic cells were assessed by FACScalibur flow cytometry (Becton Dickinson, San Jose, CA, USA). Samples for cell cycle were harvested by trypsin and fixed by 70% ethanol in PBS in -20°C overnight, washed twice with PBS and stained by propidium iodide (PI) with RNase (550825, Becton Dickinson, San Diego, CA, USA) for 30 min in dark. Fluorescent emissions were collected through FL2 band-pass filter. Samples for apoptosis were collected by trypsin without EDTA, washed twice with PBS, stained by 5 μL PI and 5 μL FITC-Annexin V in 100 uL binding buffer for 15 min in dark (BMS500FI, eBioscience, Belgium). Fluorescent emissions were collected

through FL1 band-pass filter for FITC-Annexin V, FL2 for PI. The apoptotic were PI and FITC-Annexin V both positive cells.

Cell invasion assay

HepG2 was growth to 70% confluent and transfected with miR-29 family mimics, inhibitor or scramble. After 24 h, harvested all the cells by trypsin, 1×10^4 cells were seeded with non-FBS medium onto a Matrigel-coated membrane matrix (Becton Dickinson, Badford, MA) present in the insert of a 24-well plate. FBS contained medium was added to lower chamber as a chemoattractant. After 24 h, medium was discarded, the non-invading cells were removed with cotton bud. Invasive cells located on the lower surface of chamber were stained with the 0.1% crystal violet (C0775, Sigma).

Protein extraction and Western blot

Cells were lysed in RIPA lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF and protease inhibitor cocktail) for 30 min on ice, protein quantificated with BCA protein assay kit (Beyotime, Shanghai, China), and 1/5 volumes of 5×SDS-loading buffer (250 mM Tris-HCl (pH 6.8), 10% SDS, 0.5% bromophenol blue, 50% glycerol, 5%β-mercaptoethanol) were added to denaturing the protein. Equal amounts of protein were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). The blots were blocked with 5% BSA (Albumin from bovine serum, Beyotime, Shanghai, China) and incubated with indicated antibodies. The Immobilon Western blot detection system (Millipore, Billerica, MA) was used to detect bound antibodies. Antibodies used in the experiment: RPS15A (sc-162101, Santa Cruz, USA), Cyclin A (ab38, Abcam, Cambridge, MA), Cyclin D1 (ab134175, Abcam, Cambridge, MA), Cyclin E (ab93161, Abcam, Cambridge, MA), p21 (2947, cell signaling, Beverly, MA), beta-actin (sc-47778, Santa Cruz, USA), Flag (ab1162, Abcam, Cambridge, MA), goat anti-mouse IgG HRP (m21001, Abmart, Shanghai, China), goat anti-rabbit IgG HRP (m21002, Abmart, Shanghai, China).

RNA extraction and quantitative real-time PCR

Total RNA was isolated from cells or human tissue samples by using TRIzol reagent (Invitrogen, Carlsbad, CA).

miR-29: the first-strand complementary miRNA was synthesized using the specific RT primer from total RNA using TaqMan MicroRNA Reverse Transcription Kit. miR-29a, b, c expression were evaluated by real-time PCR using indicated Taqman probes and TaqMan® Universal PCR Master Mix II on an ABIPRISM 7500 Real-time PCR system. And the expression level of U6 snRNA was used as the loading control. Relative mRNA was determined by using the formula $2^{-\Delta CT}$ (CT, cycle threshold) where ΔCT = CT (target miRNA)-CT (U6).

Human miR-29a, b, c and U6 RT primers, TaqMan MicroRNA Reverse Transcription Kit, miR-29a, b, c and U6 Taqman probes, TaqMan® Universal PCR Master Mix II, no UNG were all purchased from Applied systems (Foster city, CA). All procedures were carried out according manufacture's instrument.

RPS15A: the first-strand complementary miRNA was synthesized using oligo dT from total RNA using the PrimeScript RT master Mix Perfect Real Time (Takara, Dalian, China). RPS15A mRNA expression level was detected by real time PCR using SYBR green (Takara, Dalian, China) on ABIPRISM 7500 real time PCR system. GAPDH served as loading control. The primers of RPS15A as described before [3] and GAPDH primers [14] were: Forward, 5'-GGTGTGAACCATGAGAAGTATGA-3'; Reverse, 5'-GAGTCCTTCCACGATACCAAAG-3'.

Plasmids construction and luciferase reporter assay

pME18s-RP15A: RPS15A coding sequence (CDS) cloned into pME18s plasmid; pME18s-RP15A-3'UTR: RPS15A CDS and 3'UTR (position 1-3000 bp) cloned into pME18s plasmid; pME18s-RPS15A-FLAG: RPS15A CDS and flag tag 5 times repeats cloned into pME18s plasmid. pGL3-RP15A-3'UTR: RP15A-3'UTR position 1000-3000 bp cloned into pGL3 firefly luciferase expression plasmid; pGL3-RP15A-3'UTR-mut: pGL3-RP15A-3'UTR position 1831-1837 mutation. Together with pRL-TK Renilla luciferase report plasmid, all of the plasmids were cloned by Genecopoeia (Guangzhou, China).

Luciferase reporter assay: 293T, HepG2, Bel-7404 were seeded onto 24-well plate a day before transfection, 1×10^5 cells per well. Cells were co-transfected with pGL3 firefly luciferase

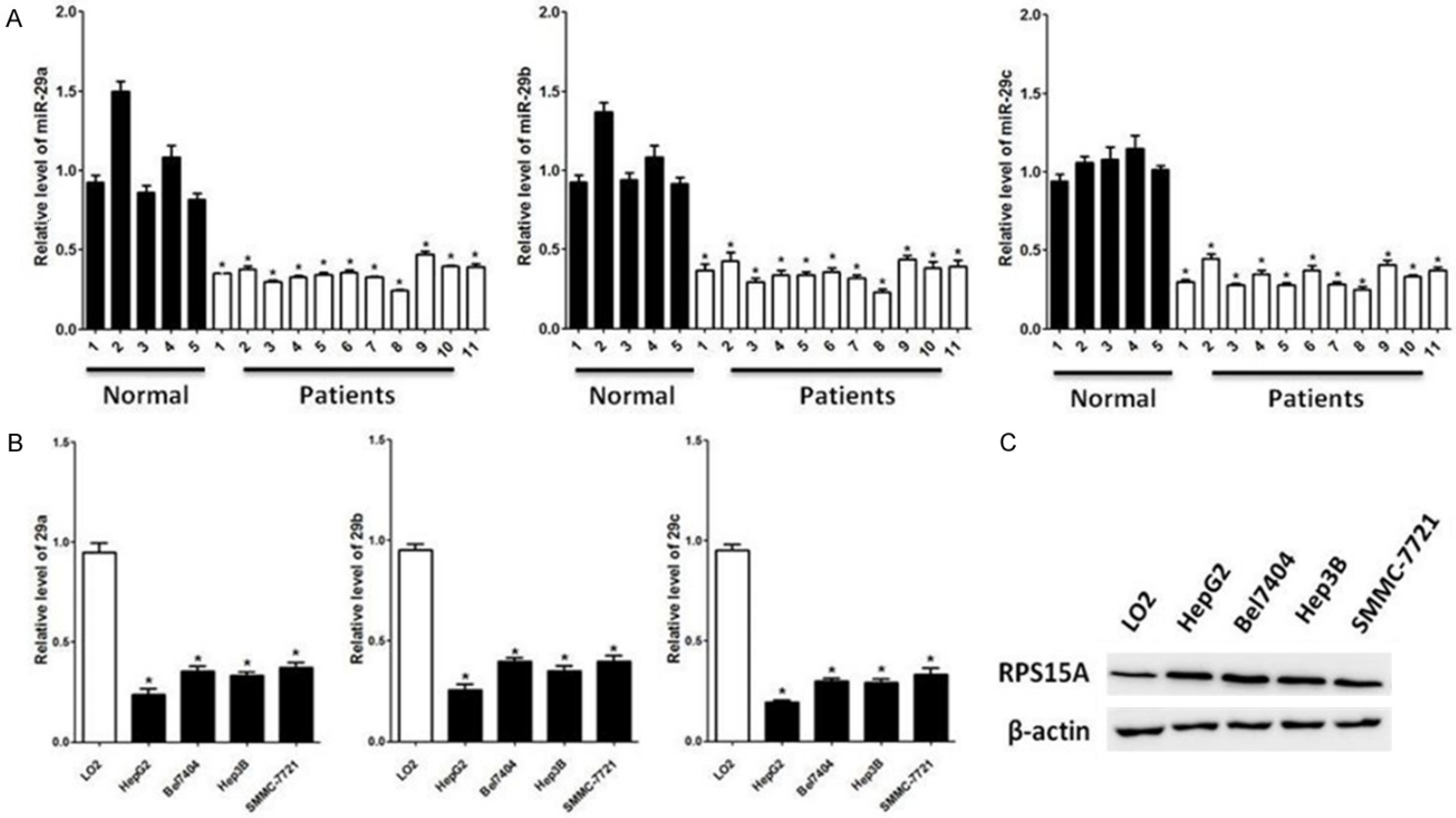

Figure 1. Reduced levels of miR-29 family expression in HCC cell lines and tissues. A. Real-time PCR analysis of miR-29 family expression in 5 nonneoplastic liver specimens and 11 human HCC tissues. The average miR-29 family expression was normalized by U6 expression. Each bar represents the mean of three independent experiments. *P<0.05. B. Real-time PCR analysis of miR-29 family expression in normal human liver cell and various tumor cell lines. C. Western blotting analysis of RPS15A in normal human liver cell and HCC cell lines, β-actin served as the loading control.

plasmid and miR-29 mimic or inhibitor separately by X-treme GENE HP DNA transfection reagent (Roche, Indianapolis, IN, USA), pRL-TK Renilla luciferase report plasmid was used as internal loading control. After transfection 48 h, poured 100 uL Passive Lysis Buffer to lysis cell and luciferase activities were measured by using the Dual-Luciferase reporter assay system (E1910, Promega).

Biotin-labelled RNA pull-down assay

HepG2 and Bel7404 cells were transfected with Bio-miR-29a, b, c or Bio-miR-control in two 60 mm dishes. After 48 hours of incubation, the cells were trypsinized and washed twice with PBS. Cells were resuspended in 0.7 mL of lysis buffer (20 mM Tris (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 0.3% IGEPAL CA-630) and then incubated on ice for 20 min. The cytoplasmic lysate was isolated by centrifugation at 10,000× for 15 min and supernatant was collected. The lysate was added to the strepatavidin-coated magnetic beads (Invitrogen) and incubated and incubated overnight at 4°C. The beads were washed with lysis buffer for 5 times and 100 μL of lysis buffer with DNaseI (2 U/μL) was added. After incubation at 37°C for 10 min, lysates were centrifuged at 5,000 g for 5 min and the supernatant was discarded. Protein kinase K (20 mg/mL) and 1 μL of 10% SDS in 100 μL of lysis buffer were added to the pellet and incubated at 55°C for 20 min. RNA bound to the beads (pull-down RNA) or from 10% of the extract (input RNA), was isolated with Trizol reagent (Invitrogen). The levels of RPS15A in the Bio-miR-29a, b, c pull-down were quantified by qRT-PCR. GAPDH was used for normalization.

Statistical analysis

The results were determined as mean ± SD of three independent experiments, in which each assay was performed in triplicate. Data were analyzed by New-man-Keuls test using Statistica software as indicated and are presented as mean ± SEM. *P*-values calculated by student's T test using SPSS 14.0 software and less than 0.05 were considered statistically significant. Results of time lapse microscopy experiments were analyzed with Wilcoxon test in R software.

Results

Reduced levels of miR-29 family expression in HCC cell lines and tissues

To investigate the miR-29 family expression level in HCC and normal tissues, we obtained 5 hepatic para-carcinoma tissues and 11 HCC tissues from newly diagnosed patients. Real-time PCR was conducted to measure the mRNA level of miR-29a, b, and c. We found miR-29a, b, and c were much lower in HCC tissues as compared with para-carcinoma tissues (**Figure 1A**). Furthermore, significant down-regulation of miR-29a, b and c were also found in HCC cell lines compared with LO2 cell (**Figure 1B**).

RPS15A played important role in HCC growth, as described before, down-regulation of RPS15A inhibit HCC growth in HCC cell lines. We validated the RPS15A protein level in LO2 or HCC cell lines representing HCC and found much higher reduction in LO2 (**Figure 1C**).

miR-29 family inhibits the growth of HCC cell line in vitro

To explore the role of miR-29 family down-regulation in the development and progression of HCC, we generated miR-29a, b, c-mimic and miR-29a, b, c-inhibitor respectively. Transfected with miR-29a, b, c-mimic and miR-29a, b, c-inhibitor and negative control microRNA, we tried to reveal the gain-of-function effect or lose-of-function effect on the proliferation of the HepG2 cell line. Results from the MTT (Methyl thiazolyltetrazolium) assay indicated that miR-29 family up-regulation significantly inhibited the proliferation rate of HepG2 cells compared with the control while this situation would be rescued by the miR-29 suppression when cells were transfected by miR-29a, b, c-inhibitor (**Figure 2A**). Results of crystal violet stained of cell colonies showed obvious reduction of cell proliferation in miR-29a, b, c overexpression cell lines (**Figure 2B**). Same results were obtained from three independent repeats (**Figure 2C**). In addition, the colony formation assays showed that the HepG2 cell was significantly increased in response to miR-29a, b, c inhibitor (**Figure 2B** and **2C**). Furthermore, these results suggested that miR-29 family up-regulation inhibits hepatic cell tumorigenicity in vitro. This result was further confirmed by FACS

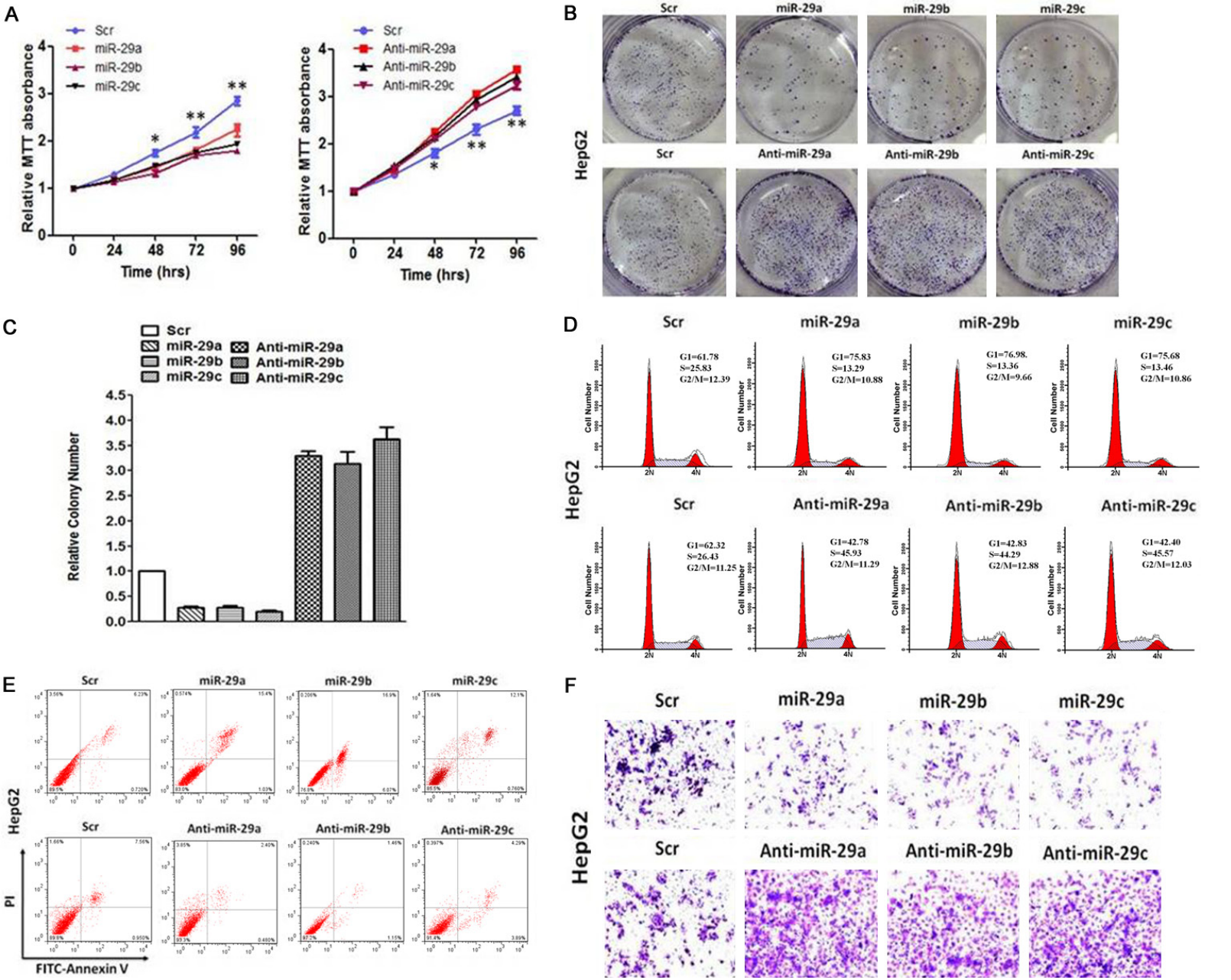
A
Scr
miR-29a
miR-29b
miR-29c
Relative MTT absorbance
Time (hrs)
Anti-miR-29a
Anti-miR-29b
Anti-miR-29c
B
HepG2
C
Relative Colony Number
D
G1=61.78
S=25.83
G2/M=12.39
G1=75.83
S=13.29
G2/M=10.88
G1=76.98.
S=13.36
G2/M=9.66
G1=75.68
S=13.46
G2/M=10.86
G1=62.32
S=26.43
G2/M=11.25
G1=42.78
S=45.93
G2/M=11.29
G1=42.83
S=44.29
G2/M=12.88
G1=42.40
S=45.57
G2/M=12.03
Cell Number
2N
4N
E
PI
FITC-Annexin V
F

Figure 2. miR-29 family inhibits the growth of HCC cell in vitro. A. Ectopic expression of miR-29 family suppressed HepG2 cell proliferation. The cell viability was determined by measuring MTT absorbance at A570. Cell growth was measured at every 24 hours. NC represents negative control microRNA (means ± SD; **P<0.01 compared to control, Student's t test). B. Representative micrographs of crystal violet stained cell colonies. C. Representative quantification of crystal violet stained cell colonies. D. After transfection of miR-29a, b, c mimics or inhibitor to HepG2, the DNA content of PI-stained cells was analyzed by flow-cytometry. E. Cells staining positive for FITC-Annexin V and negative for PI at 48 h post-transfection were considered to have undergone apoptosis. F. Representative images depicting the invasion ability of HepG2 after scramble control, or miR-29 mimics 48 h transfection.

analysis, which showed decreased the percentage of cells in S phase and increased the percentage of cells in G0/G1 phase in miR-29a, b, c-overexpressing cell and at the same time we found that transfection of the miR-29a, b, c-inhibitor drastically increased the percentage of cells in the S peak but decreased the percentage of cells in the G0/G1 peak (**Figure 2D**). Double staining of the infected HepG2 cell with Annexin V-FITC and PI showed an obvious raise ratio of apoptosis in miR-29a, b, c-overexpressing cell compared with the control cell while transfected with miR-29a, b, c-inhibitor can also rescue the apoptotic rate of HepG2 cell (**Figure 2E**). In cell invasion assays, overexpression of miR-29a, b, c significantly inhibited but miR-29a, b, c-in significantly promoted the numbers of invading cells in HepG2 cell line (**Figure 2F**). Taken together, these results indicate that miR-29 family suppresses HCC cell proliferation and the proliferative effect of inhibiting miR-29 family in HCC cells may occur through regulation of G1/S transition.

miR-29 family regulates RPS15A expression by binding 3'-UTR in HCC

Since miRNAs usually exert their function by negatively regulating the expression of their target genes and our results above indicate the tumor suppressive role of miR-29 family in HCC, putative oncotargets of miR-29 family were predicted using target prediction programs, TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microna/). Our analysis revealed that RPS15A was a potential target of miR-29 family. The 3'-UTR of RPS15A messenger RNA contains a complementary site for the seed region of miR-29 family (**Figure 3A**). Although our previously study showed RPS15A may modulate hepatic cancer growth and play a prominent role in heptocarcinogenesis, the interaction between miR-29 and RPS15A has not been experimentally validated in HCC.

To determine whether RPS15A is direct target of miR-29 family, wild-type and mutant 3'-UTR lacking miR-29 binding sites were cloned into the downstream of firefly luciferase coding region in pGL-3 luciferase reporter vector. The constructs were then co-transfected with miR-29a, b, c mimic or scramble pGL3-RPS15A-3'UTR or pGL3-RPS15-3'UTR-mut and pRL-TK renilla luciferase vector into human embryonic kidney cell line 293T, respectively. Firefly luciferase values were normalized to renilla luciferase activity. The relative luciferase activity was reduced by 50% in pGL-3 vectors with wild-type RPS15A 3'-UTR, but not in those with respective mutant 3'-UTRs (**Figure 3B**). In addition, the endogenous miR-29s inhibited the luciferase activity of the pGL-3 vectors with wide-type RPS15A 3'-UTR but did not affect the luciferase activity of the pGL-3 vectors with mutant 3'-UTR (**Figure 3C**) in HepG2 and Bel7404, suggesting that miR-29 family cognate sites are essential for negative regulation of luciferase expression driven by RPS15A-3'-UTR. Consistent with this observations, the Biotin-labeled miR-29a, b, c-mRNA pull down assay results also showed obvious reduction of the amount of RPS15A protein in miR-29a, b, c-overexpressing both in HepG2 and Bel7404 cells (**Figure 3D**).

To confirm that miR-29 can indeed suppress expression of endogenous RPS15A, HepG2 and Bel7404 cells were transfected with miR-29a, b, c or scramble mimics, followed by detection of their protein levels. The protein levels of RPS15A were substantially decreased after ectopic overexpression of miR-29 family in HepG2 and Bel7404 cell lines as evidenced by western blot assays (**Figure 3D**). Opposite, knocking down of miR-29 family by anti-miR-29a, b or c in HepG2 and Bel7404 cells increased protein levels of RPS15A (**Figure 3D**). Taken together, these findings indicate that RPS15A can be negatively regulated by miR-29 family in HCC cells.

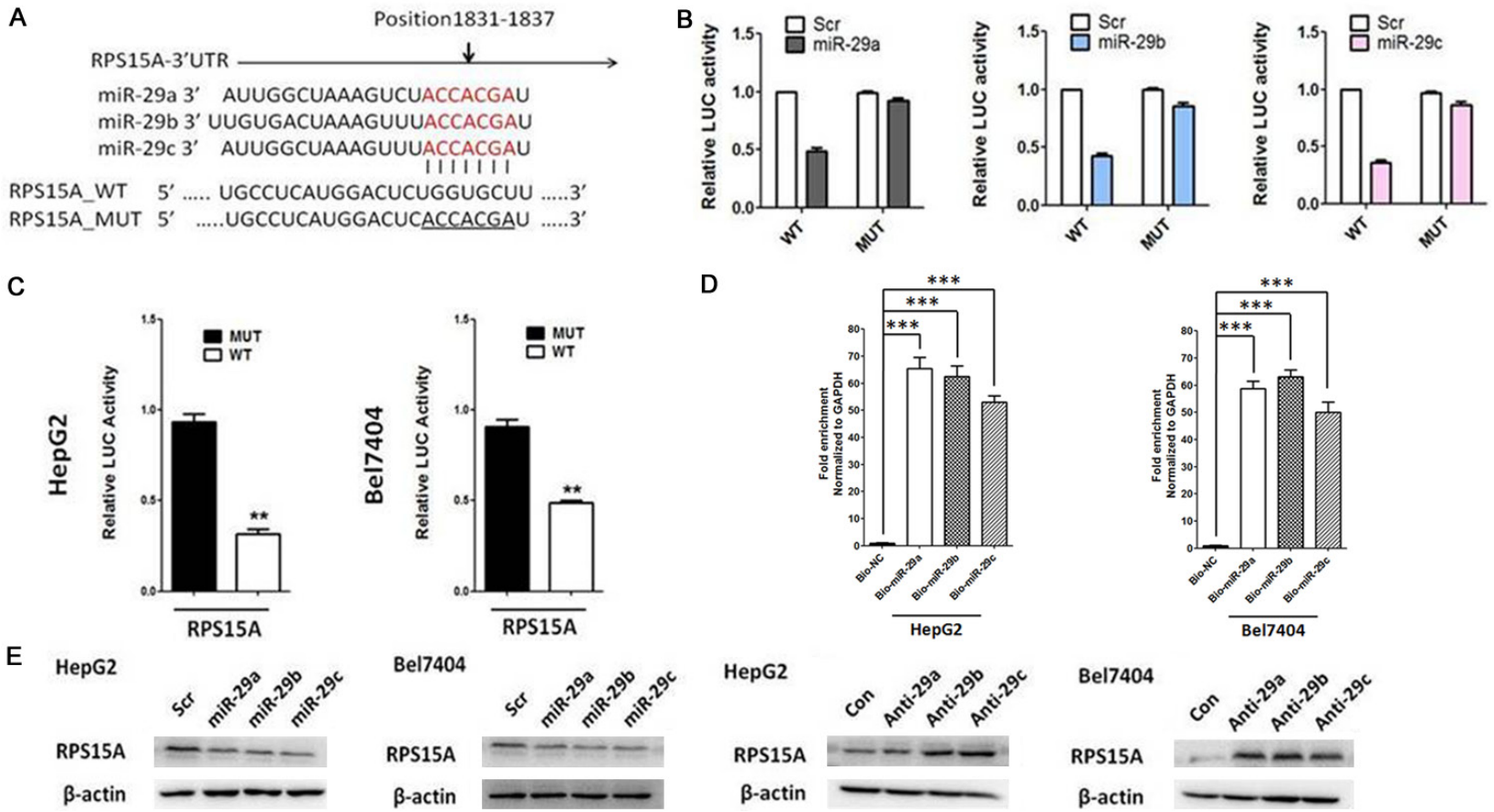

Figure 3. miR-29 family regulates RPS15A expression by binding 3'-UTR in HCC. A. Schematic representation of RPS15A 3'-UTRs showing putative miR-29 target site. B. Relative luciferase activity of the indicated RPS15A reporter constructs in 293T cells. Error bars presented standard deviation obtained from three independent experiments. C. Luciferase activity assay with wild-type RPS15A 3'-UTRs constructs and mutated luciferase constructs in HepG2 and Bel7404 cells. Firefly luciferase values were normalized to Renilla luciferase activity and plotted as relative luciferase activity (*P<0.05 and **P<0.01 compared with mutated constructs). D. The Biotin-labeled miR-29a, b, c-mRNA pull down assay. HepG2 and Bel7404 cells were transfected with Biotin-labeled microRNA control (Bio-NC) or Biotin-labeled miR-29a, b, c mimics for 48 hours. The expressions of RPS15A were measured by qRT-PCR and normalized to GAPDH (means ± SD; **P<0.05; ***P<0.001). E. Western blot analysis of RPS15A expression in HepG2 and Bel7404 cells transfected with scramble oligonucleotide or miRNA mimics/miRNA inhibitors.

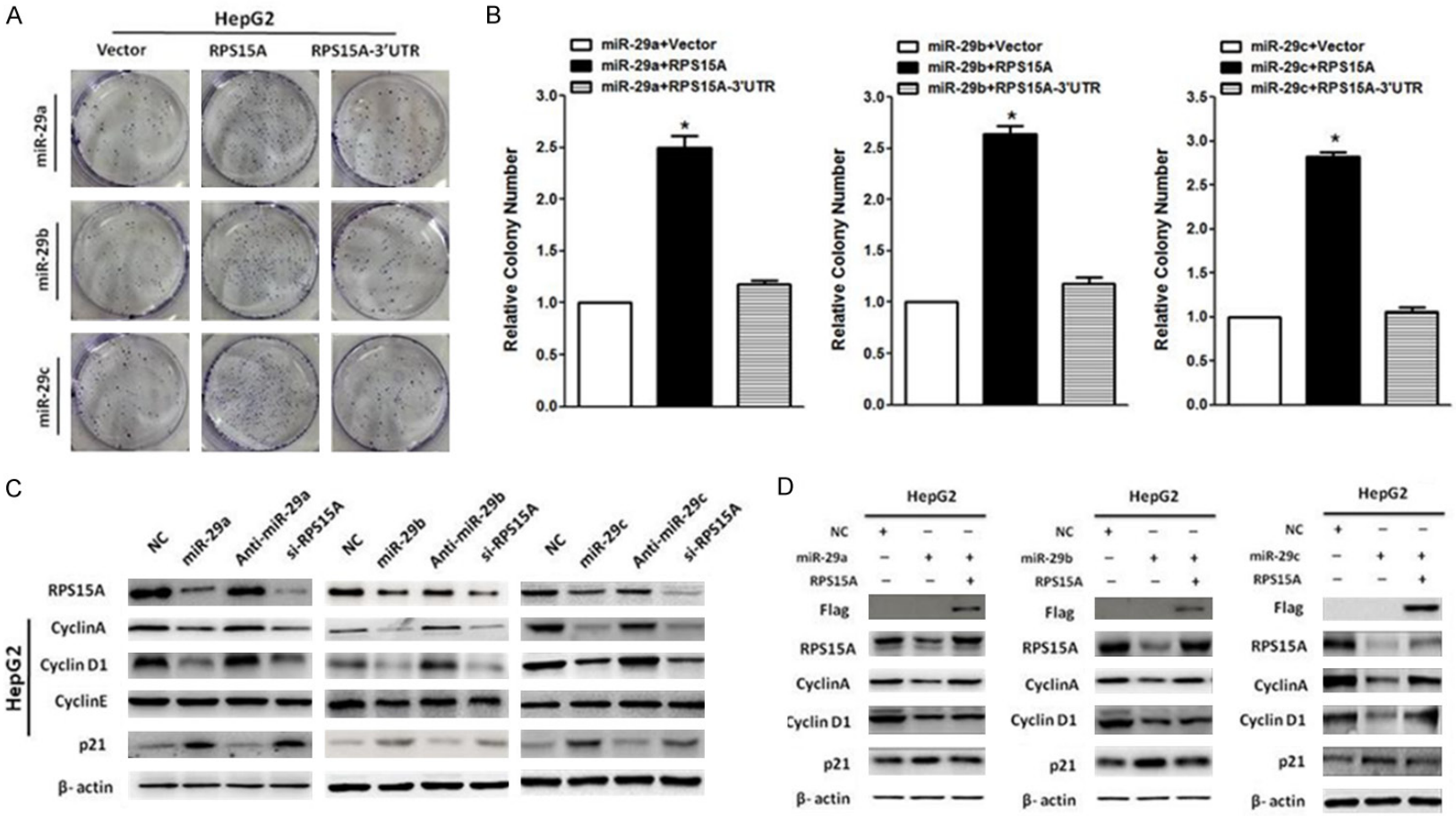

Figure 4. miR-29 family inhibits cell proliferation through targeting RPS15A 3'UTR and regulating cell cycle related molecules in HCC. A. Representative micrographs of crystal violet stained cell colonies. B. Representative quantification of crystal violet stained cell colonies. *P<0.05. C. HepG2 cell were transfected with miR-29a, b, c mimics or inhibitor. si-RPS15A were used for knockdown of miR-29 family target genes, respectively. The protein expression levels of G1/S regulatory molecules were analyzed by immunoblotting. NC represents negative control miRNA. D. Co-transfection of miR-29a, b, c with 3'UTR-deleted RPS15A plasmid (pME18s-RPS15A-FLAG) rescued the expressions of G1/S regulatory molecules. The expressions were analyzed by immunoblotting.

miR-29 family inhibits cell proliferation through targeting RPS15A 3'UTR and regulating cell cycle related molecules in HCC

To investigate whether miR-29a, b, c inhibits cell proliferation was implicated with regulation of RPS15A, RPS15A and RPS15A 3'UTR were respectively transfected into HepG2 cell with miR-29a, b, c mimics using the X-treme GENE HP DNA transfection reagent. The result of colony formation assay showed overexpressing RPS-15A significantly increased the proliferation rate of HepG2 cell compared with that cells expressing RPS15A 3'UTR (**Figure 4A**) and similar results were obtained from 3 independent repeats (**Figure 4B**). The rescuing experiment further confirmed that the inhibitory role of miR-29a, b, c in HCC cell may be mediated by RPS15A.

Our data showed that miR-29a, b, c exerts tumor suppressor function by regulating cell proliferation in HCC. So we examined some cell cycle regulator. Such as cyclin A, cyclin D1, cyclin E and p21, which has been implicated in the control of the G1 to S phase transition in mammals. Interestingly, we found the protein level of cyclin A and cyclin D1, a CDK regulator important for regulating the G1/S transition, was down-regulated in HepG2 cell transfected with miR-29a, b, c-mimics, which is similar as transfected the si-RPS15A, but increased in the cells transfected with miR-29a, b, c-inhibitor, compared with control cells (**Figure 4C**). On the other hand, the expression of p21 was increased in miR-29a, b, c overexpressing cells and inhibited in the miR-29a, b, c inhibited cells but the protein level of cyclin E was not affected obviously (**Figure 4C**). Co-transfection of miR-29a, b, c with 3'UTR-deleted RPS15A rescued the expressions of cyclin A and cyclin D1 while down-regulating the p21 expression (**Figure 4D**).

Discussion

It is well known that miRNAs play important roles in tumorigenesis of various human cancers, including hepatocellular cancer [14]. Recent evidence has shown that miR-29 family might exert extensive effects in the development and growth of human cancer [15]. It has also been identified that miR-29 family were significantly down-regulated in multiple cancers including lung cancer [16], acute or chronic myeloid leukemia [17, 18], nasopharyngeal cancer [19] and hepatocellular carcinoma [20]. Herein, we also found that miR-29 family was down-regulated in HCC tissues and cell lines. Moreover, we demonstrated that up-regulation of miR-29 family suppressed the growth and invasion of hepatocellular carcinoma cells, suggesting that miR-29 family may function as a tumor suppressor in HCC. The results of our study, together with the findings from these previous studies, collectively highlighted that the dysregulated miRNAs in HCC may serve as potential candidates for predicting recurrence and metastasis.

Previous studies have reported that miR-29c significantly inhibits the proliferation of squamous cell carcinoma cell and induces cell apoptosis [21]. Consistent with previous studies, our MTT assay and FACs analysis showed that overexpression of miR-29 family inhibits the proliferation of hepatocellular carcinoma and promotes apoptosis. Nevertheless, the biological function and underling mechanisms of miR-29 family in HCC are largely unknown.

To address the molecular mechanisms involved in miR-29 family in inhibition of proliferation and invasion, we searched for potential target genes with an established or potential function in HCC. Interestingly, a perfect match between the miR-29 seed region and the 3'UTR of RPS15A was identified. RPS15A gene, which encodes a ribosomal protein, is a component of the 40S ribosomal subunit. Emerging evidence has shown that RPS15A correlate with the development and progression of various cancers [22-24]. Our previous study also demonstrated down-regulation of RPS15A inhibits proliferation of HCC and regulates cell cycle progress. In our current study, our data firstly show that miR-29 family interacts with RPS15A by directly binding RPS15A 3'UTR. Our luciferase reporter assay and western blotting analysis consistently confirmed that the protein level of RPS15A is upregulated in HCC cells and negatively correlated with miR-29 family expression.

Our data showed the defect in G1/S phase transition in miR-29 family-overexpressing cells (**Figure 2C**). Then we examined the expression of several cell cycle regulator molecules which are reported to be important for regulating the G1/S transition. The results showed that the

important molecules in the regulation at G1/S transition, such as cyclin A and cyclin D1, were significantly down-regulated while p21 was upregulated in miR-29 family-overexpressing cells.

Taken together, our analysis revealed that the expression of miR-29 was decreased in HCC and miR-29 inhibited HCC proliferation and cell cycle progression via directly suppressing RPS15A expression. Targeting to the miR-29 family/RPS15A interaction or rescuing miR-29 family expression may be a new therapeutic application to treat hepatocellular carcinoma patients in the future. Our findings may have important implications for the development of personalized medicine, as this allows identification of those HCC patients who have a high risk of recurrence and require adjuvant chemotherapy, from those who may benefit from curative surgery alone.

Acknowledgements

The study was supported by the Scientific Research Fund of Jiangsu Provincial Education Department in China (11521184) and A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, PAPD.

Disclosure of conflict of interest

None.

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