Am J Transl Res 2017;9(5):2394-2402
www.ajtr.org /ISSN:1943-8141/AJTR0040576

Original Article

MicroRNA-142-5p promotes cell growth and migration in renal cell carcinoma by targeting BTG3

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Received September 24, 2016; Accepted February 14, 2017; Epub May 15, 2017; Published May 30, 2017

Abstract: Purpose: Some microRNA (miRNA) levels have been found to be dysregulated in cancer patients, suggesting the potential usefulness of miRNAs in cancer therapies. The purpose of this study was to investigate the expression of miR-142-5p in human renal cell carcinoma (RCC) and its potential role in tumor growth and metastasis. Methods: The expression level of miR-142-5p in human RCC tissue and cell lines was determined by quantitative reverse transcription polymerase chain reaction analysis. MTT, colony formation, Transwell, and cell cycle assays were performed to explore the potential functions of miR-142-5p in human RCC cells. The potential target gene of miR-142-5p was identified and confirmed via luciferase reporter assays. Results: miR-142-5p expression was elevated in RCC tissues and cell lines. Overexpression of miR-142-5p significantly promoted cell proliferation and colony formation and could prevent G1 phase arrest among RCC 786-O cells. Meanwhile, the migration potential of 786-O cells was greater than that of control cells. BTG3 was identified as a direct target of miR-142-5p, and re-expression of BTG3 reversed the miR-142-5p-induced cell proliferation. Conclusion: miR-142-5p promoted the proliferation and migration of RCC cells by targeting BTG3. With this potential onco-miRNA role in the progression of RCC, miR-142-5p may be a therapeutic target for the treatment of RCC.

Keywords: miR-142-5p, renal cell carcinoma, BTG3, proliferation

Introduction

Renal cell carcinoma (RCC) is the most common urologic malignancy, accounting for 2-3% [1] of all adult urologic malignancies and approximately 5% [2] of epithelial cancers worldwide. Among the five subtypes, clear cell RCC (ccRCC) is the most frequently encountered (~75% of cases) and was over-represented (~90% of cases) in a series of metastatic RCC patients [3]. Approximately 30% of patients with RCC are found to have metastatic disease on staging investigations, and roughly one-third of patients with organ-confined disease who are undergoing nephrectomy eventually develop metastases [4]. Either partial or radical nephrectomy of the affected kidney remains the mainstay of curative treatment [5]. RCC is generally resistant to chemotherapy and radiotherapy; however, the introduction of novel molecular-targeted agents has revolutionized the management of patients with metastatic RCC [6, 7]. RCC accounts for 0.5% of all cancer-related deaths, ranking 16th among all cancers [1]. Because most patients are diagnosed when the tumor is still relatively localized and amenable to surgical removal, the 5-year survival rate for RCC is approximately 73% [8]. However, the prognosis for metastatic RCC is poor with a 5-year survival typically less than 10% [9]. Therefore, a better understanding of the molecular mechanisms of RCC progression could lead to the identification of new therapeutic targets for altering the natural history of metastatic disease.

MicroRNAs (miRNAs) are highly conserved, endogenous small (~22nt), noncoding RNA molecules that regulate post-transcriptional gene expression through the epigenetic mechanism of RNA interference. They function via sequence-specific binding of a seed sequence to the 3' end of the untranslated region (UTR) of a

target mRNA, which causes it to either be degraded or translationally inhibited [10]. miRNAs are thought to be involved in the regulation of various cellular biological events, including cell proliferation and differentiation, signal transduction, organ development, tumorigenesis, and progression. Global miRNA expression studies have identified miRNAs that are consistently dysregulated across various types of human cancers, including RCC [11]. Numerous miRNAs have been reported to perform specific functions in the regulation of tumor progression either as tumor suppressors or oncogenes. The potential use of miRNAs in cancer therapies is an emerging and promising field, with research finding that miRNAs play roles in cancer initiation, tumor growth, and metastasis [12].

In previous research, miR-142-5p was revealed to be a diagnostic hallmark of acute rejection after renal transplantation based on its up-regulation in biopsies from patients experiencing acute rejection [13]. Moreover, miR-142-5p and miR-142-3p are downregulated in hepatocellular carcinoma and exhibit synergistic effects on cell motility [14]. However, miR-142-5p is significantly upregulated in metastatic RCC, and this finding suggests a special role for this miRNA in RCC progression [15]. In addition, a member of the same family, miR-142-3p, was reported to act as a tumor suppressor gene or an oncogene in many cancers including RCC. miR-142-3p was found to be specifically upregulated during angiogenesis within forming islets, which suggested a specific role for this miRNA in angiogenesis [16]. Additional research demonstrated that miR-142-3p is upregulated in RCC in comparison with expression levels in normal renal cells [17, 18]. A recent study found consistent, significant upregulation of miR-142-3p in ccRCC of high stage or high grade and concluded this miRNA might be associated with the progression of RCC [15]. miR-142-3p is upregulated in T-cell acute lymphoblastic leukemia (T-ALL) [19]. Previous studies also showed that adenomatous polyposis coli (APC) is a conserved target of miR-142-3p, and the Wnt signaling pathway may be a major target of aberrant hypermethylation in RCC [20]. In contrast to findings in RCC and T-ALL patients, miR-142-3p was shown to be downregulated in patients with non-small-cell lung carcinoma, and it might be a tumor suppressor through the downregulation of high mobility group box 1 (HMGB1) in non-small cell lung cancer [21]. Although previous studies also have demonstrated that a miR-142 family member might function as an oncogene in RCC [17, 18], studies investigating the role of miR-142-5p in RCC are not found in the literature.

In this study, we investigated the expression of miR-142-5p in human RCC tissue and cell lines and assessed the influence of miR-142-5p on cell proliferation and migration. BTG3 was identified as a direct target of miR-142-5p that might mediate its biological effects. The data in the present study suggest that miR-142-5p functions as a tumor oncogene by down-regulating BTG3 expression, providing a potential diagnostic and therapeutic target for the treatment of RCC.

Materials and methods

Ethics statement and tissue samples

This study was approved by the ethical board of Renmin Hospital of Wuhan University and complied with the Declaration of Helsinki. Patients gave written informed consent. RCC tissues and adjacent normal renal tissues were collected from 30 patients undergoing radical nephrectomy at Renmin Hospital of Wuhan University between January 2012 and December 2015. The samples were immediately snap-frozen in liquid nitrogen, and RCC was confirmed in all samples by trained pathologists.

Cell lines, cell culture and transfection

The human RCC cell lines A498, Caki-1, and 786-O cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). A normal renal cell line (HK-2) was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cells were grown under standard conditions in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS, Gibco), 1% glutamine, and 1% penicillin/streptomycin (Invitrogen), and maintained in a humidified incubator with 5% CO_2 at 37°C. The miR-142-5p mimic and negative control were purchased from GenePharma (GenePharma, China). Cells were transfected with miR-142-5p mimic or negative control using Lipofectamine

2000 (Invitrogen, Canada) following the manufacturer's instructions. The pXJ40-myc-BTG3 plasmid was prepared by subcloning after PCR amplification of the coding region of BTG3 and confirmed by sequencing. The culture medium was replaced by fresh medium 6 h after transfection, and the cells were incubated for 48 h prior to further testing.

Quantitative reverse transcription PCR

Total RNA from tissue samples and cell lines was extracted using TRIzol reagent (Invitrogen) and purified with an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The SYBR-Green PCR master mix (Applied Biosystems, Foster City, CA, USA) was used with a 7500 Real-time PCR system (Applied Biosystems). All reactions were performed in triplicate. PCR primers included: miR-142-5p: 5'-AACTCCAGCTGGTCCTTAG-3' and 5'-TCTTGA-ACCCTCATCCTGT-3'; U6: 5'-CTCGCTTCGGCAGC-ACA-3' and 5'-AACGCTTCACGAATTTGCGT; 18S: 5'-CATTCGTATTGCGCCGCT-3' and 5'-CGACGGT-ATCTGATCGTC-3'; BTG3: 5'-ATGAAGAATGAAAT-TGCTG-3' and 5'-TTAGTGAGGTGCTAACATGTG-3'. The relative quantification value for each gene was calculated by the $2^{-\Delta\Delta Ct}$ method using U6 as an internal control.

Western blotting

Western blotting was performed as previously-described [22]. Briefly, cells were lysed in a modified radioimmunoprecipitation (RIPA) buffer, and protein was separated by electrophoresis before transfer to membranes. Membranes were probed with anti-β-actin or anti-BTG3 primary antibodies at 4°C overnight, before horseradish peroxidase (HRP)-linked secondary antibodies were added for 1 h at room temperature. Western blots were developed using an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Piscataway, NJ, USA).

Cell proliferation assay

Cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay performed according to the manufacturer's instructions (Sigma-Aldrich, St.Louis, MO, USA). Cells were cultured at a density of 5,000 cells/well in 96-well plates and transfected with various concentrations of miR-142-5p mimic or negative control. Cells were cultured for 72 h, and MTT reagent was added and incubated for 4 h. Then the reaction was stopped by addition of 150 μl DMSO, and the optical density (OD) was measured at 490 nm by an enzyme immunoassay plate reader instrument (BioRad, Hercules, CA, USA).

In addition, after a transfection period of 48 h, cells were seeded in 24-well plates in triplicate at a density of 10,000 cells per well for generation of growth curves. Cells were then trypsinized and stained with the Trypan Blue Staining Cell Viability Assay Kit (Beyotime, Biotechnology, Shanghai, China), and the numbers of viable cells were counted at 24, 48, and 72 h.

Colony formation assay

For the colony formation assay, cells were transfected with miR-142-5p mimic or negative control for 48 h and then grown in 6-well plates in growth medium. After culture for 7 days, the colonies formed were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde, and then stained with 0.5% crystal violet. Stained colonies larger than 1 mm in diameter were counted. Triplicate samples were tested, and each colony formation assay was repeated at least two times.

Cell migration assay

Cell migration activity was evaluated with the Transwell system (24-well plates, 8-μm pore size, BD Bioscience, Franklin Lakes, NJ, USA). After 48 h of transfection with miR-142-5p mimic or negative control, aliquots of 100,000 786-0 cells were resuspended in RPMI-1640 medium without FBS and seeded into the upper chamber coated with collagen IV. The lower chamber was filled with 0.4 ml RPMI-1640 medium containing 10% FBS. After incubation for 24 h, the cells were fixed with 10% formalin and stained with 0.1% crystal violet solution. Images were taken of the migrating cells, and cell counts were obtained for five randomly selected fields of view under a light microscope at ×100 magnification. All experiments were performed in triplicate.

Cell cycle assay

Cells were harvested after transfection with miR-142-5p or negative control for 48 h and

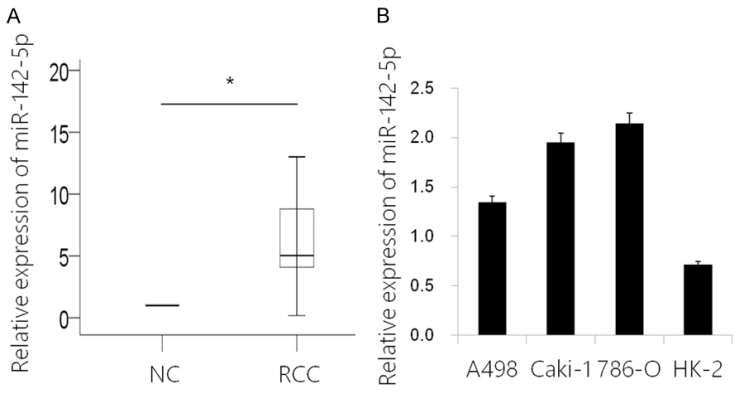

Figure 1. miR-142-5p was commonly upregulated in RCC specimens and cell lines. A. Relative expression of miR-142-5p in 30 paired clinical RCC specimens and adjacent normal tissues as measured by qRT-PCR. B. Expression level of miR-142-5p in 3 RCC cell lines (786-0, A498, and Caki-1) and one normal renal cell line (HK-2) as analyzed by qRT-PCR. U6 served as an internal reference. All data are expressed as mean ± SD for three independent experiments. *P<0.05.

washed three times with cold PBS. Ice-cold 70% ethanol was then added, and the cells were fixed at 4°C overnight. After resuspension in fluorescence-activated cell sorting (FACS) solution with RNase and propidium iodide (PI), cells in different phases were detected using a FACScan flow cytometer (BD Biosciences).

Dual-luciferase reporter assay

The BTG3 3'-UTR containing the putative miR-142-5p binding site was cloned into the p3× Flag-CMV 10 vector, and the plasmid was confirmed by sequencing. 786-0 cells were seeded in 12-well plates, and once they reached 60-80% confluency, they were transiently co-transfected with the vector along with miR-142-5p mimic or negative control. After a transfection period of 30 h, luciferase activity was measured using a Dual-Luciferase Reporter assay kit (Promega, Madison, WI, USA).

Statistical analysis

The data are expressed as mean ± standard deviation (SD) from three separate experiments. Statistical comparisons between two data samples were carried out using Student's t test, and for multiple samples, one-way analysis of variance (ANOVA) with a post-hoc test was performed using SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered statistically significant.

Results

Elevated expression of miR-142-5p in RCC specimens and RCC cell lines

To examine whether miR-142-5p is differentially expressed in RCC, we analyzed the expression level of miR-142-5p in 30 paired RCC specimens and pair-matched adjacent normal renal tissues by qRT-PCR. The data demonstrated that miR-142-5p expression was significantly higher in clinical RCC specimens than in normal renal tissues (**Figure 1A**). Moreover, we detected miR-142-5p expression in a series of human RCC cell lines, and miR-142-5p expression was higher in all RCC cell lines than in the control normal renal cell line (**Figure 1B**).

miR-142-5p promotes proliferation of RCC cells

To study the effects of the miR-142-5p mimic on the proliferation of RCC cells, 786-0 cells were exposed to different concentrations of miR-142-5p mimic for 72 h, and cell proliferation was evaluated by MTT assay. We observed significant time- and dose-dependent increases in 786-0 cell proliferation after miR-142-5p transfection, compared to the proliferation of cells transfected with the negative control (**Figure 2B**, **2C**). The colony formation assay revealed that colony numbers also were significantly increased after miR-142-5p transfection (**Figure 2D**).

Because the miR-142-5p mimic promoted 786-0 cell proliferation, we examined the effects of miR-142-5p on 786-0 cell cycle progression by flow cytometry. As shown in **Figure 2E**, transfection with the miR-142-5p mimic decreased the proportion of cells in G1 phase, while the percentage of S phase cells was greater in the miR-142-5p group than in the negative control group. Thus, these results indicated that RCC cell proliferation could be significantly increased by miR-142-5p and overexpression of miR-142-5p can prevent G1 phase arrest among 786-0 cells.

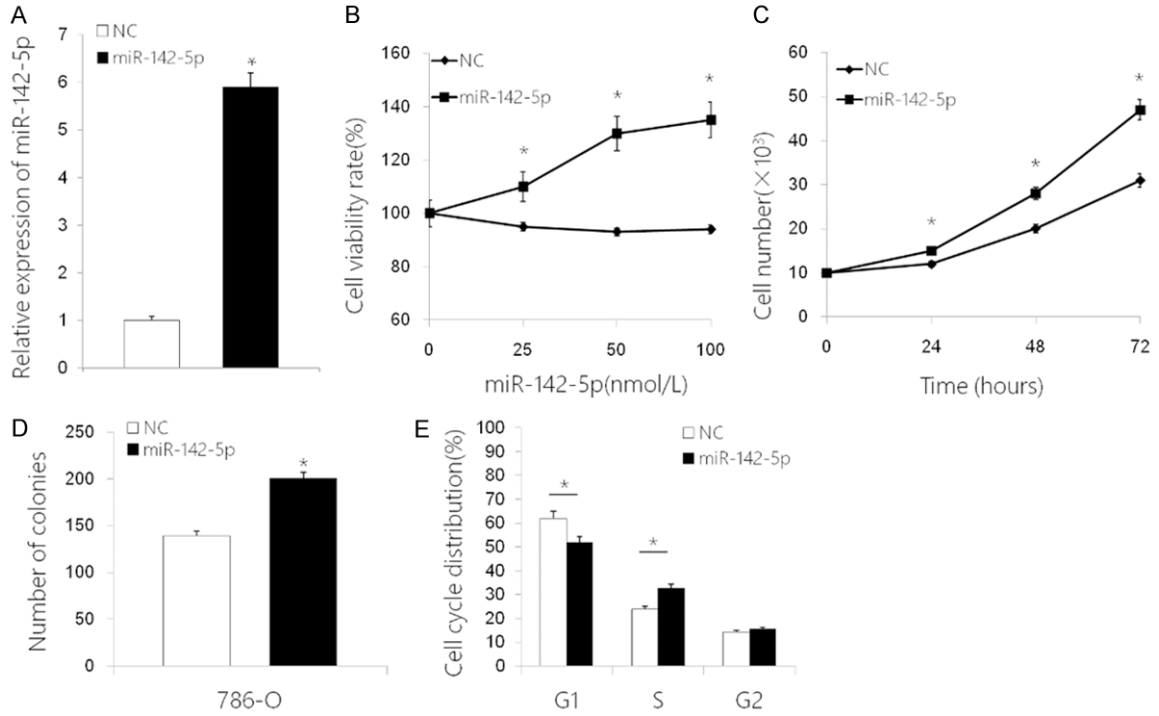

Figure 2. miR-142-5p promotes proliferation of RCC cells. A. miR-142-5p expression by qRT-PCR after transfecting of 786-O cells with miR-142-5p or negative control (NC). B. Cell proliferation of 786-O cells by MTT assay after transfection with different concentrations of miR-142-5p or NC for 72 h. C. Growth curves for 786-O cells after 48 h of transfection with miR-142-5p or NC. D. Numbers of colony in miR-142-5p- and NC-transfected groups. E. Cell cycle distribution based on flow cytometry 48 h after transfection of 786-O cells with miR-142-5p or NC. All data are expressed as mean ± SD for three independent experiments. *P<0.05.

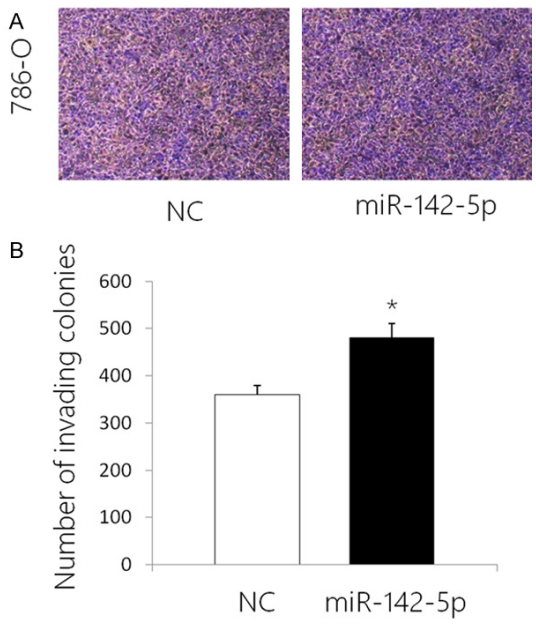

Figure 3. miR-142-5p enhances the migratory ability of RCC cells. A. Representative images (at 100× magnification) of crystal violet-stained migrating 786-O cells after transfection with miR-142-5p or NC. B. Quantification of 786-O cell migration. The data represent mean ± SD. *P<0.05.

miR-142-5p enhances migration of RCC cells

Cell migration assay was performed to observe the role of miR-142-5p in cell migration. As shown in **Figure 3A** and **3B**, cell migration was significantly increased in the group transfected with miR-142-5p compared with that of cells transfected with the negative control. These data suggest that the migration ability of 786-O cells was enhanced by miR-142-5p.

miR-142-5p downregulates BTG3 expression by directly targeting its 3'-UTR

In general, each miRNA regulates the expression of multiple mRNAs, which results in the formation of a complex regulatory network. To identify possible target genes of miR-142-5p, we performed a computational screen using TargetScan software and focused our attention on BTG3 as the potential putative target (**Figure 4A**). A luciferase reporter assay revealed a marked reduction in luciferase activity in cells co-transfected with miR-142-5p and the reporter vector compared with that in cells co-transfected with the negative control and the report-

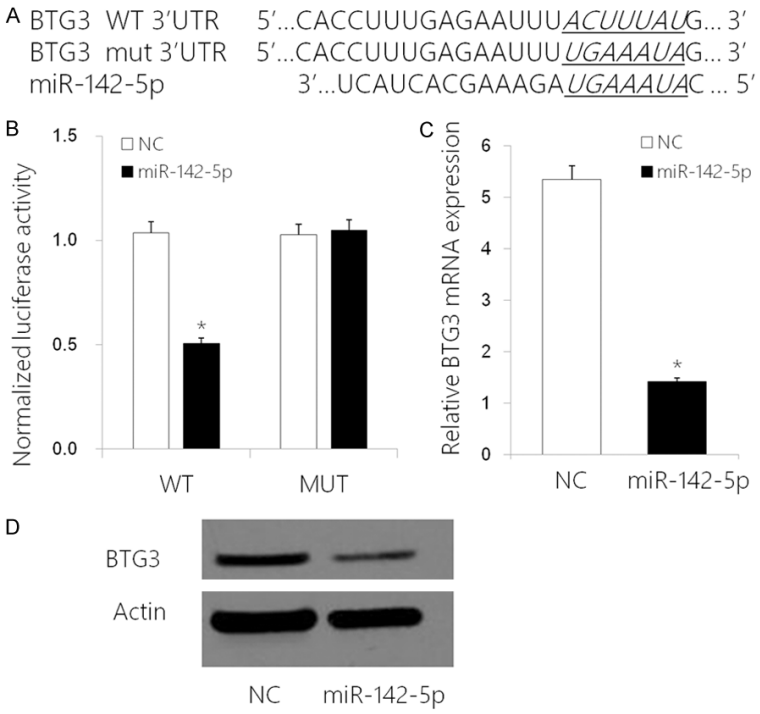

Figure 4. miR-142-5p inhibits BTG3 expression in 786-0 cells by directly targeting its 3'-UTR. A. Binding sites for miR-142-5p in the 3'-UTR of BTG3 mRNA. B. Luciferase reporter assay showing reduced luciferase reporter activity in 786-0 cells containing the BTG3 WT 3'-UTR fragment. C. qRT-PCR analysis of BTG3 mRNA expression in 786-0 cells transfected with miR-142-5p or NC. D. Western blot analysis of BTG3 protein expression in 786-0 cells transfected with miR-142-5p or NC. The data represent mean ± SD. *P<0.05.

er vector, miR-142-5p failed to inhibit luciferase activity with the mutated luciferase construct (**Figure 4B**). Moreover, we demonstrated that overexpression of miR-142-5p significantly decreased BTG3 expression at both the mRNA and protein levels via qRT-PCR and Western blot analyses, respectively (**Figure 4C**, **4D**). These data further indicate that BTG3 is a direct target of miR-142-5p in 786-0 cells.

BTG3 suppresses cell proliferation and reverses miR-142-5p-induced cell proliferation

Because the BTG family, a family of tumor suppressor genes, has been implicated in the regulation of cell cycle progression, we tested the effect of BTG3 in 786-0 cells. As shown in **Figure 5B**, treatment with BTG3 reduced cell proliferation as determined by cell counting. To further determine the effect of BTG3 on miR-142-5p-induced proliferation, direct cell counting was conducted after co-transfection of 786-0 cells with miR-142-5p and BTG3. We found that BTG3 completely inhibited the increase in cell proliferation induced by miR-142-5p (**Figure 5C**).

Discussion

The present study provides the first evidence that miR-142-5p enhances RCC progression by regulating BTG3 expression. We observed that miR-142-5p expression was elevated in RCC tissues and cell lines. Our further experiments suggested that overexpression of miR-142-5p significantly promoted cell proliferation and colony formation among the 786-0 RCC cells. In addition, the migration potential of 786-0 cells expressing miR-142-5p was greater than that of the control group. BTG3 was identified as a direct target of miR-142-5p, and re-expression of BTG3 reversed the miR-142-5p-induced cell proliferation. Taken together, our results indicate that miR-142-5p may play an onco-miRNA role in the progression of RCC.

miR-142 has been reported to be a critical regulator in carcinogenesis and tumor progression via its function as either an oncogene or tumor suppressor gene in various cancers, including RCC. Specifically, miR-142-3p was shown to upregulated in RCC [17, 18]. Another study found that both miR-142-3p and miR-142-5p were significantly upregulated in metastatic RCC, which suggested their special roles in RCC progression [15]. Consistent with these findings, our results indicate that miR-142-5p might function as an oncogene in RCC. The miR-142-5p expression was significantly higher in clinical RCC specimens and human RCC cell lines than in normal renal tissues and a normal renal cell line, respectively. Moreover, exogenous overexpression of miR-142-5p promoted cell proliferation and migration as well as prevented G1 phase arrest in 786-0 cells. These

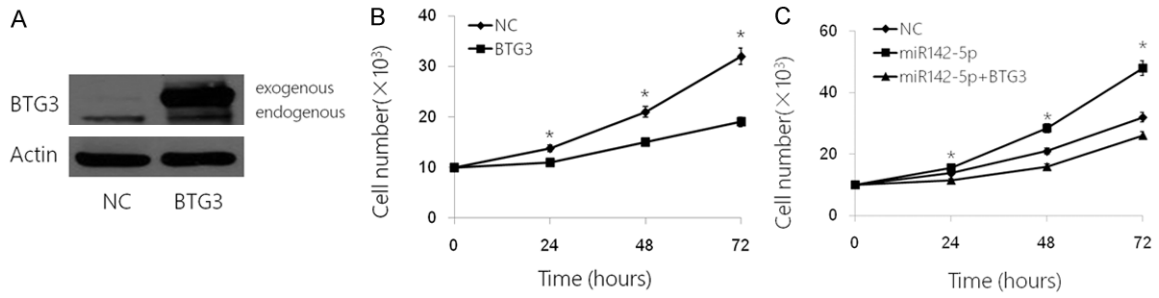

Figure 5. BTG3 reverses miR-142-5p-induced cell proliferation. A. BTG3 protein expression by Western blot analysis. B. Effect of BTG3 on 786-O cell proliferation. C. Growth curve showing that re-expression of BTG3 reversed the miR-142-5p-induced cell proliferation. The data represent mean ± SD. *P<0.05.

results demonstrate that miR-142-5p acts as an oncogenic miRNA in RCC.

BTG3 is a member of the B-cell translocation gene family and is thought to be a negative regulator of cell growth via its binding to E2F1, Smad8 receptor-regulated Smad transcription factor, and CCR4 transcription factor-associated protein Caf1 and ultimate suppression of proliferation and cell cycle progression [23-25]. In addition, BTG3 binds and suppresses Akt and Ras/MAP kinase signaling [26]. BTG3 expression was negatively correlated with lymph node metastasis in lung cancer [27] as well as with distant metastasis in gastric [28] and hepatocellular [29] cancer. Another study also reported that BTG3 is downregulated in renal cancer and the responsible mechanism may involve promoter hypermethylation [30]. Consistent with a previous study [31], our results indicated that BTG3 is a direct target of miR-142-5p in RCC cells. Moreover, miR-142-5p overexpression was correlated with BTG3 down-regulation, leading to the promotion of cell proliferation and migration. Furthermore, re-expression of BTG3 reversed the miR-142-5p-induced promotion of cell proliferation. Thus, our data revealed that the oncogene role of miR-142-5p in RCC might be mediated via the regulation of BTG3 expression.

Conclusion

In summary, our study demonstrated that miR-142-5p is upregulated in RCC specimens and cell lines. Overexpression of miR-142-5p promoted RCC cell proliferation and colony formation, increased RCC cell migration, and induced 786-O cell entry into S phase. The oncogene function of miR-142-5p was mediated by the down-regulation of its downstream target gene BTG3. These findings suggest that miR-142-5p may be a promising target for the development of new treatments for RCC.

Acknowledgements

This work was supported by the National Scientific Foundation of China (No. 81101948) and the Fundamental Research Funds for the Central Universities (No. 2042014kf0624).

Disclosure of conflict of interest

None.

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