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Original Article

miR-221 promotes growth and invasion of hepatocellular carcinoma cells by constitutive activation of NFκB

Zimin Liu[1*], Chenghong Wang[2*], Xuelong Jiao[3], Shanna Zhao[1,2], Xudong Liu[2], Yun Wang[2,4], Jian Zhang[3]

Departments of [1]Onocology, [3]General Surgery, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China; [2]Department of Clinical Laboratory, Yantaishan Hospital, Yantai, Shandong, China; [4]Lab of Tumor Immunology, Department of Anatomy and Histology & Embryology, Shanghai Medical College of Fudan University, Shanghai, China. [*]Equal contributors.

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Abstract: Background and Objective: microRNAs (miRs) are small noncoding RNAs that modulate a variety of cellular processes by regulating multiple targets, which can promote or inhibit the development of malignant behaviors. Accumulating evidence suggests that microRNA-221 (miR-221) plays important roles in human carcinogenesis. It has recently found that miR-221 was overexpressed in hepatocellular carcinoma (HCC), and overexpression of miR-221 has a bad prognosis in these patients. Thus, down-regulation of miR-221 expression in HCC would provide new treatment approaches. This study aimed to study the role of miR-221 on HCC cell growth, apoptosis, invasion and metastasis in vitro and vivo, and explored the possible mechanisms involved. Methods: Effects of miR-221 upregulation or miR-221 downregulation by miR-221 inhibitor (anti-miR-221) or miR-221 mimic (miR-221) transfection on growth, apoptosis and invasion of HepG2 cells in vitro was detected. Using p65 siRNA and p65 cDNA transfection to examine the NFκB signaling pathway. A subcutaneously implanted tumor model of HepG2 cells in nude mouse was used to assess the effects of anti-miR-221 or miR-221 overexpression on tumorigenesis development. Using an intravenously injected tumor model of HepG2 cells to assess the effects of anti-miR-221 or miR-221 overexpression on lung metastasis. The signaling pathway was analyzed in vivo. Results: Anti-miR-221 inhibited growth, invasion and induced apoptosis of HepG2 cells in vitro. This was accompanied by concomitant attenuation of NFκB, and downregulation of NFκB downstream genes such as Bcl-2, VEGF and MMP-9. In addition, miR-221 overexpression promoted growth and invasion of HepG2 cells in vitro, and accompanied by activation of NFκB, and upregulation of NFκB downstream genes Bcl-2, VEGF and MMP-9. Targeting P65 or P65 overexpression reversed the effect of miR-221, and inhibited or induced miR-221 expression, creating a positive feedback loop in human HepG2, respectively. Morever, stable overexpression of anti-miR-221 in HepG2 cells inhibits establishment of xenografts and lung metastasis in nude mice; Stable overexpression of miR-221 in HepG2 cells promotes establishment of xenografts and lung metastasis in nude mice. Conclusions: Therapies targeting the miR-221 signaling pathway may be more effective to prevent primary tumor formation and organ metastasis. The ability of this therapy to decrease tumorigenesis and metastasis may be related to NFκB signals.

Keywords: Hepatocellular carcinoma, metastasis, apoptosis, microRNA-221, NFκB

Introduction

Hepatocellular carcinoma (HCC) is the most common liver cancer, accounting for 90% of primary liver cancers [1]. The incidence rate of HCC in Asian countries is increasing, since chronic hepatitis or liver diseases are prevalent in these areas [2]. Currently, there are no well-established effective adjuvant therapies for HCC and control of HCC at the initial stage is the most effective therapeutic strategy available. However, this cancer is difficult to diagnose and confirm at the initial stage. Most HCC patients are diagnosed at advanced/symptomatic stages when limited therapeutic options are available. Therefore innovative research findings are necessary to understand the etiology of cancer and to improve the treatment and survival of patients.

The development of successfully targeted therapies for HCC is dependent on the identification

of signalling pathways used by tumour cells to proliferate, invade or metastasize during the progress of tumour growth. MicroRNAs (miRs) are small non-coding RNAs, regulating gene expression post-transcriptionally. They mediate fundamental cellular processes such as proliferation, differentiation and apoptosis and are actively involved in carcinogenesis [3].

A recent study has shown that miR-221 stimulates the onset of tumors and promotes tumor progression, significantly shortening the mean time to death in a mouse model of liver cancer [4]. Another study offers preclinical proof for the efficacy of chol-anti-miR-221 in a valid orthotopic mouse model of HCC, suggesting that this targeted agent could improve treatment for patients with advanced HCC [5]. The dysregulation roles of miR-221 in cellular apoptosis and differentiation indicate the negative regulatory effect of miR-221 [4]. Other studies have found that suppression of oncogenic miR-221 resulted in prolonged survival and significant reduction in the number and size of tumors in comparison with untreated animals [6]. Similarly, HCC cells transfected with anti-miR-21 were significantly sensitive to chemotherapy with combined interferon-α and 5-FU [7]. Thus, the use of synthetic miR-221 inhibitors may be a promising approach for HCC treatment.

The promotive roles of miR-221 in tumor progression may result from the interfering function of miR-221 against some cellular anti-oncogenes. The NFκB pathway has been recognized as an underlying link between inflammation and malignancy [8]. In recent years, several results have established strong support for the critical role of NFκB in many types of cancer, including HCC [9-13]. NFκB is aberrantly expressed and activated in both human HCC tissue and HCC cells [14, 15].

miRs not only directly regulate NFκB expression, but also up- or down-regulate NFκB activity via upstream and downstream signaling pathways of NFκB. Several miRNAs have been reported to modify cell behavior by regulating the NFκB pathway [16, 17]. In human colorectal cancer cell cells, miR-221 and miR-222 act in a positive feedback loop to increase expression levels of RelA and STAT3, resulting in increase of growth of colon tumors in mice with colitis [18]. In HUVECs, overexpression of miR-221 inhibited adiponectin-stimulated nitric oxide (NO) production, and miR-221 abolished the inhibitory effect of adiponectin on NFκB activation and the expression of adhesion molecules [19]. However, in prostate carcinoma and glioblastoma cells, NFκB contribute to tumorigenesis by inducing the expression of the miR-221/222 [20]. Although SND1 regulates NFκB and miR-221, two important determinants of HCC controlling the aggressive phenotype, the regulation between NFκB and miR-221 in HCC cells is not very clear [21].

In the present study, we assessed the effect of miR-221 on invasion, apoptosis and growth of HCC cells in vitro and vivo, and to explore its molecular mechanisms. Our findings demonstrate that miR-221 enhanced growth at least in part via activation of the NFκB signaling pathway. This study reveals a novel mechanism of miR-221 on growth and invasion of HCC cells and may also provide a novel therapeutic target.

Materials and methods

Cell line and culture

The HepG2 cell line was purchased from the Insitute of Biochemistry and Cell Biology (Shanghai, China). The cell line was maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) at 37°C in a humid atmosphere of 5% CO_2. Stable p65 siRNA and p65 cDNA transfection.

Control siRNA and siRNAs for p65 were obtained from Santa Cruz Biotechnology. P65 cDNA plasmid was kindly gifted by Dr Chen (General surgery, the affiliated hospital of Qingdao University). siRNA or cDNA or control constructs were transfected into HepG2 cell cells using Lipofectamine 2000 according to the manufacturer's protocol. 24 hrs after transfection, the cells were split into 96-well plates and subjected to the G418 (1 mg/ml) selection for 2 weeks. All transfection experiments were done at least three times.

Anti-miR-221 and miR-221 mimics transfection

miR-221 mimics (miR-221) and scrambled oligonucleotides were purchased from Geneco-

poeia (China); miR-221 inhibitor (anti-miR-221) and or non targeting negative control stranded RNA were synthesized in Ribobio Inc (Guangzhou, China) based on the sequence of miR-221 in miRBase database.

miR-221 or anti-miR-221 or control constructs were transiently transfected into HepG2 cells for 72 h using Lipofectamine 2000 according to the manufacturer's protocol. For stable transfection, 24 hrs after anti-miR-221 or miR-221 or their control transfection, the cells were split into 96-well plates and subjected to the G418 (1 mg/ml) selection for 2 weeks. All transfection experiments were done at least three times.

The stably siRNA or cDNA transfected HepG2 cells were transiently transfected with miR-221 or anti-miR-221 or control constructs for 72 hrs using Lipofectamine 2000 according to the methods above.

The stable miR-221 or anti-miR-221 or control constructs were transiently transfected with siRNA or cDNA for 48 hrs using Lipofectamine 2000 according to the methods above.

Invasion assay

The upper chamber of each transwell was coated with Matrigel (BD Biosciences, MA, USA) 1:6 diluted with DMEM at 37°C for 3 h. Cells (2×10^4) were seeded in upper chambers in DMEM and incubated in 24-well-plates with 10% FBS supplemented DMEM. After 48 h of incubation, cells remaining on the upper surface of the membrane were removed with a cotton swab. Cells that invaded through the Matrigel-precoated membrane filter were fixed. The cells were counted at × 100 magnification in 10 random fields of view under a microscope. Three independent experiments were performed in each case.

Cell viability assay

Cell viability was detected by LDH release assay. Briefly, culture medium was collected and LDH activity was assessed using a LDH cytotoxicity assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's protocol. LDH activity was quantified by measuring absorbance at 490 nm with a microplate reader (MRXII photometer, Thermo Scientific, Courtaboeuf, France). The ratio of released LDH over total LDH was calculated and presented as relative LDH release compared to non-treated cells. All experiments were performed in triplicate and repeated 3 times.

Cell proliferation assay

The BrdU assay was performed using a BrdUrd cell proliferation assay kit from Oncogene (San Diego, CA) according to manufacturer's instructions. The BrdU-labeling index, reported as the percentage of cells labeled with BrdU, was determined by counting 10,000 cells from two independent reactions using the Zeiss Axiovert 40 inverted microscope and the AxioVision Rel. 4.8.2 software (Carl Zeiss, New York, USA).

Colony formation assay

Stable transfected HepG2 cells were seeded at a density of 300/ml on 35-mm dishes. Colonies were allowed to grow for 14 days. The medium was discarded and each well was washed twice with phosphate buffered saline (PBS) carefully. The cells were fixed in methanol for 15 min, and then stained with crystal violet for 20 min. Finally, positive colony formation (more than 50 cells/colony) was counted.

Apoptosis assay

Apoptosis induction was quantified by Annexin V/PI double staining followed by flow cytometry. Annexin V/PI double staining was performed using an apoptosis detection kit (Biovision, Mountain view, CA) following the manufacturer's instruction. Briefly, after various transfection, cells were gently detached by brief trypsinization, and then washed with ice cold PBS. After another wash with binding buffer, cells were suspended in 300 μL binding buffer containing Annexin V and propidium iodide, and incubated for 5 min at room temperature. Early apoptotic cells were identified as Annexin V positive/PI negative cells, while late apoptotic/necrotic cells were identified as Annexin V positive/PI positive cells using a BD LSR II cell analyzer.

qRT-PCR analysis

At every experimental end point, RNA was extracted from cells using TRIzol® (Invitrogen). Following TRIzol® RNA preparation, 350 μl of

RLT buffer from RNeasy mini kit (Qiagen) was added to dissolved RNA. Then add 500 μL of RPE buffer to the RNeasy Mini spin column and repeat the 12,000 × *g* centrifugation for 15 s. Again discard the flow-through from this wash step. Repeat the wash step of the RNeasy Mini spin column with another 500 μL of RPE buffer. To ensure all buffer is removed from the column, centrifuge for 2 min 12,000 × *g* and discard the flow-through. To ensure the column is completely dry before eluting the RNA, discard the old collection tube with the flow-through and place the RNeasy Mini spin column into a new collection tube. Transfer the RNeasy Mini spin column to a new 1.5 mL collection tube. Add 30 μL of RNase-free water directly onto the RNeasy Mini spin column membrane, wait 1 min. To elute the RNA, centrifuge the RNeasy Mini spin column for 2 min at 12,000 × *g*. For each sample, pool the 30 μL of eluates from the multiple spin columns and measure mir-221 concentration.

The cDNA was synthesized from total RNA using a TaqMan miRNA Reverse transcription kit (Applied Biosystems, USA). Briefly, 10 μL of the supplied 5 × Reverse Transcription (RT) Primer for miR-221 (5'-TGAGCCAGCGGTGTATGGC-3' and 5'-CGTCGTAGACCTAGCTAACGAGGCA-3') and endogenous control U6 (5'-CTCGCTTCGGCAGCACA-3' and 5'-AACGCTTCACGAATTTGCGT-3') was added to the pools. The RT Primer Pool was to be prepared at a final volume of 1 mL. If the resultant solution is less than 1 mL, adjust volume to achieve 1 mL with molecular grade water. The RT Primer Pools are to be stored at -20°C. In a 200 μL PCR tube, add 100 ng RNA in a final volume of 12.75 μL then add 2.25 μL of DNase master mix. Vortex then centrifuge tube for 30 s in a microcentrifuge. Incubate the reaction in a thermal cycler for 10 min at 37°C followed by an incubation at 90°C for 5 min. To the denatured RNA, add 10 μL of the appropriate RT Primer Pool and 25 μL of Reverse Transcription Master Mix, final volume is 50 μL. Mix and centrifuge tube for 30 s in a microcentrifuge. Transfer the RT reaction to a thermal cycler and perform the following incubations: 95°C for 10 min; 55°C for 2 min; 72°C for 2 min; 95°C for 15 s; 60°C for 1 min; Repeat 95°C for 15 s and 60°C for 1 min for 12 cycles followed by a 4°C hold. Dilute the amplification product 1:50 with molecular grade water and store at -20°C until needed or proceed immediately to qRT-PCR

qRT-PCR was performed using a miR-221 MicroRNA Taqman assay (Applied Biosystems) following the manufacturer's instruction , with slight modification. Briefly, 1 μg of total RNA was reverse-transcribed to cDNA using AMV reverse transcriptase (TaKaRa, Dalian, China) and a stem-loop RT primer (Applied Biosystems). The reaction conditions were: 16°C for 30 min, 42°C for 30 min, 85°C for 5 min. Real-time PCR was performed using a TaqMan PCR kit on an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems). The reactions were incubated in a 96-well optical plate at 95°C for 10 min, followed by 38 cycles of 95°C for 15 s and 55°C for 1 min. All reactions were run in triplicate. After the reactions, the threshold cycles (C_T) values were determined using fixed threshold settings, and the mean C_T was determined from the triplicate PCRs. In the experiments presented here, a comparative C_T method was used to compare each condition with controls. miRNA expression in cells was normalized to that of the U6 snRNA. The amount of miR-221 relative to the internal control U6 was calculated with the equation $2^{-\Delta\Delta CT}$, in which $\Delta\Delta C_T=(C_{T\ miR\text{-}221}-C_{T\ U6})_{target}-(C_{T\ miR\text{-}221}-C_{T\ U6})_{control}$.

Electrophoretic mobility-shift assay (EMSA)

After transfection, nuclear extracts were prepared from cells, and the NFκB activity was measured by electrophoretic mobility shift assay (EMSA) according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). The NFκB oligonucleotide was end-labeled with [γ-^{32}P] ATP (Free Biotech, Beijing, China) using T4-polynucleotide kinase. Binding reactions were performed by adding 10 μg of nuclear extracts to 7 μl of binding buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 4% glycerol, 0.05 g/l polydeoxyinosinic deoxycytidylic acid, and 1 μl of [γ-^{32}P]ATP-labeled oligonucleotide probes (5'-AGTTGAGGGGACTTTCCCAGGC-3') (Sigma-Aldrich). The binding specificity of the DNA/protein binding was determined by competitive reactions in which a 100 fold molar excess of unlabeled NFκB oligonucleotide was added to the binding reaction 10 min before the addition of the biotin probe. Mixed samples were incubated at room temperature (25°C) for 30 min and fractionated by electrophoresis on a 4% non-denaturing polyacrylamide gel in a TBE (Tris-borate EDTA) buffer that was pre-electrophoresed for 1 h at 100 V. After electropho-

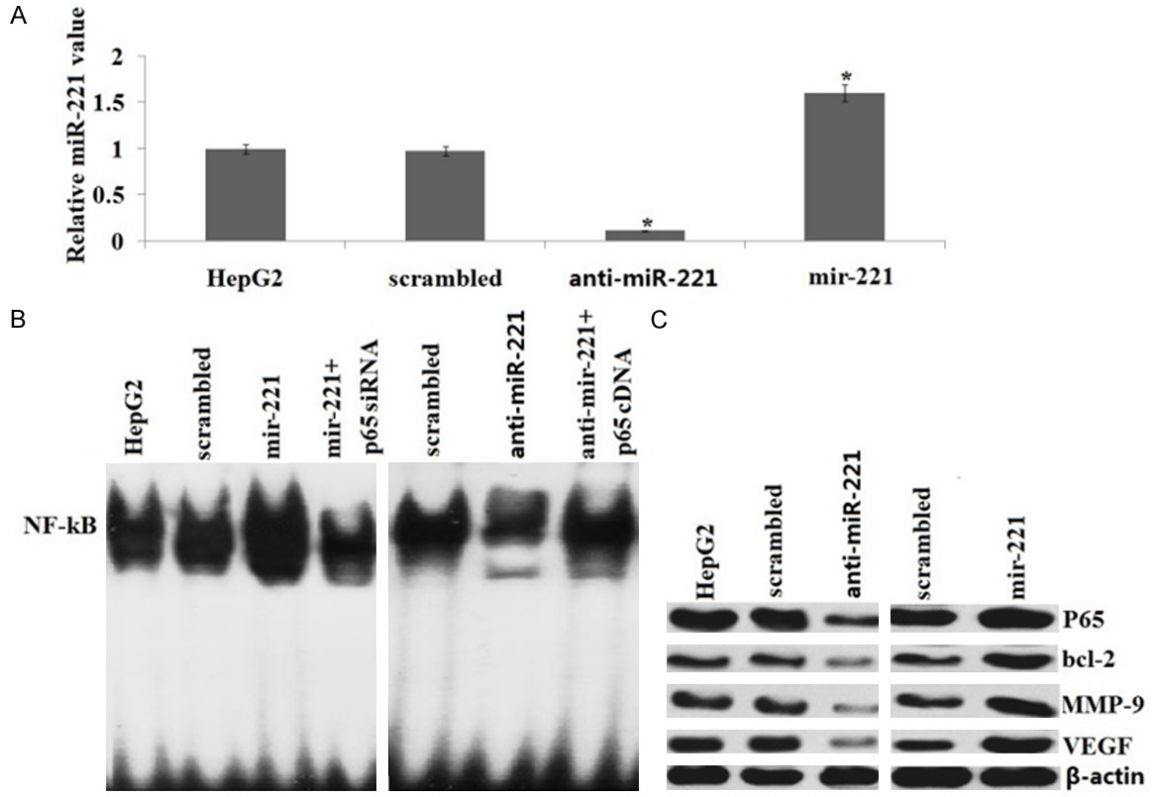


Figure 1. Effect of mir-221 on NFκB and its signals. HepG2 cells were transfected with mir-221 inhibitor or mir-221 mimics for 72 hrs. A: miR-221 mRNA was detected by qRT-PCR assay; B: NFκB activity was detected by EMSA assay; C: NFκB signals molecular was detected by western blot assay.

resis, gels were dried and autoradiographed to detect NFκB activity.

Western blot assay

For total protein extraction, cells were washed once with phosphate-buffered saline (PBS) and lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% Nonidet P-40, supplemented with complete protease inhibitor tablets; Roche Diagnostics) for 30 minutes on ice. Protein concentration was quantified with Coomassie Plus (Bradford) Protein Assay Reagent according to manufacturer's instructions. Extracts (40 μg) were resolved on 10% SDS-PAGE and transferred to Hybond-C Extra nitrocellulose membrane (GE Healthcare; Germany). Membranes were probed with primary antibodies against p65, MMP-9, VEGF and Bcl-2 followed by incubation for 1 hour at room temperature with HRP-conjugated anti-rabbit IgG or anti-goat IgG, respectively. Immunoblotting for β-actin served as protein loading control. All experiments were performed at least three independent times.

Xenograft model in nude mice

Xenograft tumors were generated via the subcutaneous injection of HepG2 cells (2×10^6, HepG2/scramble or HepG2/anti-miR-221/or miR-221 mimics) into the hind limbs of 4-6 week-old Balb/C athymic nude mice. All mice were housed and maintained under specific pathogen-free conditions, and all experiments were approved by the Use Committee for Animal Care and performed in accordance with institutional guidelines. Tumor size was measured by a slide caliper, and tumor volume was determined using the formula: $0.44 \times A \times B^2$ (A is the diameter of the base of the tumor, B is the corresponding perpendicular value). The tumors were excised after euthanasia, fixed in 10% neutral buffered formalin, and embedded in paraffin before preparing 4 μm sections, which were stained with hematoxylin.

For metastasis assay, 1×10^6 HepG2/anti-miR-221/mimics or control were intravenously injected through the tail vein. Mice were sacrificed, the lungs were fixed, paraffin-embedded,

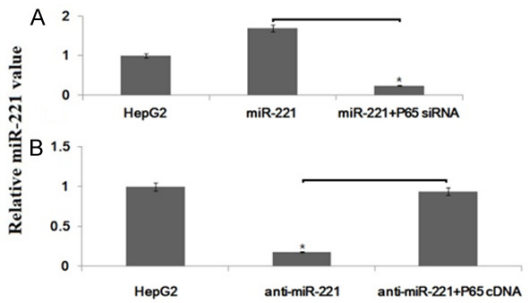


Figure 2. Effect of NF-κB on miR-221 expression. A: The stable miR-221 transfected HepG2 cells were transiently transfected with P65 siRNA for 48 hrs, miR-221 mRNA was detected by qRT-PCR assay; B: The stable miR-221 transfected HepG2 cells were transiently transfected with P65 siRNA for 48 hrs, miR-221 mRNA was detected by qRT-PCR assay. *P<0.01.

cut, and stained with H&E staining after 2 weeks.

TUNEL assay

TUNEL was performed with an In situ Cell Death Detection Kit (Roche). Cell apoptosis was quantified by determining the percentage of positively stained cells for all of the nuclei in 20 randomly chosen fields/section at 200 × magnification. Slides of the apoptosis studies were quantified in a blind manner by two independent reviewers two different times.

Statistical analysis

All data are presented as mean ± S.E. Statistical analyses were performed using SPSS.21 Software. The significance was determined by two tailed Student's *t* test or one-way analysis of variance with Bonferroni post tests where applicable. Experiments were performed in triplicate. *p* value less than 0.05 was considered statistically significant.

Results

Mir-221 regulates the NFκB signaling pathway in HepG2 cells

After a 72 hrs transient transfection of anti-miR-221, mir-221 mRNA expression in HepG2 cells lines was decreased by more than 90% as compared to noninduced cells, or those transduced with negative control stranded RNA using qRT-PCR assay (**Figure 1A**). In addition, qRT-PCR demonstrated that after a 72 hrs transient transfection of mir-221, mir-221 mRNA expression in HepG2 cells lines was increased by more than 50% as compared to noninduced cells, or those transduced with scrambled oligonucleotides (**Figure 1A**).

Overexpression of miR-221 significantly increased the NFκB activity (**Figure 1B**), and downregulation of miR-221 decreased NFκB activity (**Figure 1B**). In addition, western blotting demonstrated that the protein expression levels of numerous well-characterized NF-κB downstream genes, such as MMP-9, VEGF and Bcl-2 was upregulated in miR-221-upexpressed HepG2 cells (**Figure 1C**) and downregulated in miR-221-downregulated HepG2 cells (**Figure 1C**), suggesting that mir-221 may contribute to activation of NF-κB.

NF-κB regulates miR-221 expression

We examined whether the expression of miR-221 is regulated by NF-κB. First, we observed that the expression of miR-221 was markedly downregulated when the stable miR-221 transfected HepG2 cells were transiently transfected with P65 siRNA for 48 hrs (**Figure 2A**). Second, we observed that the expression of miR-221 was markedly upregulated when the stable anti-miR-221 transfected HepG2 cells were transiently transfected with P65 cDNA for 48 hrs (**Figure 2B**). These results collectively demonstrate that miR-221 positively regulate NF-κB signaling pathways, which in return induce the expression of miR-221, creating a miR-221-mediated positive feedback loop.

miR-221-induced invasion involves NFκB activation

To investigate the biological function of miR-221 during the pathogenesis of HCC, we established stable miR-221/HepG2 cells and stable miR-221 inhibitor/HepG2 cells. As shown in **Figure 3A**, overexpression of miR-221 significantly increased the invasive ability of HepG2 cells in vitro. However, downregulation of miR-221 significantly decreased the invasive ability of HepG2 cells in vitro (**Figure 3B**). Negative control stranded RNA and scrambled oligonucleotides did not affect invasive ability of HepG2 cells.

We now determined whether miR-221 mediated NFκB-induced invasion in HepG2 cells. We transfected P65 cDNA or P65 siRNA into the

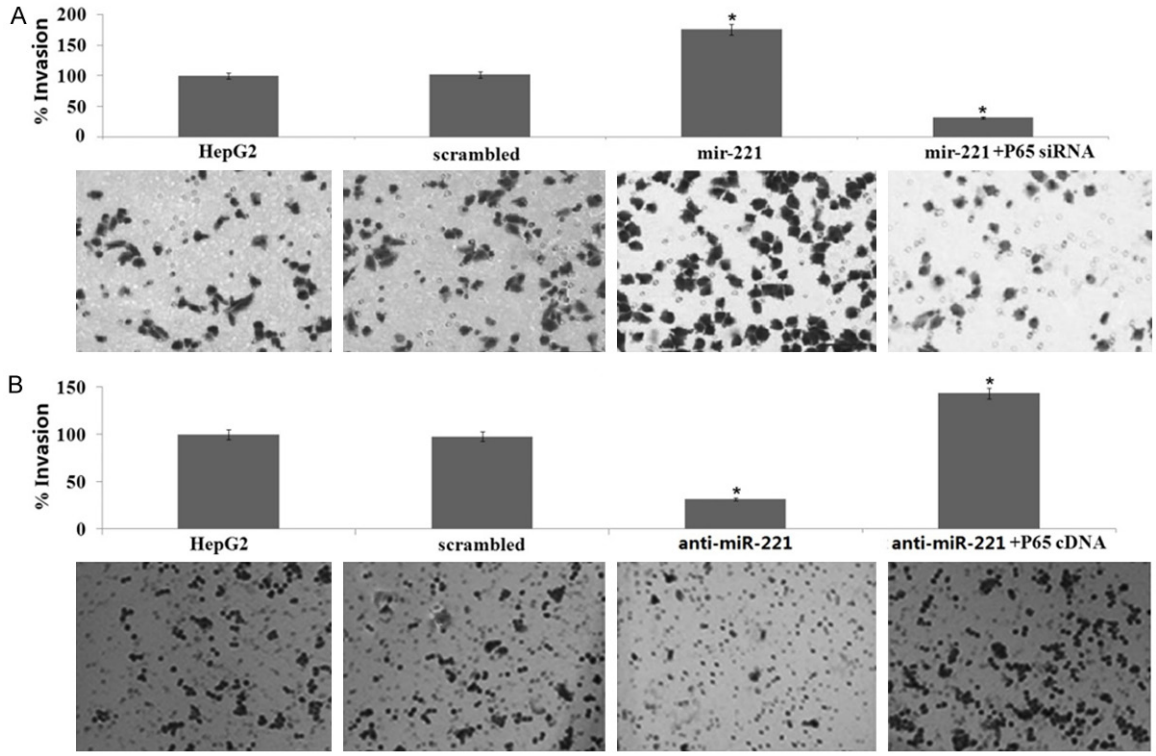


Figure 3. Effect of Mir-221 on invasion of HepG2 cells in vitro. A: HepG2 cells was transfected with mir-221 minics or combined with p65 siRNA transfection, HepG2 cells invasion was detected by matrigel invasion assays; B: HepG2 cells was transfected with mir-221 inhibitor or combined with p65 cDNA transfection, HepG2 cells invasion was detected by matrigel invasion assays; *P<0.01 compared with respective controls.

stable anti-mir-221/HepG2 cells or stable mir-221/HepG2 for 48 h. Compared with the stable anti-miR-221/HepG2 cells, cell invasive ability was significant increased when P65 cDNA was transfected into the stable anti-miR-221/HepG2 cells (**Figure 3B**). On the contrary, the cell invasive ability of stable miR-221/HepG2 cells was significant decreased when p65 siRNA was transfected into the stable miR-221/HepG2 cells (**Figure 3A**).

Targeting miR-221 induces apoptosis and inhibits growth of HepG2 cells by inactivation of NFκB

To investigate the function of miR-221 silencing on HepG2 cells in vitro, we transiently transfected anti-miR-221 to the HepG2 cells for 72 h. As shown in **Figure 4A**, anti-miR-221 significantly showed a significant increase in the percentage of apoptotic cells present. In addition, cell viability (**Figure 4B**) and proliferation (**Figure 4C**) inhibited compared to cells expressing negative control stranded RNA. Furthermore, the stable anti-miR-221/HepG2 cells showed decreased number of colony compared to cells expressing negative control stranded RNA (**Figure 4D**), supporting the hypothesis that miR-221 is necessary to sustain HepG2 cell growth.

We now determined whether NFκB mediated anti-miR-221-induced apoptosis and growth inhibition in the HepG2 cells. We transfected anti-miR-221 into the stable P65 cDNA/HepG2 cells for 72 h. We found that P65 cDNA transfection inhibited apoptosis in anti-miR-221-transfected HepG2 cells (**Figure 4A**). In addition, transfection of P65 cDNA increased anti-miR-221-induced cell viability and proliferation (**Figure 4B**, **4C**). The oligonucleotides did not affect the effect of P65 cDNA on HepG2 cells (data not shown). These results were in favor of our claim that the apoptosis-inducing effect by targeting miR-221 is partly mediated through the inactivation of NFκB pathway.

miR-221 promotes growth of HepG2 cells in vitro by activation of NFκB

After a 72 hrs transfection of miR-221, the relative growth of the HepG2 cells was then mea-

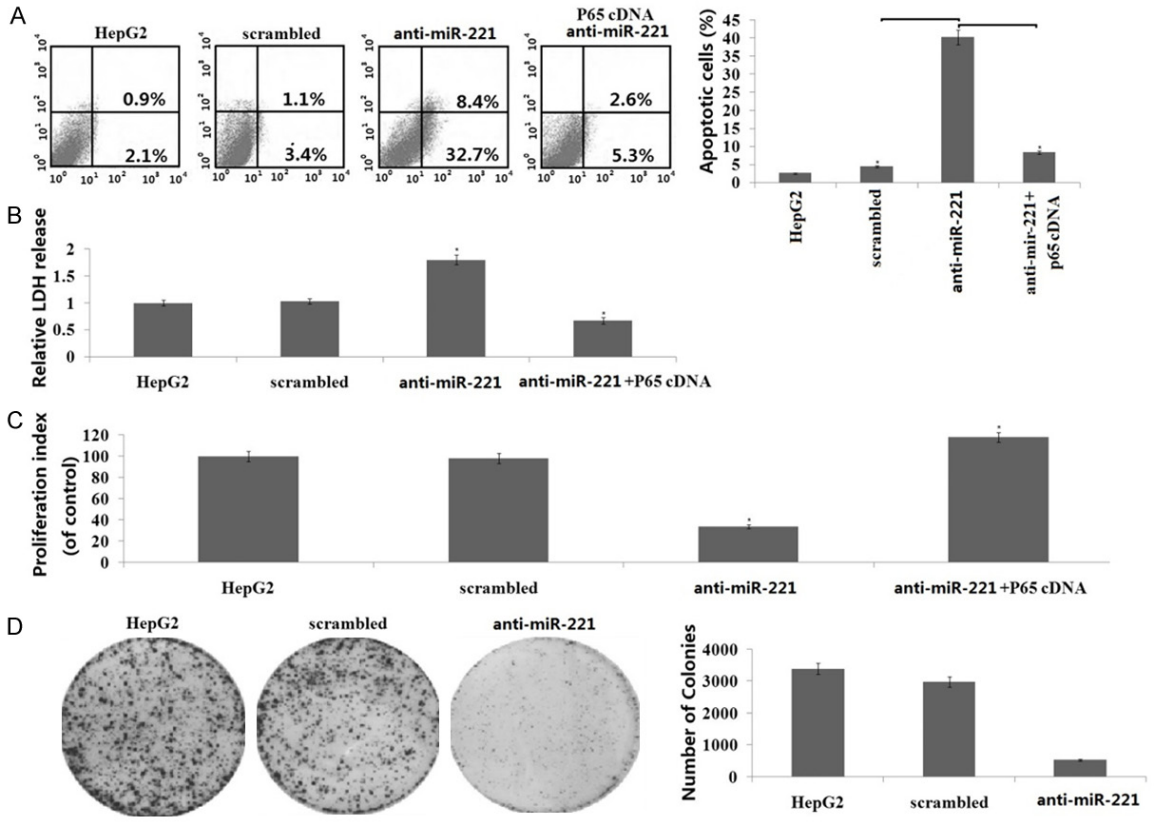


Figure 4. Targeting Mir-221 induces apoptosis and inhibits growth and colony formation of HepG2 cells in vitro. HepG2 cells were transfected with mir-221 inhibitor or combined with p65 cDNA transfection. A: HepG2 cells apoptosis was detected by FCM; B: Cell viability by LDH toxicology assay; C: Cell proliferation by BrdUrd cell proliferation assay; D: Cell growth by colony formation assay. The data are shown as the mean ± SD. The experiments were all repeated at least 3 times to confirm the reproducibility of the results. *P<0.01 versus the control.

sured over time by LDH release and BrdU assay. HepG2 cells expressing miR-221 showed significant growth promotion compared to HepG2 cells expressing scrambled oligonucleotides (**Figure 5A**, **5B**). In addition, the stable miR-221/HepG2 cells showed increased number of colony compared to cells expressing scrambled oligonucleotides (**Figure 5C**).

We transfected miR-221 into the stable P65 siRNA/HepG2 cells for 72 h. We found that P65 siRNA transfection enhanced apoptosis induced by miR-221 transfection (**Figure 5D**). The negative control stranded RNA did not affect the effect of P65 siRNA on HepG2 cells (data not shown).

miR-221 contributes to the progression of HCC in vivo

Given that suppression of anti-miR-221 inhibited the proliferation and invasion of HepG2 cells, and vice versa in vitro, we next investigated the effect of miR-221 on the tumorigenicity and metastasis of HepG2 cells using an in *vivo* mice model. As shown in **Figure 6A**, the tumors formed by miR-221-transfected HCC cells grew more rapidly and were larger in size, while the tumors formed by anti-miR-221-transfected cells were smaller in size, compared to the tumors formed by control cells (**Figure 6A**). Moreover, the tumors established using anti-miR-221-transfected HepG2 cells showed increased TUNEL-positive cells (**Figure 6B**), the tumors established using miR-221-transfected HepG2 cells showed decreased TUNEL-positive cells (**Figure 6B**). We next determined whether miR-221 is involved in regulation of the NFκB signaling pathway *in vivo*. The anti-miR-221-transfected tumors showed decreased miR-221 mRNA expression (**Figure 5C**), NFκB activity (**Figure 6E**) and P65, MMP-9, VEGF expression (**Figure 5D**). On the contrary, the miR-

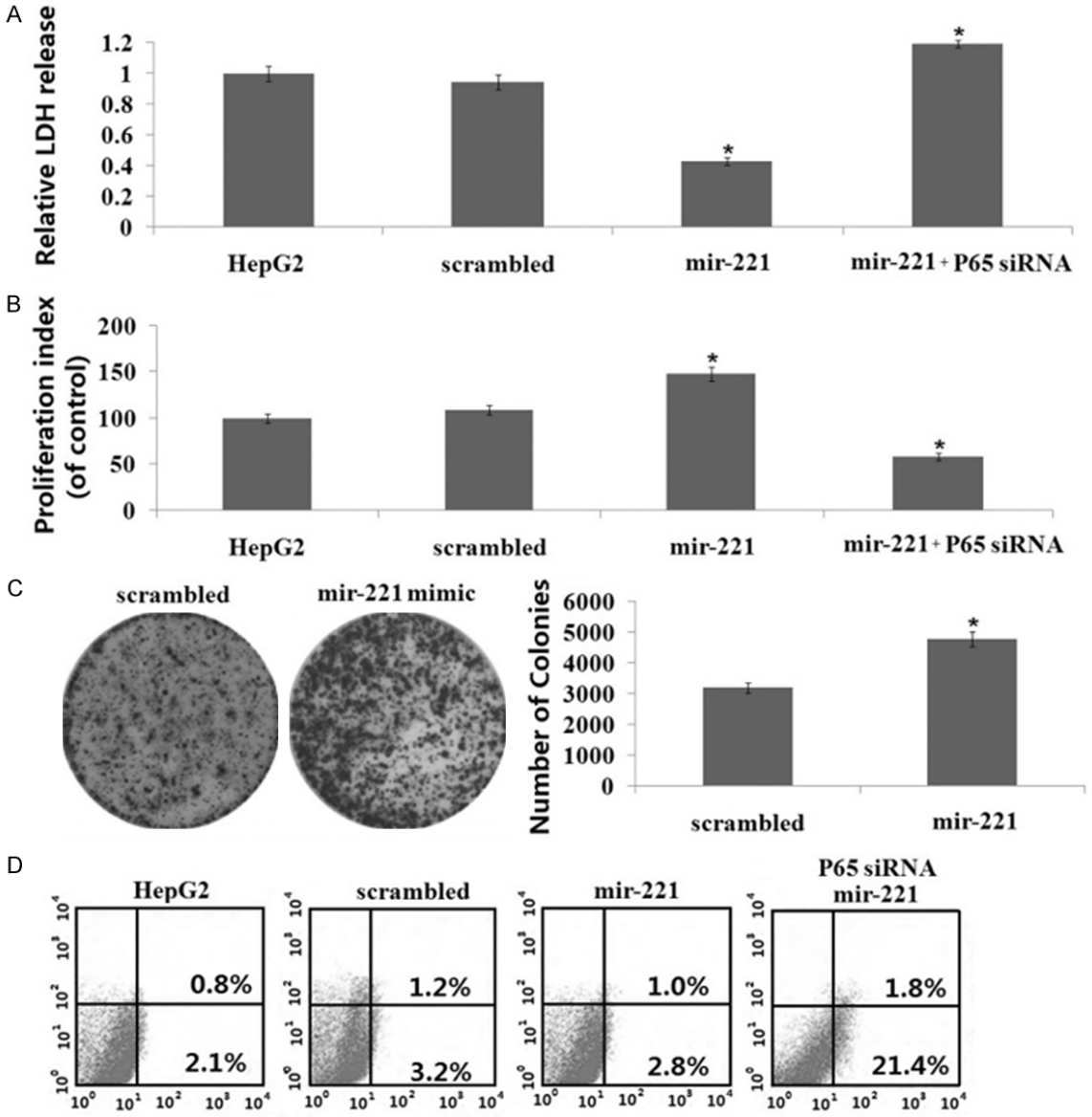
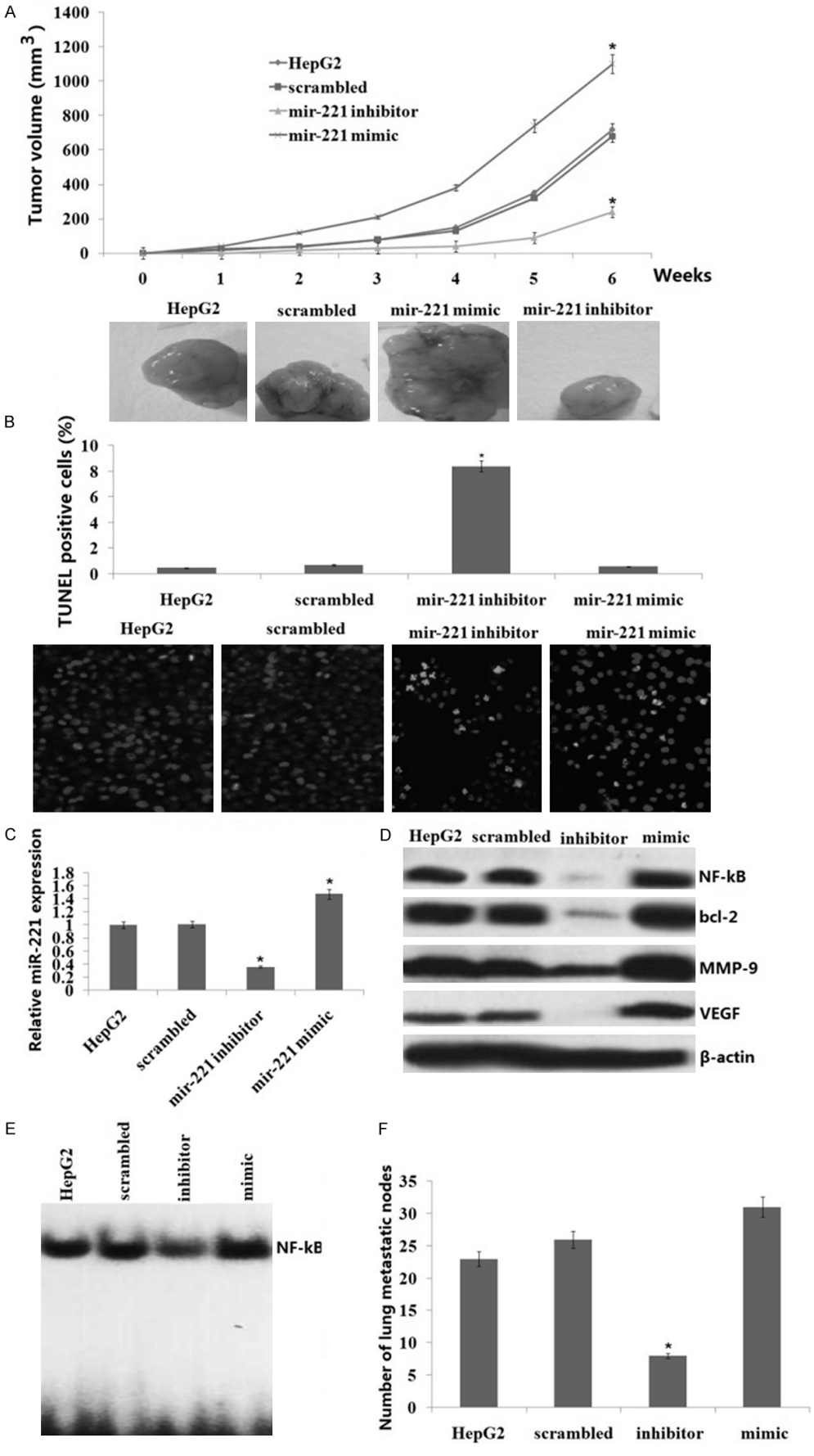


Figure 5. Mir-221 promotes growth and colony formation of HepG2 cells in vitro. HepG2 cells was transfected with mir-221 mimics or/and p65 siRNA. A: Cell viability by LDH toxicology assay; B: Cell proliferation by BrdUrd cell proliferation assay; C: Cell growth by colony formation assay. D: Cells apoptosis was detected by FCM. The data are shown as the mean ± SD. The experiments were all repeated at least 3 times to confirm the reproducibility of the results. *P<0.05 versus the control.

221-transfected tumors showed increased miR-221 mRNA expression (**Figure 6C**), NFκB activity (**Figure 6E**) and P65, MMP-9, VEGF expression (**Figure 6D**). Lung metastatic nodes formed on the surface of lungs in the anti-miR-221-transfected groups was significantly decreased compared to the controls ($P<0.01$, **Figure 6F**). Although more metastatic nodes were formed on the surface of lungs in the miR-221-transfected groups than the control group, there was not significantly different between the two groups ($P>0.05$, **Figure 6F**).

Discussion

Downregulation of subsets of miRNAs, such as miR-122 and miR-199, is a common finding in HCC, suggesting that some of these miRNAs may act as putative tumor suppressor genes. Restoration of tumor suppressive miRNAs leads to cell cycle block, increased apoptosis, and reduced tumor angiogenesis and metastasis by inhibiting migration and invasion [22-24]. Oncogenic miRNAs that are upregulated in HCC potentially target many tumor suppressive


A
Tumor volume (mm3)
HepG2
scrambled
mir-221 inhibitor
mir-221 mimic
Weeks
HepG2
scrambled
mir-221 mimic
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B
TUNEL positive cells (%)
HepG2
scrambled
mir-221 inhibitor
mir-221 mimic
C
Relative miR-221 expression
HepG2
scrambled
mir-221 inhibitor
mir-221 mimic
D
HepG2
scrambled
inhibitor
mimic
NF-kB
bcl-2
MMP-9
VEGF
β-actin
E
HepG2
scrambled
inhibitor
mimic
NF-kB
F
Number of lung metastatic nodes
HepG2
scrambled
inhibitor
mimic

Figure 6. Effect of miR-221 on HepG2 cells xenograft tumor growth and lung metastasis in vivo. A: Representative growth curves of tumor volume. B: Tissue apoptotic cells by TUNEL assay; C: miR-221 mRNA expression by qRT-PCR assay; D: NFκB signals molecular was detected by western blot assay. E: NFκB activity was detected by western blot assay. F: The number of metastatic nodes in each group. Tumors were counted with the naked eye. The data represent the mean and the standard deviation (n=6). Data is presented as the mean ± SD; P values were calculated with the Student's t-test. $^{*}P<0.05$.

genes. Experimental suppression of oncogenic miRNAs helps restoring expression of tumor suppressive genes that initiates apoptosis and inhibits cell proliferation, angiogenesis, and metastasis in HCC. Among the oncogenic miRNAs, miR-221 has found to be upregulated in HCC, there is evidence in support of the tumor-promoting activity of miR-221 [25, 26].

Fu et al. has reported that miR-221 was overexpressed in HCC. Moreover, miR-221 expression was positively correlated with metastasis and tumor size in HCC [27]. Pineau et al. also found that miR-221 was overexpressed in HCC. In addition, they showed, using a mouse model of liver cancer, that miR-221 overexpression stimulates proliferation, migration, and invasion capability of tumorigenic murine hepatic progenitor cells [28]. Yuan et al. has demonstrated that miR-221 enhances proliferation of cultivated primary hepatocytes *in vitro* and *in vivo* [29].

The present study showed targeting miR-221 using anti-miR-221 inhibits cell proliferation, invasion, metastasis and induced apoptosis *in vitro* and *in vivo*. Re-expression of miR-221 by miR-221 transfection reversed the anti-tumor role, implicated its potential pro-tumorigenic function in HCC. The down-regulation of miR-221 suggests the potential therapeutic application against hepatocarcinogenesis.

As shown above, miR-221 inhibition induced apoptosis and inhibited invasion, but which of the many miR-221-regulated genes are required has remained unknown. NFκB is often activated in numerous types of tumors and promotes cancer development and chemoresistance. Therefore, miRNAs that possess the NFκB inhibitory activity may provide novel targets for anti-cancer therapy. Here, we show that miR-221 is a central positive regulator of NFκB activation as it promotes several NFκB-activating pathways.

In this study, we found that overexpression of miR-221 by miR-221 transfection increased NFκB activity in vitro and vivo. On the contrary, downregulation of miR-221 by anti-miR-221 transfection inhibited the NFκB activity in HepG2 cells in vitro and vivo. NFκB has been shown to regulate transcriptionally the expression of several members of the bcl-2 gene family, and bcl-2 remains the prototypic antiapoptotic protein regulated by NFκB [30]. Here we found that overexpression of miR-221 promotes bcl-2 expression and downexpression of miR-221 inhibits bcl-2 expression, suggesting that miR-221 mediated activation of bcl-2 is through activation of NFκB. Moreover, it is suggested that miR-221 exerts its growth promoting effect by inducing bcl-2 activation. Therefore, targeting miR-221 mediated cell growth inhibition and induction of apoptosis could be mediated via inactivation of NFκB/bcl-2 signals.

To further investigate whether the enhanced cell growth inhibition and apoptosis by miR-221 inhibition was mediated through the NFκB pathway, we conducted p65 cDNA and p65 siRNA transfection studies. We found that p65 cDNA transfection induced the activity of NFκB. However, p65 siRNA was functioning similarly as miR-221 inhibition, which inhibited NFκB activity. Moreover, anti-miR-221 combined with p65 cDNA transfection reversed the inhibitory effect of anti-miR-221 on apoptotic cell death; However, miR-221 overexpression combined with p65 siRNA transfection rescued the inhibitory effect of miR-221 overexpression on apoptotic cell death, suggesting that anti-miR-221-induced cell growth inhibition and apoptosis is mediated through the NFκB pathway.

NFκB activation has been reported to regulate several genes such as VEGF and MMP-9, which are directly associated with metastatic processes [31-33]. Indeed, in this study, we showed that anti-miR-221 reduced NFκB activity and concomitantly inhibited the expression of VEGF and MMP-9, and vice versa. Since we observed that anti-miR-221 inhibited the expression of MMP-9 and VEGF, and miR-221 promoted the expression of MMP-9 and VEGF,

we tested the effects of miR-221 on the invasion and metastasis of HepG2 cells *in vitro* and *in vivo*. We found that anti-miR-221 inhibited invasion and lung metastasis and miR-221 promoted invasion and lung metastasis of HepG2 cells *in vitro* and *in vivo*. These results were consistent with inactivation and activation of MMP-9 and VEGF, documenting that anti-miR-221 could inhibit cancer cell migration and invasion which is likely due in part through the downregulation of MMP-9 and VEGF, mediated by inactivation of NFκB.

Although the modulation of NFκB activity and its target genes by inhibition/overexpression of miR-221, and the rescue of phenotype by p65 transfection/inhibition indicates the influence of this miRNA on NFκB pathway, however, how miR-221 mediates this effect is not known. Zhao et al. has reported that miR-221 activates the NFκB pathway by targeting A20 [34]. Some studies have reported that miR-221 abolished the inhibitory effect of adiponectin on NFκB activation by targeting adipoR1 [35, 36], suggesting that NFκB pathway was indirectly regulated by miR-221. However, Galardi et al. has recently found that NFκB involved in cancer onset and progression and contributed to oncogenesis by inducing miR-221/222 transcription [37]. Ding et al. has found that HCVcc infection could upregulate the expression of miR-221 in NF-κB dependent manner [38], indicating that miR-221 was regulated by NFκB. Whereas, Liu et al. has found that miR-221/222 bound directly to the coding region of RelA mRNA, miR-221/222 act in a positive feedback loop to increase expression levels of RelA and STAT3 in human colorectal cancer cells [18]. In the present study, we found that miR-221 positively regulate NF-κB signaling pathways, which in return induce the expression of miR-221, creating a miR-221-mediated positive feedback loop.

Conclusion

In this study, we demonstrated that targeting miR-221 showed strongly antitumor effects in HCC in vitro and in vivo. Targeting miR-221 could potentially be an effective therapeutic agent for the inactivation of NFκB and its downstream target genes such as bcl-2, MMP-9 and VEGF, resulting in the inhibition of cell growth, invasion and metastasis of HCC. Our study suggests that miR-221 represent a promising gene target for the treatment of HCC.

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Disclosure of conflict of interest

None.

Address correspondence to: Jian Zhang, Department of Surgery, The Affiliated Hospital of Qingdao University, Qingdao, Shandong, China. E-mail: zhangthy@126.com

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