Upregulated microRNA-429 inhibits the migration of HCC cells by targeting TRAF6 through the NF-κB pathway

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Abstract. Increasing evidence indicates that miR-429 is involved in tumor suppression in various human cancers. However, its role in hepatocellular carcinoma (HCC) remains unclear. In the present study, we found that miR-429 was significantly downregulated in HCC tissue samples and cell lines. Upregulation of miR-429 markedly suppressed proliferation and migration of HCC cells. Moreover, we identified TRAF6 as a direct target of miR-429. Downregulation of TRAF6 partially attenuated the oncogenic effect of anti-miR-429 on HCC cells. Ectopic expression of miR-429 in HCC cells inhibited TCF-4 activity as well as nuclear accumulation of P65 and expression of the NF-κB targets c-Myc and phosphorylation of TAK1. In a nude xenograft model, miR-429 upregulation significantly decreased HCC growth. In conclusion, by targeting TRAF6, miR-429 is downregulated in HCC and inhibits HCC cell proliferation and motility. Our data suggest that miR-429 may serve as a potential anticancer target for the treatment of HCC.

Introduction

Worldwide, hepatocellular carcinoma (HCC) is one of the most invasive, therapy-resistant and metastatic tumors (1). Although various therapies are used to improve outcomes of HCC patients, such as hepatic resection or liver transplantation, the 5-year survival rate after diagnosis is only approximately 30% (2). The majority of HCC patients are not candidates for curative surgery largely due to distant metastasis and high recurrence ratio at the time of the diagnosis (3). Although a number of reports have demonstrated that multiple signaling pathways are involved in tumor metastasis, the molecular mechanisms governing the metastatic cascades of HCC are complex, and our current knowledge regarding these mechanisms remains limited (4). Recently, microRNAs (miRNAs) have been implicated as key regulators of these pathways (5).

miRNAs, an abundant class of endogenous, small, non-coding RNAs of 22 nucleotides in length, are post-transcriptional regulators that bind to specific cognate sequences in the 3'-untranslated region (3'-UTR) of target transcripts. These RNAs commonly result in translational repression and gene silencing (6-8). Evidence is being accumulated which indicates that miRNAs have been reported to be involved with diverse biological processes, such as development, inflammation and tumorigenesis (9). It should be noted that numerous miRNAs play crucial roles in the regulation of HCC tumorigenesis, such as miR-221, miR-122, miR-451, miR-452 and miR-1180 (7,10-13).

In the present study, we investigated the possible involvement of miRNAs in the proliferation and migration of HCC. We found that at high expression levels, microRNA-429 (miR-429) played a critical role in regulating NF-κB pathway activity in HCC to inhibit tumor progression. miR-429 inhibited the NF-κB pathway by targeting TRAF6. The targeting of TRAF6 not only reduces the ability of HCC growth but also inhibits tumor migration and metastasis by downregulating TCF4, pTAK1 and c-Myc *in vitro* and *in vivo*. These findings provide new insights into the molecular mechanisms that regulate the expansion and functions of tumor-associated HCC and into the exploration of potential targets for therapeutic intervention.

Materials and methods

Cell culture and reagents. HL7702, SK-HEP1, SMMC7721, HCCLM3 and HepG2 were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and cultured, as recommended, as monolayers in RPMI-1640 supplemented with 10% fetal bovine

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serum (FBS; HyClone Laboratories, Inc., Logan, UT, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) in a humidified incubator at 37˚C in a 5% CO_2 atmosphere.

Tissue samples. A total of 25 pairs of human primary HCC tissues, and their corresponding non-tumorous tissues were collected from The Affiliated Hospital of Qingdao University, China. These HCC cases consisted of 20 males and 5 females, and none of the patients had received radiotherapy or chemotherapy prior to surgery. For the use of these clinical materials for research purposes, prior consent from patients and approval from the Ethics Committee of The Affiliated Hospital of Qingdao University were obtained. Informed consent was provided to all study subjects. Patient anonymity has been preserved. The pathological judgment was determined by two senior pathologists with a double-blind study design. All specimens were classified according to WHO (World Health Organization) criteria.

Isolation of microRNA from HCC cells, liver tissues and reverse transcription and qRT-PCR. Total RNA was extracted from the HCC tissues and adjacent non-tumorous tissues by TRIzol reagent (Invitrogen). For miRNA isolation and detection, reverse-transcribed complementary DNA was synthesized using the PrimeScript RT reagent kit (Takara Bio, Dalian, China) and quantitative real-time-PCR (qRT-PCR) was performed using SYBR Premix Ex Taq (Takara) with the real-time PCR system (Takara Bio). The PCR cycling conditions were as follows: pre-denaturing at 95˚C for 10 min, followed by 40 cycles of denaturing at 95˚C for 10 sec, annealing at 58˚C for 20 sec and extension at 72˚C for 10 sec. Expression levels were normalized to the endogenous snRNA U6 control. The relative expression ratio of miR-429, in each paired tumor and non-tumorous tissue, was calculated by the $2^{-\Delta\Delta CT}$ method. For the mRNA analysis, cDNA was synthesized using reverse transcriptase kit (Takara Bio). qRT-PCR was performed with SYBR Premix Ex Taq with the Takara SYBR real-time PCR system. GAPDH was used as an internal control for mRNA quantification. The relative expression ratio of mRNA was calculated by the $2^{-\Delta\Delta CT}$ method. PCR reactions for each gene were repeated three times. Independent experiments were performed in triplicate.

Cell viability assay. Cell viability was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability kit following the instructions of the manufacturer. Cells were cultured in plastic 96-well plates under 200 μl of growth medium and at an initial density of 10,000 cells/well. After the treatment at the indicated time, 20 μl of 5 mg/ml MTT was added. After 2 h, cells were lysed with 150 μl isopropanol/HCl (0.05 M HCl in 100% isopropanol), and the optical density of each well was determined at 450 nm on a Dynatech MR5000 microplate reader (Dynatech Corp., Burlington, MA, USA). For each measurement, both treatment and control, eight replicate wells were recorded.

Transwell migration assays. The migration assays were performed using 24-well Transwell® chambers (8 μm; Corning Incorporated, Corning, NY, USA). For the migration assay, tumor cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) and $2x10^5$ cells were seeded into the upper chambers. Then, 0.5 ml DMEM containing 10% FBS was added to the bottom chambers. Following a 24-h incubation, cells on the upper surface of the membrane were scrubbed off, and the migrated cells were fixed with 95% ethanol, stained with 0.1% crystal violet and counted under a light microscope (Olympus Corp., Tokyo, Japan).

Transient transfection with siTRAF6 or pcDNA-TRAF6. SiTRAF6 were designed and synthesized by Shanghai GenePharma, Co., Ltd. (Shanghai, China). The sequence of the negative control (siCON) was also designed by Shanghai GenePharma. The TRAF6-overexpressing plasmid vector (pcDNA-TRAF6) was previously constructed and preserved in our laboratory. Twelve hours prior to the transfection, the cells were plated in a 6-well or a 96-well plate (Corning Incorporated). Lipofectamine 2000 reagent (Invitrogen) was then used to transfect siTRAF6 or pcDNA-TRAF6 into the cells according to the manufacturer's protocol.

Western blot analysis. As previously described, target protein levels were evaluated by western blot assays. Briefly, cells were lysed in 100 μl cold Triton lysis buffer (20 mM Tris pH 7.4, 2 mM PPiNa, 2 mM EDTA pH 7.4, 137 mM NaCl, 25 mM β-glycerophosphate pH 7.4, 10% glycerol, 1% Triton X-100) supplemented with 1 mM Na_3VO_4, 0.5 mM DTT, 1 mM PMSF, and 1X complete protease inhibitor cocktail (Roche) for 15 min at 4˚C. Lysates were spun at 14,000 rpm for a period of 10 min at 4˚C. Protein concentration was determined using the Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total cell extracts (30 μg) were separated in 12% SDS-PAGE gels, transferred to PVDF membranes (Millipore, Temecula, CA, USA) and probed with antibodies against c-Myc (1:1,000 dilution; Abcam, Cambridge, UK), total TAK1 (1:1,000 dilution; Abcam), phosphorylation of TAK1 (1:1,000 dilution; Abcam), TRAF6 (1:1,000 dilution; Abcam) or GAPDH (1:2,000 dilution; Abcam). After incubation with the primary antibodies, the membranes were washed with TBS/0.05% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Signals were detected using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

3'-UTR luciferase reporter assays. To generate the 3'-UTR luciferase reporter, the partial sequence of the 3'-UTR from TRAF6 was cloned downstream of the firefly luciferase gene in the pGL3-control vector (Promega, Madison, WI, USA). Mutation of the miR-429 target sites in the 3'-UTR of TRAF6 was used as a control. pRL-TK containing *Renilla* luciferase was co-transfected for data normalization. For the luciferase reporter assays, $3x10^5$ HEK293 cells were seeded in 35-mm culture plates, allowed to adhere overnight, and then transfected with either 100 ng of empty pGL3-control vector, 100 ng of the pGL3/TRAF6 construct, or 100 ng of the pGL3/TRAF6-mut construct together with either 200 ng of miR-CON or miR-429 using Lipofectamine 2000 (Invitrogen). Two days later, the cells were harvested and assayed with the Dual-luciferase assay (Promega) according to the manufacturer's instructions. Each treatment was performed in triplicate in three independent

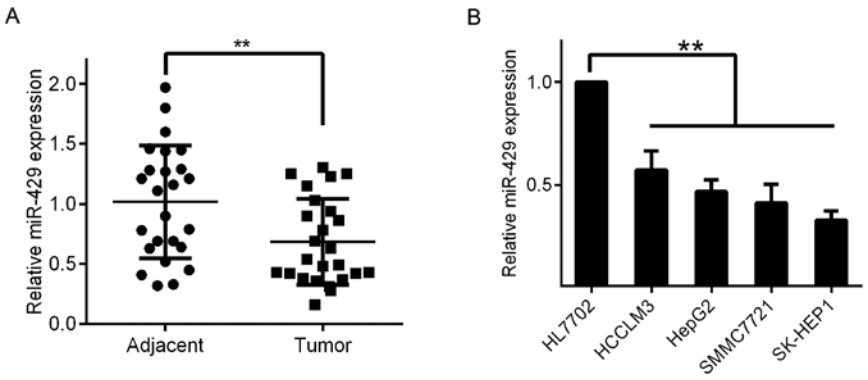

Figure 1. miR-429 is downregulated in HCC tissues and cell lines. (A) Relative expression level of miR-429 in HCC tissue samples and adjacent normal tissue samples detected by qRT-PCR. (B) Relative expression level of miR-429 in HCC cell lines and the normal liver cell line HL7702 detected by qRT-PCR. Data is expressed as the mean ± SD of three independent experiments. *P<0.05; **P<0.01.

experiments. The results are expressed as the relative luciferase activity (Firefly LUC/*Renilla* LUC). After luciferase activity was calculated for each miRNA, all were normalized taking miR-CON data as 1.

Tumorigenicity assays in nude mice. BALB/c athymic nude mice (male, 6-weeks old, 16-20 g) were purchased from Beijing HFK Bioscience, Co., Ltd. (Beijing, China) and bred under pathogen-free conditions. All animal experiments were approved by the Affiliated Hospital of Qingdao University Institutional Animal Care and Use Committee. Briefly, athymic nude mice were randomly divided into two groups, each containing 6 mice. Each mouse injected the right flank with 5x10^{6} LV-miR-429- or LV-miR-CON-infected SK-HEP1 cells in 150 μl phosphate-buffered saline (PBS). Thereafter, tumor sizes were measured every other day. When the sizes of the tumors were above the limit described by the animal protocol approved by the Affiliated Hospital of Qingdao University Medical College Institutional Animal Care, the mice were sacrificed and the tumors were removed. Tumors were fixed in 10% buffered formalin (HistoChoice Tissue Fixative MB; Amresco, Solon, OH, USA) then processed and embedded in paraffin. Immunohistochemistry was performed on 5-μm-thick paraffin sections mounted on charged slides. The sections were stained with H&E and immunostained with Ki-67 to observe proliferation. The sections were also immunostained to detect the expression of P65. For immunohistochemistry (IHC) staining, endogenous peroxidase was blocked using a 3% hydrogen peroxide solution. Primary antibodies (P65 with 1:100 dilution, Ki-67 with 1:100 dilution) were incubated overnight at 4˚C in antibody diluent with background reducing components (Beijing Zhongshan Jinqiao Biotechnology, Co., Ltd., Beijing, China). Secondary antibody, 1:250 HRP labeled anti-mouse/rabbit (Abcam), incubation was performed at room temperature for 30 min, and bound peroxidase was detected using the ABC Peroxidase kit (Beijing Zhongshan Jinqiao Biotechnology) and DAB (Beijing Zhongshan Jinqiao Biotechnology). All IHC slides were counterstained with hematoxylin.

Statistical analysis. Data were analyzed using the two-tailed Student's t-test. A P<0.05 was considered statistically significant.

Results

Downregulation of miR-429 is correlated with the progression of HCC. To measure the expression level of miR-429 in HCC, quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using 25 pairs of snap-frozen human primary HCC and corresponding adjacent tissue. As shown in Fig. 1A, miR-429 expression, in HCC tissues, was significantly lower than that in pair-matched adjacent tissue (P<0.01). These results suggested that miR-429 downregulation showed a positive correlation with the progression of HCC. Next, we determined whether miR-429 expression was similarly correlated in HCC cells lines. We compared miR-429 expression in five selected cell lines: HL7702 (human normal liver cells), SK-HEP1, HepG2, SMMC7721 and HCCLM3. Indeed, miR-429 was downregulated in the HCC cell lines compared with the normal liver cells HL7702 (Fig 1B). Among these HCC cell lines, miR-429 was upregulated in HCCLM3, which is used for anti-miR-429 experiment in the following process. On the contrary, miR-429, expressed at a low-level in the SK-HEP1, was upregulated in the miR-429-overexpression experiment. Collectively, the data indicated that miR-429 downregulation was significantly associated with the proliferation of HCC.

Overexpression of miR-429 suppresses HCC cell proliferation and migration. To investigate the effect of miR-429 on HCC cell proliferation and motility, we successfully constructed a recombinant lentiviral vector named LV-miR-429, LV-anti-miR-429 (miR-429 downregulation), and LV-miR-CON (and LV-miR-CON as control) to infect HCCLM3 or SK-HEP1 cells. After successful infection, miR-429 expression was confirmed by qRT-PCR (Fig. 2A). The results of the MTT assay showed that overexpression of miR-429 significantly suppressed HCC cell proliferation (Fig. 2B). Then, cell motility was measured by wound-healing assay and Transwell migration assays. The results showed that overexpression of miR-429 effectively suppressed HCC cell migration (Fig. 2C-F). These results revealed that miR-429 suppressed HCC cell proliferation and motility.

TRAF6 is a direct target of miR-429. To further elucidate the mechanism by which miR-429 affected cell proliferation and

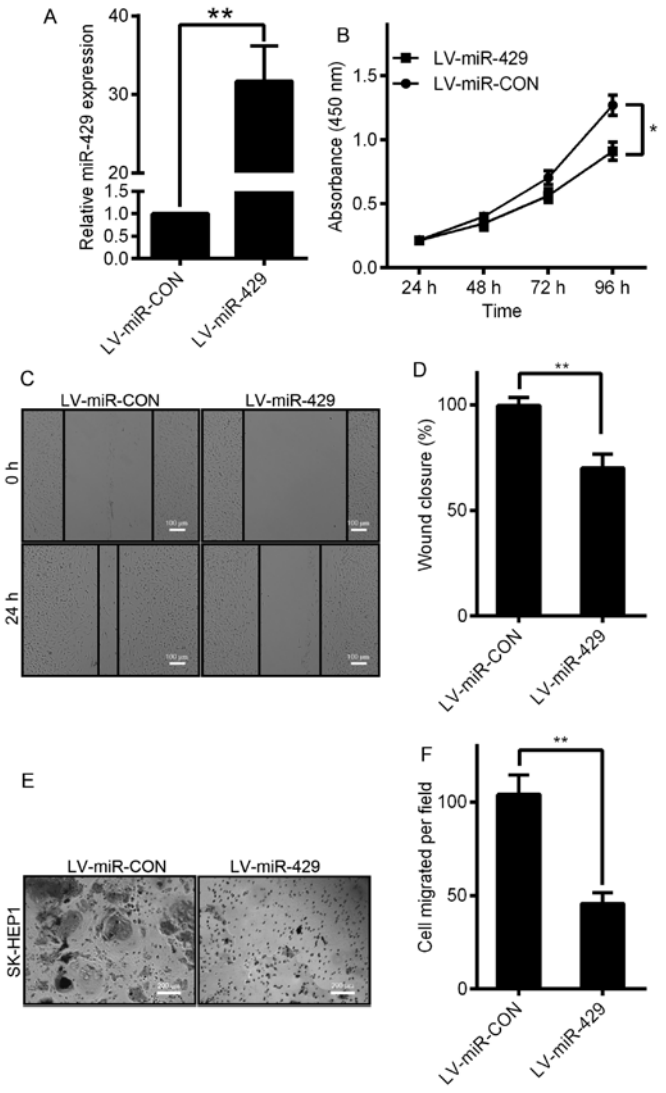

Figure 2. Upregulation of miR-429 suppresses HCC cell proliferation and migration. (A) SK-HEP1 cells were infected with LV-miR-429 or LV-miR-CON and the relative expression levels of miR-429 were measured by qRT-PCR. (B) Proliferation of pretreated SK-HEP1 cells was evaluated using the MTT assay. (C and D) Motility of pretreated SK-HEP1 cells was determined by wound-healing assays. (E and F) The effects of miR-429 on cell migration were confirmed by Transwell migration assay in SK-HEP1 cells infected with LV-miR-429 or LV-miR-CON. Data are expressed as the mean ± SD of three independent experiments. *P<0.05; **P<0.01.

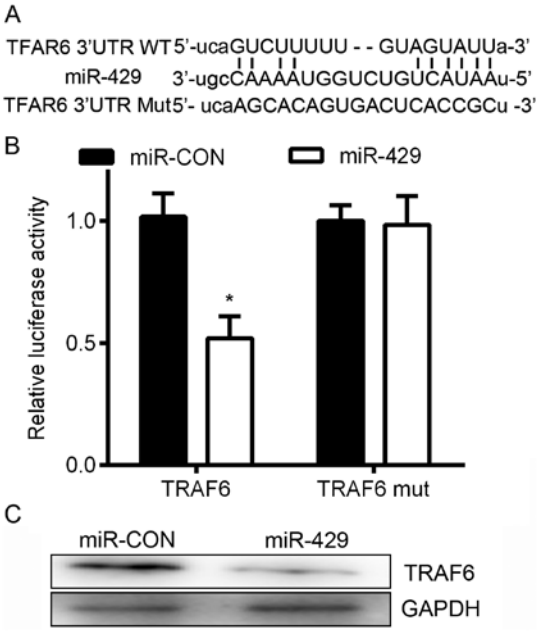

Figure 3. Prediction and confirmation of the miR-429 target. (A) Schematic diagram for construction of the miR-429 binding site in the pGL3 control vector. Mutation analysis of the miR-429 binding site. (B) Confirmation of the target gene of miR-429. HEK293 cells were co-transfected with miR-CON, miR-429 and pGL3/TRAF6 for the Dual-luciferase assay. PRL-TK containing *Renilla* luciferase was co-transfected with 3'-UTR of TRAF6 for data normalization. (C) TRAF6 protein levels in miR-429 treated HEK293 cells were detected by western blot analysis. The expression of GAPDH served as an internal control.

migration, we screened for potential targets of miR-429 using three target prediction programs with different algorithms: TargetScan, miRanda and PicTar. Based on the representation of the miR-429 sites in their 3'-UTRs, >100 mRNAs were predicted to be regulated by miR-429. Genes with >2-fold changes in expression were considered of interest. Among these candidates, the following six genes, TNF receptor associated factor 6 (TRAF6), zinc finger E-box binding homeobox 2 (ZEB2), WAS/WASL interacting protein family member 1 (WIPF1), neuregulin 1 (NRG1), heat shock protein family A (Hsp70) member 9 (HSPA9) and leber congenital amaurosis 5 (LCA5) were involved in the cancer proliferation and migration. TRAF6, a tumor promoter in human tumorigenesis, was the most downregulated among all miR-429 target genes (Fig. 3A). To confirm this finding, we subcloned the 3'-UTR region of TRAF6 mRNA, including the predicted miR-429 recognition site (wild-type, WT) or the mutated site (mutant type, Mut) into luciferase reporter plasmids. A Dual-luciferase reporter system was used. We found that the overexpression of miR-429 significantly suppressed WT, but not Mut 3'-UTR of TRAF6 (Fig. 3B). In addition, the western blot results showed that the overexpression of miR-429 significantly decreased the protein level of TRAF6 (Fig. 3C). Collectively, these results suggest that miR-429 downregulates TRAF6 expression by directly targeting its 3'-UTR.

TRAF6 downregulation partially attenuates the effect of miR-429. To further investigate whether downregulation of TRAF6 could attenuate the effect of miR-429 on HCC cells, siCON or siTRAF6 or LV-anti-miR-429 was transfected into HCCLM3 cells and the effect was validated by western blot analysis (Fig. 4A). The results of the MTT (Fig. 4B), wound-healing and Transwell migration assays (Fig. 4C-F) all showed that TRAF6 downregulation could significantly attenuate the effect of miR-429 on HCC cells. The data suggest that miR-429 inhibits HCC cell proliferation and motility, partially by targeting TRAF6.

Overexpreesion of miR-429 inhibits cell proliferation and NF-κB/TCF-4 signaling. To further study the role of miR-429 in inhibiting HCC cancer cell proliferation, miR-429 was overexpressed in SK-HEP1 cells. We found that the protein expression of pTAK1 (phosphorylation TAK1) and c-Myc was significantly decreased in cells infected with LV-miR-429 in

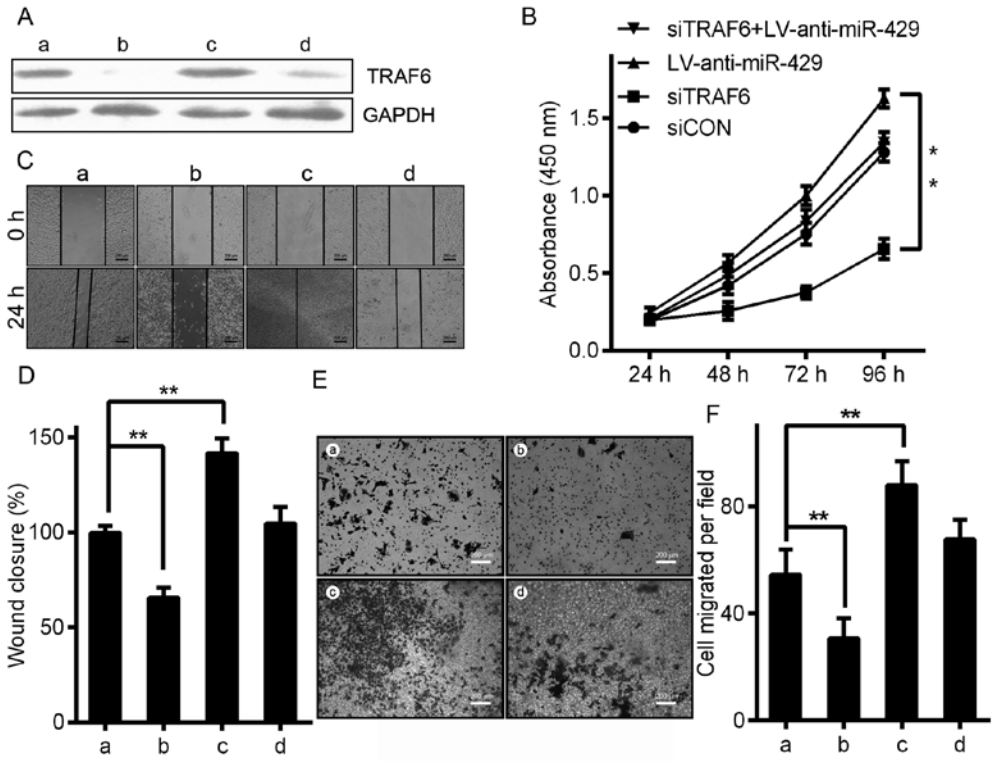

Figure 4. TRAF6 downregulation partially attenuates the effect of miR-429. (A) HCCLM3 cells were transfected with siTRAF6 or/and LV-anti-miR-429, and the relative protein levels of TRAF6 expression were detected by western blot analysis. (a) siCON group; (b) siTRAF6 group; (c) LV-anti-miR-429 group; (d) siTRAF6 and LV-anti-miR-429 group. (B) HCCLM3 cells were co-transfected with siTRAF6 or/and LV-anti-miR-429, and cell proliferation was examined by the MTT assay. (C and D) Cell motility of pretreated HCCLM3 cells were determined by wound-healing assays. (a) siCON group; (b) siTRAF6 group; (c) LV-anti-miR-429 group; (d) siTRAF6 and LV-anti-miR-429 group. (E and F) Cell motility of pretreated HCCLM3 cells were determined by Transwell migration assays. (a) siCON group; (b) siTRAF6 group; (c) LV-anti-miR-429 group; (d) siTRAF6 and LV-anti-miR-429 group. Data are expressed as the mean ± SD of three independent experiments. *P<0.05; **P<0.01.

comparison with those transfected with the LV-miR-CON (Fig. 5A and B). In contrast, co-transfection of LV-miR-429 and pCDNA-TRAF6 resulted in opposite effects on the levels of pTAK1 and c-Myc. Analysis of NF-κB localization by immunofluorescence indicated that the fraction of cells with nuclear P65 also decreased following upregulation of miR-429 (Fig. 5C and D). Furthermore, nuclear localization of P65 was increased in response to pCDNA-TRAF6 transfection (Fig. 5C and D), suggesting that miR-429 upregulation is sufficient to decrease cell proliferation and NF-κB signaling in at least a portion of HCC cancer cells.

miR-429 inhibits HCC tumor growth in nude mice. Considering the important role of miR-429 in HCC, we next used HCC xenograft models to further confirm the above findings *in vivo*. Because miR-429 was significantly downregulated in HCC, we successfully infected SK-HEP1 cells with LV-miR-429 or LV-miR-CON. The LV-miR-429 or LV-miR-CON-infected SK-HEP1 cells were then injected into the right flank of athymic nude mice to establish subcutaneous HCC xenografts. Twenty-four days after the injection, the tumors were removed and measured. The tumor sizes in the LV-miR-429 group were much smaller than those in the LV-miR-CON group (Fig. 6A and B). To clarify the cellular mechanisms underlying miR-429-mediated tumor growth, resected tissues from the subcutaneous xenograft tumors were analyzed to verify the expression of Ki-67 and P65. As shown in Fig. 6C and D, the LV-miR-429 group displayed reduced Ki-67 and P65 expression in the tumor tissues. The data provide strong evidence that miR-429 can inhibit HCC growth *in vivo*.

Discussion

Accumulated studies have shown that miRNAs play a crucial role in the process of tumor formation (14). It is well known that dysregulation of miRNAs is frequently observed in multiple types of cancers and plays fundamental roles in tumor initiation and progression. miRNAs impact the dynamic balance between oncogenes and tumor suppressor genes by degrading target genes, thereby, contributing to cancer progression (15). In the present study, we demonstrated that miR-429 was frequently downregulated in HCC tissues in comparison to non-tumorous liver tissues. In addition, its expression level is also decreased in HCC cell lines compared with normal liver cells. However, the role of miR-429 in HCC proliferation and migration remains unknown.

Previous studies have shown that the expression of miR-429 is significantly downregulated in several types of cancers, such as gastric (16), bladder (17), breast cancer (18), glioma (19), colorectal carcinoma (20) and oral squamous cell carcinoma (21). Ye and colleagues (18) found that overexpression of miR-429 in MDA-MB-231 cells remarkably suppressed invasion *in vitro*. Additionally, Zhang and colleagues (16) found that miR-429 is significantly downregulated in gastric

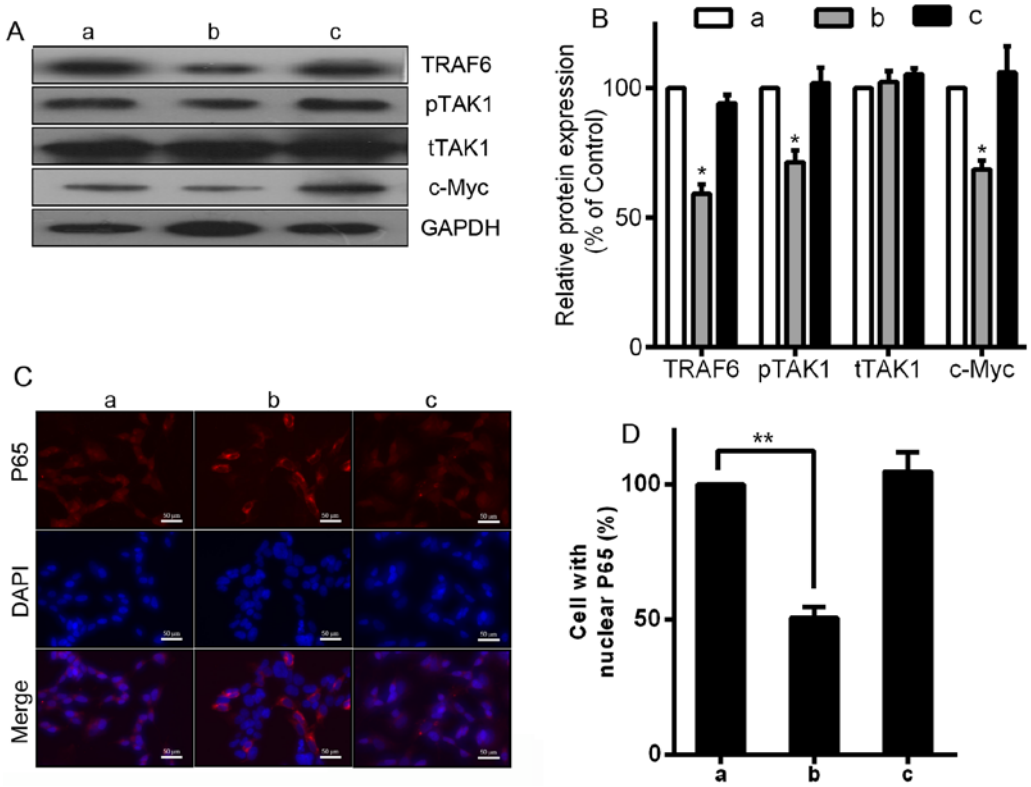

Figure 5. miR-429 inhibits nuclear translocation of NF-κB, and expression of pTAK1 and c-MYC. (A) pTAK1 and c-Myc expression at the indicated time-points after LV-miR-429 or LV-miR-429 plus pcDNA-TRAF6 transfection in SK-HEP1 cells was analyzed by western blot analysis. GAPDH was used as the loading control. (a) miR-CON group; (b) LV-miR-429 group; (c) LV-miR-429 plus pcDNA-TRAF6 group. (B) The bands were analyzed using the Quantity One analysis system (Bio-Rad Laboratories). The black histogram represents the optical densities of the signals quantified by densitometric analysis and presented as tTAK1, pTAK1, or c-Myc intensity/GAPDH intensity to normalize for gel loading and transfer. (a) miR-CON group; (b) LV-miR-429 group; (c) LV-miR-429 plus pcDNA-TRAF6 group. (C and D) SK-HEP1 cells were transfected with LV-miR-429 or LV-miR-429 plus pcDNA-TRAF6. Localization of P65 was analyzed by immunofluorescence 48 h after transfection. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). (a) miR-CON group; (b) LV-miR-429 group; (c) LV-miR-429 plus pcDNA-TRAF6 group. *P<0.05; **P<0.01.

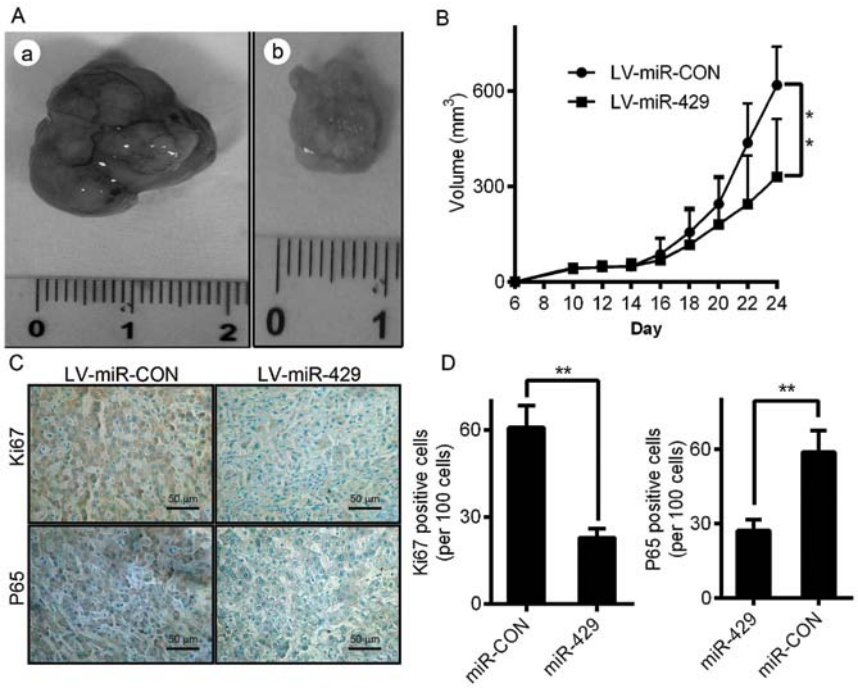

Figure 6. miR-429 inhibits HCC xenograft tumor growth *in vivo*. (A) SK-HEP1 cells infected with LV-miR-429 or LV-miR-CON lentivirus were injected subcutaneously into nude mice. From day 6, the tumor sizes were measured every other day, and tumor growth curves were obtained. Data are presented as the mean ± SD tumor volume. (a) LV-miR-CON group; (b) LV-miR-429 group. (B) Twenty-four days after injection of SK-HEP1 cells, the mice were sacrificed and photographed. (C) Immunohistochemical (IHC) staining of Ki-67 and P65 was performed using serial sections of subcutaneous xenograft tumors. Scale bar, 50 μm. (D) Ki-67 and P65 protein levels were measured by IHC in tissue sections from (C). **P<0.01.

cancer tissues in comparison to matched non-tumorous tissues. Overexpression of miR-429 in gastric cancer cells suppressed cell proliferation. Moreover, Sun and colleagues (20) showed that miR-429 was significantly downregulated in colorectal carcinoma (CRC) tissues and cell lines. The results reveal that miR-429 inhibited the proliferation and growth of CRC cells both *in vitro* and *in vivo*, suggesting that miR-429 could play a role in CRC tumorigenesis.

miR-429 significantly decreased the migration and proliferation of HCC cells *in vitro*, and reduced their capacity to develop HCC tumors *in vivo*. However, some investigators observed an opposite pattern of miR-429 expression in other cancers. For example, Lang *et al* (22) found that miR-429 are often upregulated in non-small cell lung cancer (NSCLC) in comparison to normal lung tissues, and its expression level is also increased in NSCLC cell lines in comparison to normal lung cells. High expression levels of miR-429 in A549 NSCLC cells significantly promoted cell proliferation, migration, and invasion, whereas inhibition of miR-429 inhibits these effects *in vitro* (22). It has been reported that the overexpression of miR-429 leads to a decrease in PTEN, RASSF8 and TIMP2 expression, whereas an adverse effect is observed when miR-429 is downregulated in NSCLC. Moreover, miR-429 has been found to be a potential target for NSCLC therapy. It was recently reported that ectopic expression of miR-429 markedly induced the expression of MMP2/7/9 and enhanced HCC migration and invasion *in vitro* and *in vivo* via regulating the classic Wnt pathway (23). Epigenetic modification of miR-429 can manipulate liver tumour-initiating cells by targeting the Rb binding protein 4 (RBBP4)/E2F1/OCT4 axis (24). However, miR-429 directly targeted NOTCH1 and reduced both mRNA and protein levels of NOTCH1 which stimulated proliferation and suppressed apoptosis in HCC cells in HBV-related HCC (25). Therefore, we conducted these experiments in order to answer this question regarding the relationship between miR-429 expression and HCC.

Through the use of TargetScan, miRanda and PicTar, we were able to predict that TRAF6 was a probable target of miR-429 in the 3'-UTR of TRAF6. We demonstrated that miR-429 overexpression resulted in the downregulation of TRAF6 at the protein level, whereas functional inhibition of miR-429 led to the upregulation of TRAF6. These results strongly suggest that TRAF6 is regulated by miR-429 in HCC. Moreover, a Dual-luciferase reporter assay identified TRAF6 as a direct target of miR-429.

Tumor necrosis factor receptor-associated factors (TRAFs) are a family of adaptor proteins that couple tumor necrosis factor receptor families to signaling pathways (26). As a key activator of nuclear factor-κB (NF-κB), TNF receptor-associated factor 6 (TRAF6) is known to transduce activating signals from the TNFR or Toll-like receptor (TLR)/IL-1 receptor family to NF-κB (27). At the molecular level, TRAF6 functions as an E3 ubiquitin ligase that activates IκB kinase (IKK), resulting in degradation of IκB, as well as nuclear translocation and activation of NF-κB as a physiologic response.TRAF6 has also been intermittently investigated in various cancer cells (28,29). Activation of some signaling mechanisms, including the NF-κB pathway, has been suggested to be involved in TRAF6-mediated oncogenesis (30). The generation of TRAF6 is essential for the recruitment of the downstream kinase TGFβ-activated kinase 1 (TAK1), which forms a complex with the ubiquitin-binding TAK1-binding proteins, TAB1, -2 and -3 (31). Subsequently, TAK1 activates the NF-κB pathway. In the present study, we provide solid evidence demonstrating a strong relationship between TRAF6 expression and HCC oncogenicity both *in vitro* and *in vivo*. Previously, it has also been shown that TRAF6 is able to modulate the degradation of TAK1 (32), which could impair the downstream effectors for pathogenesis. Therefore, a potential linkage between miR-429 and the phosphorylation of TAK1 was investigated. In HCC cells, TRAF6 gene silencing by short hairpin RNA led to a significant increase in NF-κB-dependent gene activity.

NF-κB is frequently activated in various types of tumors and promotes cancer development (33). Therefore, miRNAs that possess the NF-κB inhibitory activity may provide novel targets for anticancer therapy. Our data reveal a significant inhibitory role of miR-429 on NF-κB signaling and a reduced nuclear accumulation of NF-κB p65. Therefore, our results suggest that miR-429 downregulation is a novel mechanism that contributes to the abnormal activation of the NF-κB pathway in HCC cells. We demonstrated that miR-429 inactivated NF-κB signaling in order to inhibit migration and decrease motility of HCC cells. Previously, the level of TRAF6 also revealed a positive correlation with P65 expression (34). Therefore, the present study revealed a novel mechanism by which miR-429 regulates NF-κB signaling and contributes to cancer proliferation *in vitro* and *in vivo*. It is exciting to find that a single miRNA may suppress tumor growth via NF-κB signaling, which makes miR-429 a promising anticancer target in HCC.

Acknowledgements

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