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MicroRNA-21 Regulates the Proliferation, Differentiation, and Apoptosis of Human Renal Cell Carcinoma Cells by the mTOR-STAT3 Signaling Pathway

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MicroRNA-21 (miRNA-21), a kind of short, noncoding RNAs, functioned as a tumor marker and was upregulated in renal cell carcinoma (RCC). However, the underlying mechanisms of miRNA-21 in RCC were uncertain. Therefore, the effects and mechanisms of miRNA-21 on the proliferation, differentiation, and apoptosis of cultured human RCC cells were further investigated in this study. After slicing miRNA-21 in RCC cells, the viability, mRNA expression of C/EBPα and PPARγ, caspase 3 activity, and protein expression of mTOR, STAT3, and pSTAT3 were determined. It was found that knockdown of miRNA-21 downregulated the optical density (OD) value of cells, inhibited mRNA expression of PPARγ and C/EBPα, and enhanced activity of caspase 3. Furthermore, protein expression of pSTAT3 was also decreased in the absence of miRNA-21. Notably, miRNA-21-changed proliferation, differentiation, and apoptosis of human RCC cells were partially regulated following the block of the mTOR-STAT3 signaling pathway by the mTOR inhibitor (XL388). It was indicated that miRNA-21 promoted proliferation and differentiation and decreased apoptosis of human RCC cells through the activation of the mTOR-STAT3 signaling pathway.

Key words: miRNA-21; Human renal cell carcinoma (RCC); Proliferation; Differentiation; Apoptosis; mTOR; STAT3

INTRODUCTION

Renal cell carcinoma (RCC) is the most common cancer in humans and has three common types including clear cell RCC (80%–90%), papillary RCC (10%–15%), and chromophobe RCC (3%–5%) (1). It has been reported that approximately 30% of patients with RCC exhibit metastasis, and less than 10% of these patients achieve 5-year survival. The incidence of RCC is highest in Western and Eastern Europe, followed by Australia, Scandinavia, Southern Europe, and Japan (2). For years, a great deal of effort has been made to produce a number of new agents and approaches for treating RCC (3).

The widely accepted idea is that microRNAs (miRNAs) are a class of small endogenous gene regulators that have been implicated in initiation, progression, and metastasization of various cancers (4). Recent work has highlighted the importance of miRNAs in cancer development and regards them as novel biomarkers for diagnosing various kinds of malignancies. Studies have shown that the expression of several miRNAs is deregulated in adult kidneys (5), indicating the involvement of miRNAs in kidney cancer pathogenesis. Of note, among a large number of miRNAs, miRNA-21 is one of the most studied miRNAs due to its ubiquitous role in various biological processes, especially in cancer, inflammation, and cardiovascular diseases. Deregulated expression of miRNA-21 is detected in various cancer tissues such as liver, stomach, colon, lung, breast, prostate, and pancreas (6–8). In particular, miRNA-21 plays an important role in cancer development, progression, and metastasis (9) and is established as an oncogenic miRNA (10,11). Moreover, upregulation of miRNA-21 expression is associated with lymph node metastasis, aggressive progression, and poor prognosis in cervical cancer (12).

During recent years, the role of miRNA-21 in RCC progression has been experimentally described. Downregulation of miRNA-21 is found in RCC patients in comparison with healthy human cells, and a significant difference exists in miRNA-21 expression levels between ccRCC and pRCC subtypes, suggesting that the miRNA-21 expression level can be used as a diagnostic marker in distinguishing RCC subtypes (13). In particular, the expression level of miRNA-21 is found to be correlated with a span of 5-year survival and pathological stage in RCC patients (13,14). The findings above suggest that miRNA-21 might play a crucial role in the biological functions of RCC. However, the precise functions and mechanisms of miRNA-21 involved in antitumor processes are not yet well understood. In the present study, we investigate the

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influence of miRNA-21 on the proliferation, differentiation, and apoptosis of human RCC cells and elucidate the mechanisms by which miRNA-21 exerts its functions.

MATERIALS AND METHODS

Cells and Treatments

The human RCC cell line ACHN (ScienCell, Carlsbad, CA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum, 10 mg/ml streptomycin, and 100 IU/ml penicillin (Gibco, New York, NY, USA) at 37°C in a humidified atmosphere of 5% CO_2 in air. Furthermore, the experiments were randomly divided into the following groups: control, miRNA-21 inhibitor treatment, miRNA-21 mimic treatment, XL388 treatment, and miRNA-21 mimic plus XL388 treatment. XL388 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). miRNA-21 mimics and miRNA-21 inhibitors were purchased from GenePharma (Shanghai, China).

XTT Assay

As the method described by Huyck et al. (15), cell viability was evaluated using a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) Cell Viability Assay Kit (Sigma-Aldrich, Carlsbad, CA, USA). In brief, human RCC cells at a density of 2.0×10^5/ml were seeded in 96-well plates. When cells attained 80% confluency, cells were transfected with miRNA-21 mimics or inhibitors. At 24, 48, 72, and 96 h, 100 µl of fresh medium and 25 µl of XTT solution were added to PBS-washed human RCC cells. The plates were then incubated in an incubator for 5 h, and optical density (OD) in each well was determined by a microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm.

Flow Cytometry

Cells seeded onto a 96-well plate were treated with miRNA-21 inhibitors, miRNA-21 mimics, XL388, and miRNA-21 mimics plus XL388 for 24, 48, and 72 h, and the cell apoptotic rate was measured by flow cytometry according to the manufacturer's instructions. Briefly, human RCC cells were collected and fixed in 70% ethanol for 30 min. Cells were then stained with 50 µg/ml FITC, annexin V, and PI (BD Biosciences, San Jose, CA, USA), respectively. Cell apoptotic rate was analyzed using a FACScan Vantage SE (BD Biosciences).

Caspase 3 Activity Analysis

Caspase 3 activity was analyzed using Caspase 3 Activity Assay Kits according to the manufacturer's instructions. Briefly, the reaction buffer and the specific enzyme DEVD-pNA were added to each cell plate and further cultured in an incubator for 1 h at 37°C. The developed colorimetric reaction was measured at 405 nm in a 96-well Bio-Rad 680 microplate reader.

Western Blot Analysis

The examination of the protein expression levels of mTOR, STAT3, and pSTAT3 was performed separately using Western blot analysis as described in a previous study (16). Antibodies were purchased from Santa Cruz Biotechnology. Band density was quantitated using the ImageJ software.

Gene Expression Analysis

Expression of miRNA-21, CCAAT/enhancer-binding protein-α (C/EBPα), and peroxisome proliferator-activated receptor-γ (PPARγ) in human RCC cells was determined at indicated times by RNA preparation and quantitative reverse transcription polymerase chain reaction (RT-PCR) according to the previous study (16). β-Actin expression was used as the control. Specific primer sequences (BIOSUNE Biological Technology Corp, Shanghai, China) were as follows: β-actin, 5′-GACGTGGACATCCGCAAAG-3′ (forward) and 5′-CTGGAAGGTGGACAGCGAGG-3′ (reverse); C/EBPα, 5′-GTTAGCCATGTGGTAGGAGAC A-3′ (forward) and 5′-CCCAGCCGTTAGTGAAGAGT-3′ (reverse); miRNA-21, 5′-TGACATCGCATGGCTGTA-3′ (forward) and 5′-GATGCTGGGTAATGTTTGAAT-3′ (reverse); C/EBPα, 5′-CTCCGATCTCTTTGCCGTGA A-3′ (forward) and 5′-GGACCGGAGCGCTAGGCG-3′ (reverse); PPARγ, 5′-CATTCTGGCCCACCAACTTTG G-3′ (forward) and 5′-TGGAGATGCAGGCTCCACTT TG-3′ (reverse).

Transfection

For plasmid transfections, human RCC cells in the exponential growth phase were grown to 65% confluence and then transfected with 2 µg of miRNA-21 mimics or inhibitors using HiPerFect (Qiagen, Valencia, CA, USA) according to the manufacturer's protocols. After cells were cultured in medium for 48 h, the efficiency of miRNA-21 slicing was determined by RT-PCR. Furthermore, cells grown to 65% confluence were treated with XL388 for 48 h and then transfected with miRNA-21 mimics for 48 h; cell proliferation and apoptosis were investigated.

Statistical Analysis

Statistical analysis was carried out with one-way analysis of variance (ANOVA) using SPSS 15.0 software. Data are presented as the mean ± standard deviation (SD). A value of $p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

miRNA-21 Expression Was Upregulated in Human RCC Cells

We investigated the cellular distribution of miRNA-21 expression in normal renal cells and RCC cells by

RT-PCR. As shown in Figure 1, there was detectable upregulation of miRNA-21 expression in RCC cells compared with normal renal cells at 24, 48, 72, and 96 h, and the difference was significant ($p < 0.05$).

Downregulated miRNA-21 Suppressed the Proliferation of Human RCC Cells

We first checked the knockdown effect of miRNA-21 inhibitors in RCC cells. As demonstrated in Figure 2A, the expression levels of miRNA-21 in RCC cells were reduced significantly ($p < 0.01$). Because the knockdown efficiency of miRNA-21 inhibitors was obvious, we used the inhibitors in the latter experiments. We next examined the effect of miRNA-21 knockdown on cell growth in RCC cells using the XTT method. It was found that the proliferation of RCC cells was significantly suppressed when compared with that of the control after culture for 24, 48, 72, and 96 h (Fig. 2B).

Downregulated miRNA-21 Suppressed the Differentiation of Human RCC Cells

Expression of two differentiation-related factors, PPARγ and C/EBPα, in RCC cells was determined by RT-PCR after knocking down cellular miRNA-21. Results showed that, compared to the control without miRNA-21 stimulation, PPARγ (Fig. 3A) and C/EBPα (Fig. 3B) mRNA expression was decreased within 72 h in miRNA-21-deleted RCC cells, suggesting that downregulated miRNA-21 inhibited differentiation of human RCC cells.

Downregulated miRNA-21 Promoted the Apoptosis of Human RCC Cells

We assessed whether miRNA-21 knockdown induced apoptosis in RCC cells using flow cytometry after transfection with miRNA-21 inhibitors or inhibitor control for 24, 48, and 72 h. Knockdown of miRNA-21 markedly increased the rate of apoptotic cells when compared with the control ($p < 0.001$). The rate of apoptotic cells reached 16.24 ± 3.42%, 38.75 ± 5.88%, and 56.37 ± 8.39% at 24, 48, and 72 h, respectively, in miRNA-21 downregulated RCC cells (Fig. 4A). Furthermore, analysis kits were used to test the activity of an apoptosis relative factor, caspase 3. Results in Figure 4B suggested that caspase 3 activity was heavily enhanced in miRNA-21 slicing in RCC cells in comparison with the control.

Downregulated miRNA-21 Inhibited the Activation of the mTOR-STAT3 Signaling Pathway

To evaluate the effects of downregulated miRNA-21 on the cellular mTOR-STAT3 signaling pathway, protein expression of mTOR, STAT3, and pSTAT3 in RCC cells was determined separately by Western blot analysis using their antibodies (Fig. 5A). Band ratio analysis suggested that downregulation with miRNA-21 inhibitors markedly inhibited the protein expression of pSTAT3, but showed no significant changes in the expression level of mTOR and STAT3 (Fig. 5B). It was suggested that downregulated miRNA-21 suppressed the activation of the mTOR-STAT3 signaling pathway.

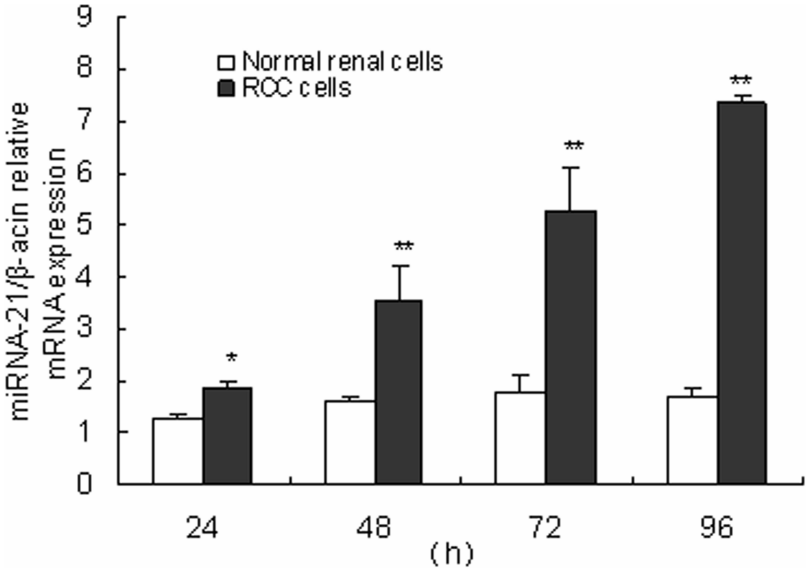

Figure 1. miRNA-21 expression was upregulated in human renal cell carcinoma (RCC) cells. Expression of miRNA-21 was measured by reverse transcription polymerase chain reaction (RT-PCR) in human RCC cell line and normal renal cells at 24, 48, 72, or 96 h. Results are presented as mean ± SD. *$p < 0.05$ compared with the control.

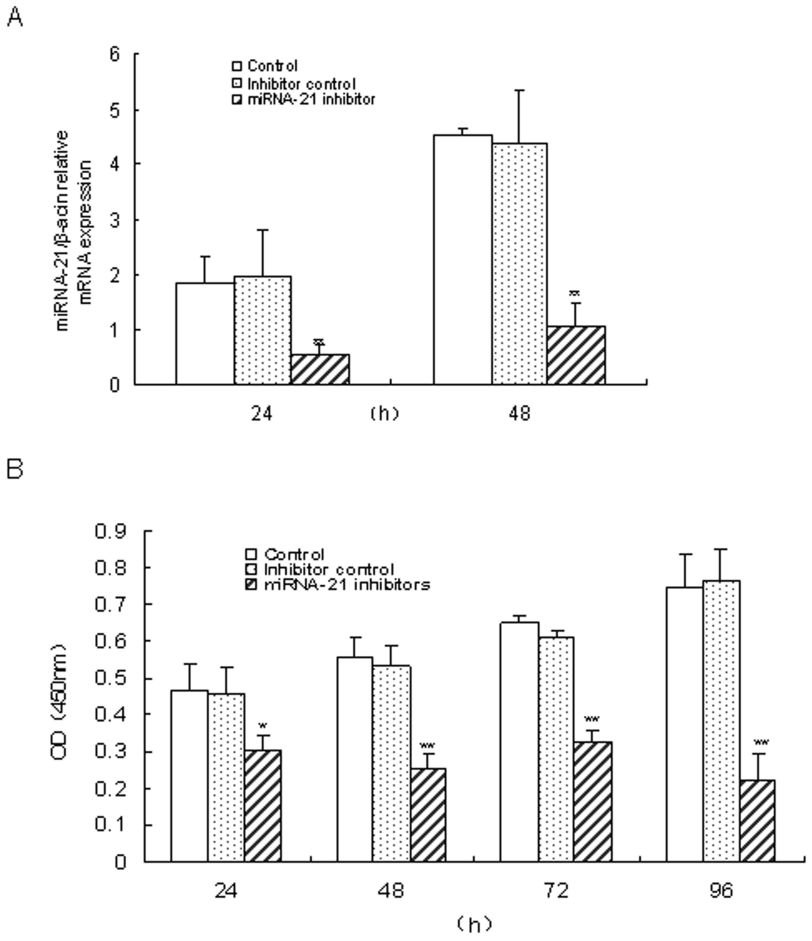

Figure 2. Inhibition of the proliferation of human RCC cells by miRNA-21 knockdown. Expression of miRNA-21 in human RCC cell line after knockdown of endogenous miRNA-21 (A). After treatment with control inhibitor or miRNA-21 inhibitors for 24, 48, 72, or 96 h, the number of viable RCC cells was determined by XTT assay (B). Three individual experiments were performed. Results are presented as mean ± SD. $*p < 0.05$ compared with control.

The mTOR-STAT3 Signaling Pathway Played an Important Role in miRNA-21-Meditated Function in Human RCC Cells

We then investigated the role of the cellular mTOR-STAT3 signaling pathway in miRNA-21-induced cellular changes in RCC cells at 48 h after transfection with miRNA-21 mimics. As shown in Figure 6A, protein expression of pSTAT3 was decreased significantly after blockade of the mTOR-STAT3 signal using XL388, indicating good efficiency of this signal inhibitor. After transfection with miRNA-21 mimics, mRNA expression of miRNA-21 had almost 3.0-fold changes at 24 h and 2.2-fold changes at 48 h when compared to the control (Fig. 6B). Of note, there was significant promotion in cell growth (Fig. 6C), and mRNA expression of PPARγ and C/EBPα (Fig. 6D) in miRNA-21 mimic-treated cells when compared with the control. Inhibitor XL388 significantly decreased cell proliferation and differentiation compared with that in control cells, but there was no difference between XL388-treated cells and XL388 plus miRNA-21 mimic-treated cells. Moreover, treatment with miRNA-21 mimics inhibited caspase 3 activity, while XL388 inhibitor caused almost a 2.7-fold increase in caspase 3 activity. However, it showed no significant difference in caspase 3 activity between XL388-treated cells and XL388 plus miRNA-21 mimic-treated cells (Fig. 6E). All results above indicated that the mTOR-STAT3 signaling pathway was involved in miRNA-21-meditated proliferation, differentiation, and apoptosis in human RCC cells.

DISCUSSION

RCC occurs in adult kidneys and is a potentially lethal urological malignancy in humans. As mentioned in the previous studies, miRNA-21 was involved in the pathogenesis of cancers such as lung cancer, prostate cancer, breast cancer, gastric cancer, ovarian cancer, and bladder

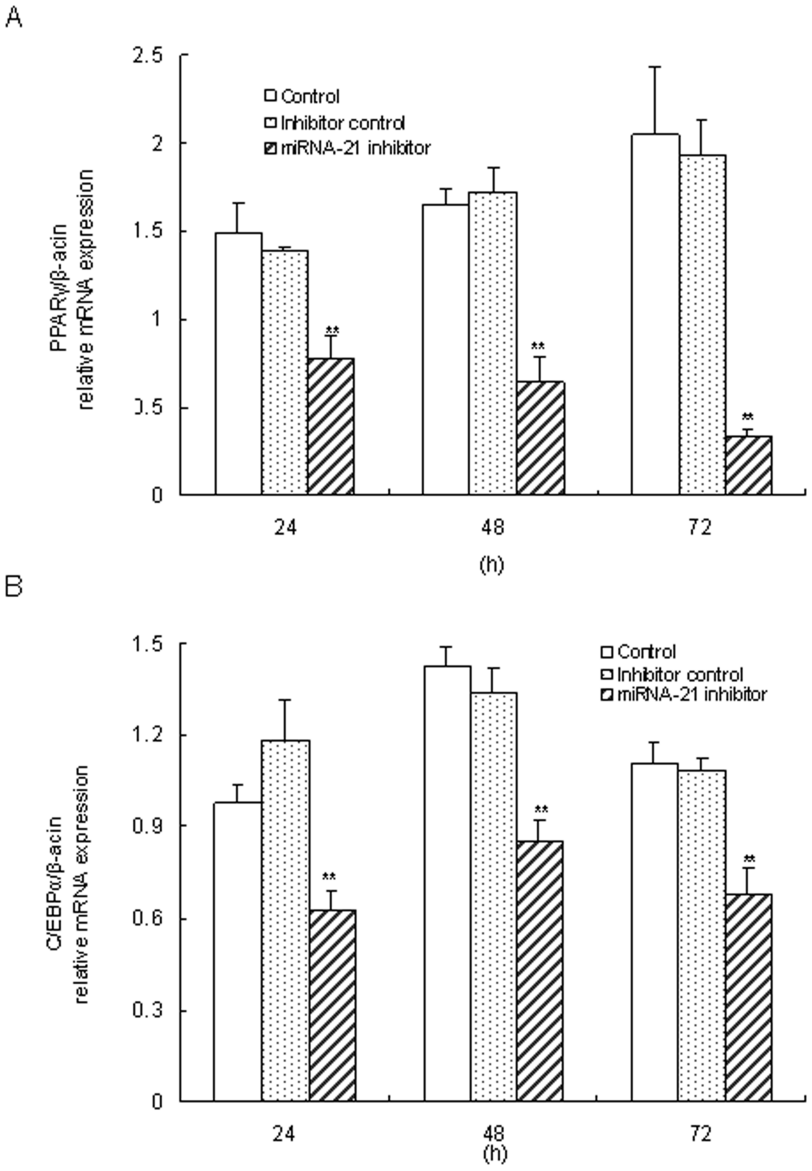

Figure 3. Knockdown of strongly suppressed human RCC cell differentiation. After treatment with control inhibitor or miRNA-21 inhibitors for 24, 48, or 72 h, the expression of PPARγ (A) and C/EBPα (B) was determined by RT-PCR. Three individual experiments were performed. Results are presented as mean ± SD. $*p < 0.05$ compared with control.

cancer (17,18). Especially, miRNA-21 was reported to be an important biomarker of RCC. This study further investigated the changes of miRNA-21 expression and its influence and underlying mechanism in human RCC cells, and proved that miRNA-21 expression was upregulated and miRNA-21 played a crucial role in the proliferation, differentiation, and apoptosis of human RCC cells through targeting the mTOR-STAT3 signaling pathway.

miRNA-21 is regarded as a biomarker of tumors and might be a potential therapeutic target for cancer therapy (19). miRNA-21 was previously reported to be upregulated in different kinds of cancers. Hatley et al. (20) have showed that incidence of lung tumors is significantly high in miRNA-21-overexpressing mice (20). miRNA-21 expression was increased in non-small cell lung cancer (NSCLC), and miRNA-21 might be considered as a potential novel target for future development of specific therapeutic interventions in NSCLC (21). The expression of miR-21 was upregulated in human gastric cancer cells (22). miRNA-21 expression was significantly higher in lung cancer cell lines including 95-D, A549, HCC827, and NCI-H282, than in normal human bronchial epithelial cells. An observation of Faragalla et al. suggested that miR-21 expression level correlated with a span of 5-year

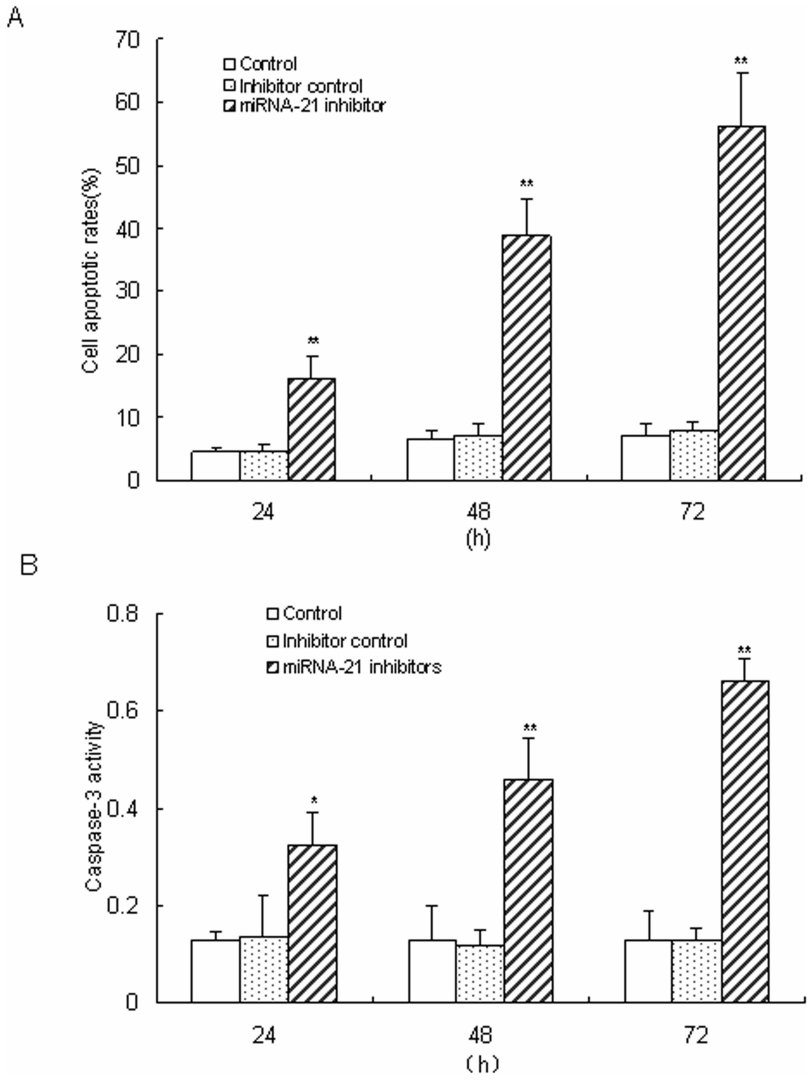

Figure 4. Induction of apoptosis in miRNA-21 knocking down human RCC cells. Following transfection with control inhibitor or miRNA-21, the apoptotic rate of human RCC cells was measured using flow cytometry (A), and caspase 3 activity was analyzed using Caspase 3 Activity Assay Kits (B). Three individual experiments were performed. Results are presented as means ± SD. $*p<0.05$ compared with the control.

survival and pathological stage in RCC and could be used as a diagnostic marker in distinguishing RCC subtypes (13). The higher expression of miRNA-21 in human RCC cells was also found in this study.

miRNAs have an important role in the regulation of cellular activities such as cell growth, proliferation, and differentiation. miRNA-21 was first noticed for its apoptotic effects in various cell lines (23). Upregulated miRNA-21 promoted tumor proliferation and inhibited cell apoptosis in breast cancer cells in vitro (24). Sun et al. examined the impact of miRNA-21 on the biological characteristics of lung cancer cells, such as proliferation, apoptosis, and invasion, and proved that cell viability and invasion of lung cancer cells were significantly lower in the miRNA-21 downregulated group than in the control. The number of apoptotic cells and caspase 3 expression were significantly higher in the miRNA-21 downregulated group than in the control (25). Downregulation of miRNA-21 inhibited proliferation and cell cycle progress of NSCLC cells and sensitized cells to radiation, but promoted the apoptosis of cells induced by irradiation (21). Inhibition of miR-21-5p or miR-21-3p resulted in a significant decrease in ovarian and prostate cancer cell proliferation and invasion (26). miRNA-21 degradation could decrease viability and induce apoptosis and necrosis of the hepatocellular carcinoma cell line (HepG2) (27). miRNA-21 silencing could inhibit the capacity of proliferation, migration, and invasion, and arrest the cell cycle and induce apoptosis of tongue squamous cell

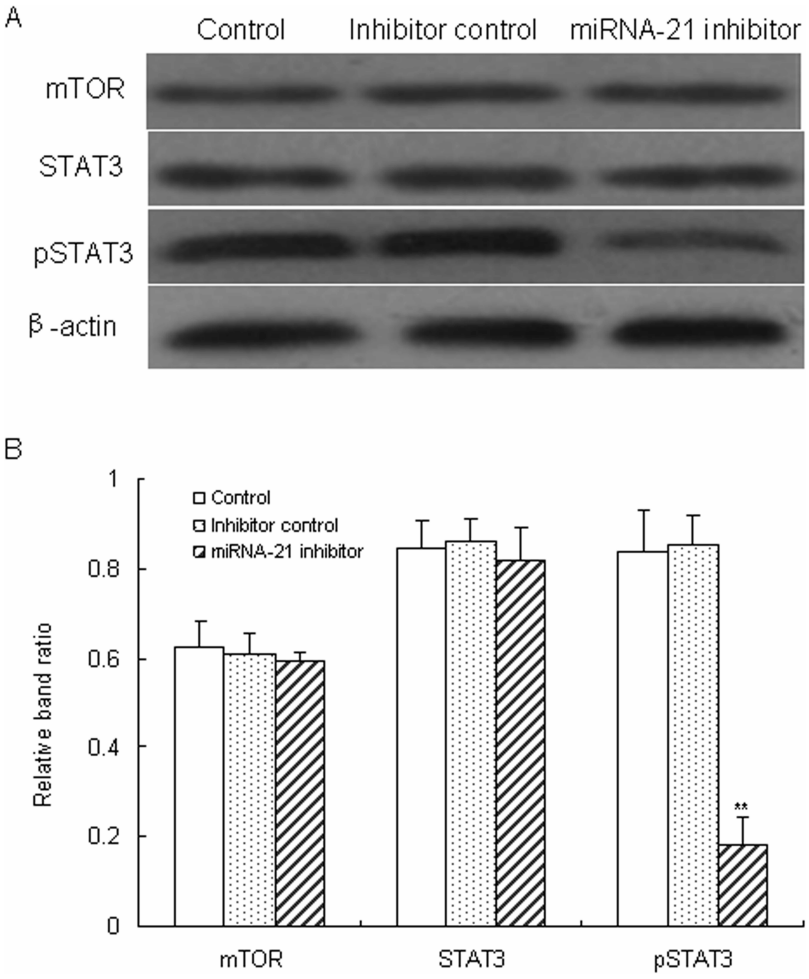

Figure 5. Suppression of the mTOR–STAT3 signaling pathway in miRNA-21 knockdown human RCC cells. After treatment with control inhibitor or miRNA-21 for 48 h, the protein expression of mTOR, STAT3, and pSTAT3 was determined by Western blot (A). Furthermore, band relative ratio was analyzed by ImageJ software. Three individual experiments were performed. Results are presented as means ± SD. $*p < 0.05$ compared with control (B).

carcinoma cell lines (28). miRNA-21 mimic-transfected cells exhibited increased cell proliferation and transformation capacity, whereas miRNA-21 inhibitor-transfected cells exhibited the opposite phenomenon in renal cancer cell lines (A498, 786-O, and caki-1) (29). In addition, overexpression of miRNA-21 significantly decreased antiproliferative effects and apoptosis induced by paclitaxel, while knockdown of miRNA-21 dramatically increased antiproliferative effects and apoptosis induction by paclitaxel in human gastric cancer cells (22). We made an assessment of the effect of miRNA-21 degradation on viability, differentiation, and apoptosis of human RCC cells and proved that miRNA-21 inhibition might inhibit cell proliferation and differentiation while promoting cell apoptosis in human RCC cells.

Because of the critical functions of its target proteins in various signaling pathways, miRNA-21 has become an attractive target for genetic and pharmacological modulation in various disease conditions (30). miRNA-21 promoted glioblastoma cell line A172 proliferation and suppressed chemosensitivity by inhibiting FOXO1 (31). miRNA-21 acts as a molecular switch to regulate aerobic glycolysis in bladder cancer cells via the PTEN/phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR pathway (18). A study reported that an miRNA-21 inhibitor enhanced the release of chemoattractants RANTES and IP-10 and resulted in increased lymphocyte migration via its inhibition of PIAS3 expression and oncogenic STAT3 signaling in the MCF-7 breast cancer cell line (32). miRNA-21 knockdown suppresses cell growth, migration, and invasion partly by inhibiting PI3K/AKT activation via direct targeting PIK3R1 and reversing EMT in breast cancer (33). miRNA-21 downregulation inhibits cell growth and cell invasion and induces cell apoptosis by targeting the FASL, TIMP3, and RECK genes in esophageal carcinoma

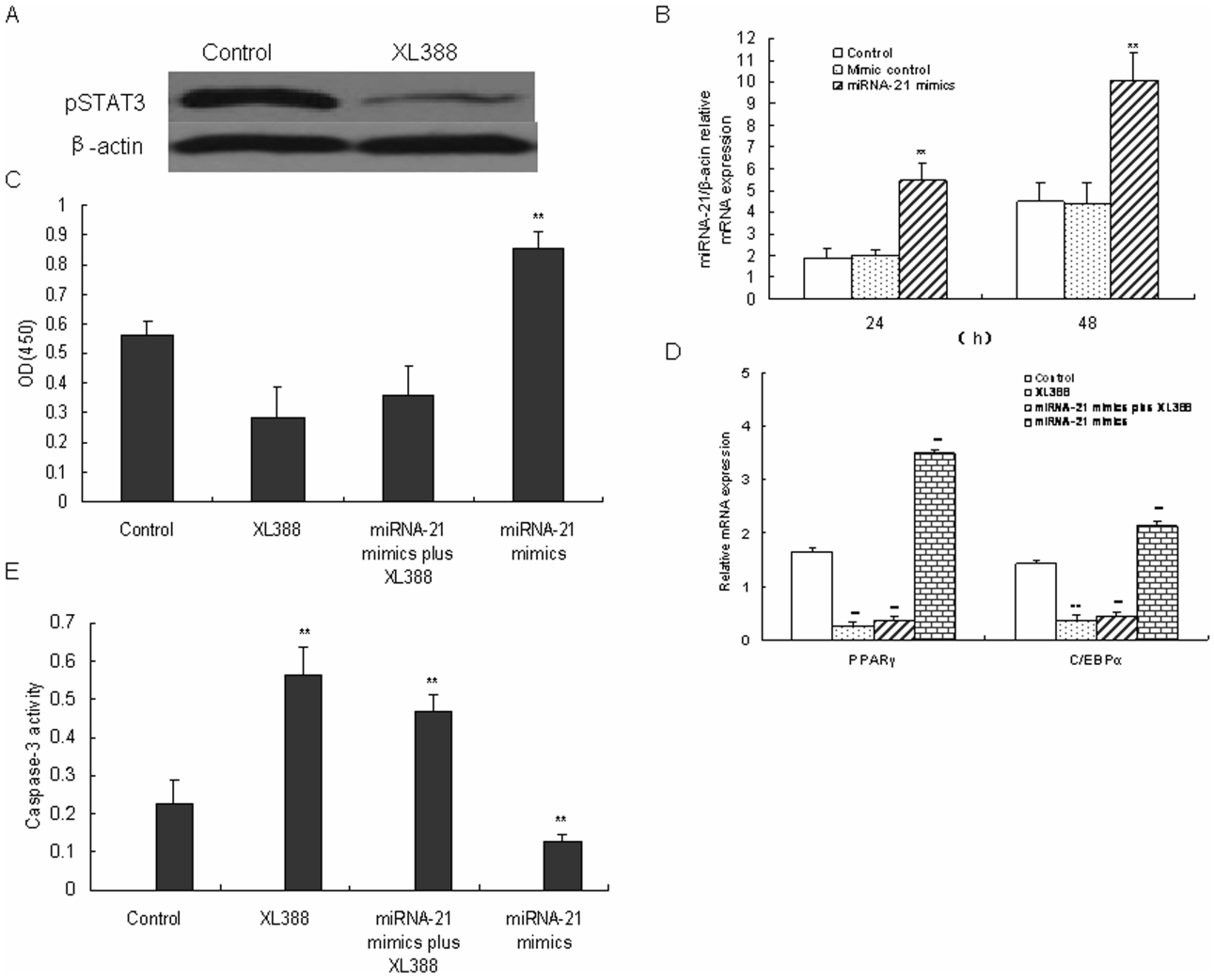

Figure 6. The mTOR-STAT3 signaling pathway played an important role in miRNA-21-induced cellular changes in human RCC cells. After blocking the mTOR-STAT3 signal using XL388, protein expression of pSTAT3 was detected by Western blot (A). After transfection with miRNA-21 mimics for 48 h, miRNA-21 expression was measured by RT-PCR (B). After treatment of cells with miRNA-21 mimics or XL388, cell growth (C), mRNA expression of PPARγ and C/EBPα (D), and caspase 3 activity (E) were demonstrated. Three individual experiments were performed. Results are presented as means ± SD. $*p < 0.05$ compared with control.

(34). miRNA-21 downregulation could significantly inhibit growth, migration, and invasion and reverse chemo- or radioresistance of NSCLC cells through modulation of the PTEN signaling pathway (17). Additionally, knockdown of miRNA-21 considerably inhibited tumor growth and diminished the expression of STAT3 and hTERT in a xenograft model, indicating that miRNA-21 regulated hTERT expression mediated by STAT3, therefore controlling glioblastoma cell growth (35). In this study, we first demonstrated that miRNA-21 enhanced viability and differentiation and decreased apoptosis of human RCC cells through the activation of the mTOR-STAT3 signaling pathway.

In conclusion, we found that miRNA-21 expression was heavily upregulated in human RCC cells. Further, miRNA-21 could activate the mTOR-STAT3 signaling pathway and exert an important role in the proliferation, differentiation, and apoptosis of human RCC cells. We speculated that miRNA-21 might be regarded as a diagnostic and prognostic marker for RCC.

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