

## ZBTB7/miR-137 Autoregulatory Circuit Promotes the Progression of Renal Carcinoma

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Renal carcinoma greatly threatens human health, but the involved molecular mechanisms are far from complete understanding. As a master oncogene driving the initiation of many other cancers, ZBTB7 has not been established to be associated with renal cancer. Our data revealed that ZBTB7 is highly expressed in renal carcinoma specimens and cell lines, compared with normal cells. The silencing of ZBTB7 suppressed the proliferation and invasion of renal cancer cells. ZBTB7 overexpression rendered normal cells with higher proliferation rates and invasiveness. An animal study further confirmed the role of ZBTB7 in the growth of renal carcinoma. Moreover, miR-137 was identified to negatively regulate the expression of ZBTB7, and its abundance is inversely correlated with that of ZBTB7 in renal carcinoma specimens and cell lines. ZBTB7 overexpression may be induced by miR-137 downregulation. Interestingly, ZBTB7 can also suppress miR-137 expression by binding to its recognition site within the miR-137 promoter region. Taken together, we identified an autoregulatory loop consisting of ZBTB7 and miR-137 in gastric cancers, and targeting this pathway may be an effective strategy for renal carcinoma cancer therapy.

**Key words:** miR-137; ZBTB7; Renal carcinoma

### INTRODUCTION

Kidney cancer is a type of malignant tumor with higher morbidity and mortality in the past decade<sup>1,2</sup>. Despite advances in therapeutic modalities, renal cell carcinoma patients benefit little from the current treatments, such as surgery, chemotherapy, and radiotherapy<sup>3,4</sup>. One of the major obstacles hampering kidney cancer treatment is that the underlying molecular mechanism has not been completely elucidated yet. Therefore, the identification of new genes and regulators involved with the initiation and progression of renal cancer is always of interest in the field of cancer research.

ZBTB7 is a member of the POK family. It is POK erythroid myeloid oncogenic factor. So its name is ZBTB7. It is a master oncogene identified to drive other genes to promote the progression of human tumors<sup>5,6</sup>. Unlike its established role in many other types of cancers<sup>7</sup>, the implication of ZBTB7 in renal cancer has not been studied yet. Furthermore, the molecular mechanism of ZBTB7 overexpression in cancers is still unknown.

miRNAs have been well recognized as a key modulator for cancer initiation and progression<sup>8,9</sup>. These noncoding RNAs can act as both oncogene and tumor suppressor, depending on their targets or the context. For renal cancer, miRNAs have also been shown to be associated with

gastric cancer<sup>10</sup>. miR-137, a tumor suppressor miRNA known for glioma and head neck cancer, is shown to be downregulated in renal cancer specimens and cell lines<sup>11–13</sup>. The restoration of miR-137 was identified to suppress the growth of kidney cancer both in vitro and in vivo<sup>13</sup>. However, mRNA molecules targeted by miR-137 are still not identified in renal cancer.

This study is aimed to investigate if ZBTB7 is overexpressed in human renal carcinoma, and if so, what is the role of ZBTB7 for the progression of kidney cancer. Also, the association between ZBTB7 and miR-137 was investigated in our study.

### MATERIALS AND METHODS

#### *Cell Lines and Cell Culture*

Renal cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), as well as normal kidney epithelial cells. These cell lines were grown in 10% FBS-containing media recommended by ATCC in a 37°C atmosphere with 5% CO<sub>2</sub>.

#### *Tumor Specimen*

Renal cancer specimens were harvested from patients with their written informed consent, following

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the procedures approved by the ethical review board in the First Affiliated Hospital of Zhengzhou University (Zhengzhou, Henan, P.R. China). The renal cell cancer specimens were cut into small pieces with scissors. After the incubation with collagenases for 2 h, these cancerous tissues were prepared as single-cell suspension. Then primary gastric cancer cells were cultured with DMEM containing 10% FBS (Hyclone, Logan, UT, USA).

#### Quantitative PCR (qPCR)

Total RNA was extracted using TRIzol solution (Invitrogen, Carlsbad, CA, USA), and then RNA was transcribed into cDNAs with Rever Tra Ace qPCR RT Kit (Toyobo, Osaka, Japan) according to the procedures provided by the manufacturer. Subsequently, qPCR was performed with TaqMan<sup>®</sup> 2× Universal PCR Master Mix (Applied Biosystems, New York, NY, USA) on CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules CA, USA). The used primers were: ZBTB7 5'-GGAGC CCAGTCCCAACTTC-3' (forward) and 5'-GTTCAGC CCACTCAGGATGT-3' (reverse); GAPDH 5'-GCGAG ATCGCACTCATCATCT-3' (forward) and 5'-TCAGT GGTGGACCTGACC-3' (reverse). qPCR for miR-137 detection was conducted following the protocols described previously<sup>14</sup>. Briefly, isolated total RNA was inversely transcribed on Applied Biosystems 9700 Thermocycler. qPCR was then performed according to a standard TaqMan<sup>®</sup> PCR kit protocol on Applied Biosystems 7300 Real-Time PCR System. The involved primer for miR-137 detection was purchased from Applied Biosystems (MIMAT0000429).

#### Lentiviral Vectors, Plasmids, and miR-137 Mimics and Inhibitors

Lentiviral vectors (Lv-shZBTB7 and Lv-scrambled) were purchased from Qingdao Higene Biotechnology Cooperation (Qingdao, P.R. China). A plasmid expressing ZBTB7 (pcDNA-ZBTB7) was constructed as follows. A DNA fragment encoding ZBTB7 was amplified with cDNAs extracted from MKN45 cells as templates using the following primers (ZBTB7-forward: 5'-CTAGCTA GCatggccggcgctggac-3'; ZBTB7-reverse: 5'-GCTCT AGAttaggcgagtcggctgtg-3'). The generating PCR product was inserted into pcDNA3.0 to get pcDNA-ZBTB7. pcDNA-GFP was used as control. mirVana<sup>®</sup> miRNA mimic (Cat. No. 4464066) and anti-miR™ miRNA inhibitor (Cat. No. AM17000) were used to increase and decrease the expression level of miR-137, respectively.

#### Immunoblotting

Total protein was extracted from cell lysate with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher, Waltham, MA, USA), followed by being separated by SDS-PAGE and transferred onto 0.45- $\mu$ m cellulose

acetate membrane. Five percent fat-free milk was used to block the membranes, and then ZBTB7-specific antibodies (1:1,000; BD, Sparks, MD, USA) were used to incubate the membranes, followed by the treatment of horseradish peroxidase (HRP)-conjugated secondary antibodies. The bands were subjected to ChemiDox XRS imaging system (Bio-Rad)-based analysis.

#### Proliferation Assays

Twenty-four hours after the indicated treatments,  $5 \times 10^3$  cells were seeded in 96-well plates. At the indicated time points, MTT solution was added to the cell culture. After 4-h incubation, the media were removed and DMSO was added to the cultures. The absorbance was determined on a model 550 microplate reader at 570 nm. The proliferation rates were calculated according to the following formula: proliferation rates (fold) = absorbance of tested cells/absorbance of control cells.

#### Invasion Assay

Transwell assay was performed to detect the invasiveness of cells under the indicated treatments. Forty-eight hours after the indicated treatments,  $3 \times 10^3$  cells were seeded on Matrigel-pretreated inserts. Another 24 h later, crystal violet (0.1%) was used to stain cells, and then stained cells were counted using a CX-22 microscope (Olympus, Tokyo, Japan). The number of stained migrated cells was recorded in five random fields (200 $\times$ ).

#### Animal Studies

Animal study was performed following the Guide for the Care and Use of Laboratory Animals, and the procedures were approved by the ethical review board in China–Japan Union Hospital of Jilin University (Changchun, Jilin, P.R. China). To establish renal cancer xenograft mouse model,  $5 \times 10^6$  786-0 cells, which were treated with Lv-shZBTB7 or Lv-scrambled, were injected into the left flanks of 4- to 6-week-old BALB/c nude mice ( $n = 16$ ). The diameters of tumors were measured with calipers every 7 days. The volumes were calculated according to the following formula: volume ( $\text{mm}^3$ ) = length  $\times$  (width)<sup>2</sup>/2. Twenty-eight days later, the mice were all sacrificed, followed by the harvesting of tumors for weighing.

#### ChIP Assay

A ChIP assay was performed using the ChIP chromatin immunoprecipitation kit (Millipore, Beijing, P.R. China). Immunoprecipitate (IP) complexes were immunoprecipitated with an anti-ZBTB7 or a rabbit IgG antibody overnight at 4°C. The isolated genomic DNA was obtained and used for quantitative PCR analysis. Ten percent of total genomic DNA from the nuclear extract was used as input. The primers used to detect the miR-137 promoter sequence are shown in the figures. The amplification

efficiency was calculated, and the data were expressed as enrichment related to input.

#### Luciferase Assay

A 233-bp ZBTB7 mRNA 3' UTR DNA fragment, which harbors miR-137 MRE (AGCAATA), was inserted into psiCheck2 to construct psiCheck2-MRE-wt. A mutant construct (ATTGGTA) was also inserted to psiCheck2 to obtain psiCheck2-MRE-mut as a control plasmid. After miR-137 mimics and inhibitors were added to the cell culture, psiCheck2, psiCheck2-MRE-wt, or psiCheck2-MRE-mut were transfected into cells. Forty-eight hours later, cell lysate was subjected to the quantification of luciferase expression level with Dual-Luciferase<sup>®</sup> Reporter Assay System/Dual-Luciferase<sup>®</sup> (Promega, Madison, WI, USA) according to the manufacturer's instructions.

#### Statistical Analysis

The involved experiments were subjected to statistical analysis based on two-tailed Student's test for two groups and ANOVA for three and more groups. The difference was regarded to be "significant" and "very significant" with a value of  $p < 0.05$  and  $p < 0.01$ , respectively.

## RESULTS

### ZBTB7 Expression Was Elevated in Renal Carcinoma Specimens

The expression level of ZBTB7 was evaluated by qPCR and immunoblot assays in kidney cancers ( $n=26$ ).

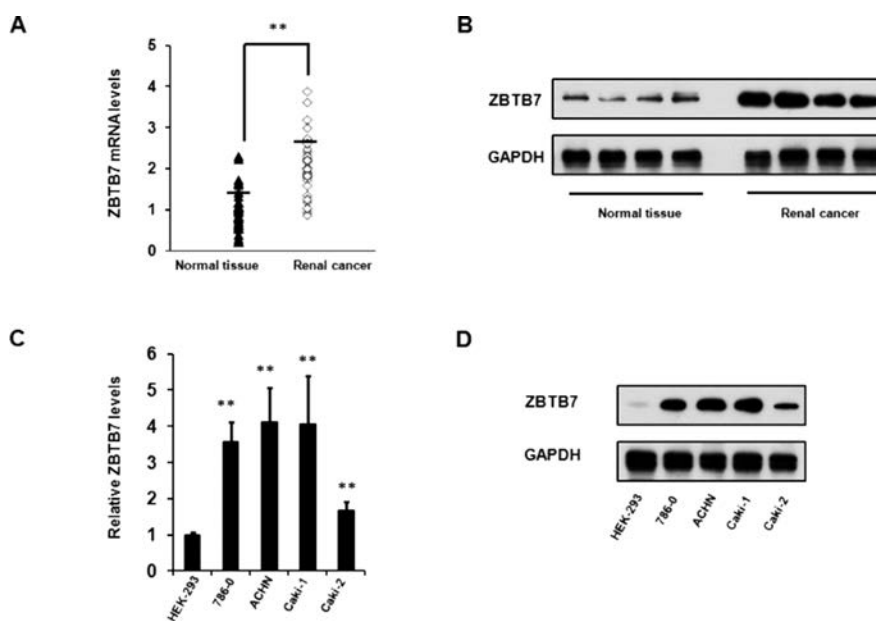
ZBTB7 mRNA abundance was increased in renal carcinoma specimens, compared with matched adjacent normal tissues (Fig. 1A). Immunoblot assay further revealed that ZBTB7 proteins were overexpressed in renal cell cancers (Fig. 1B). Similarly, ZBTB7 was also found to be overexpressed in renal cancer cell lines at the level of mRNA (Fig. 1C) and protein (Fig. 1D).

### ZBTB7 Suppression Impairs the Proliferation and Invasion of Renal Carcinoma Cells

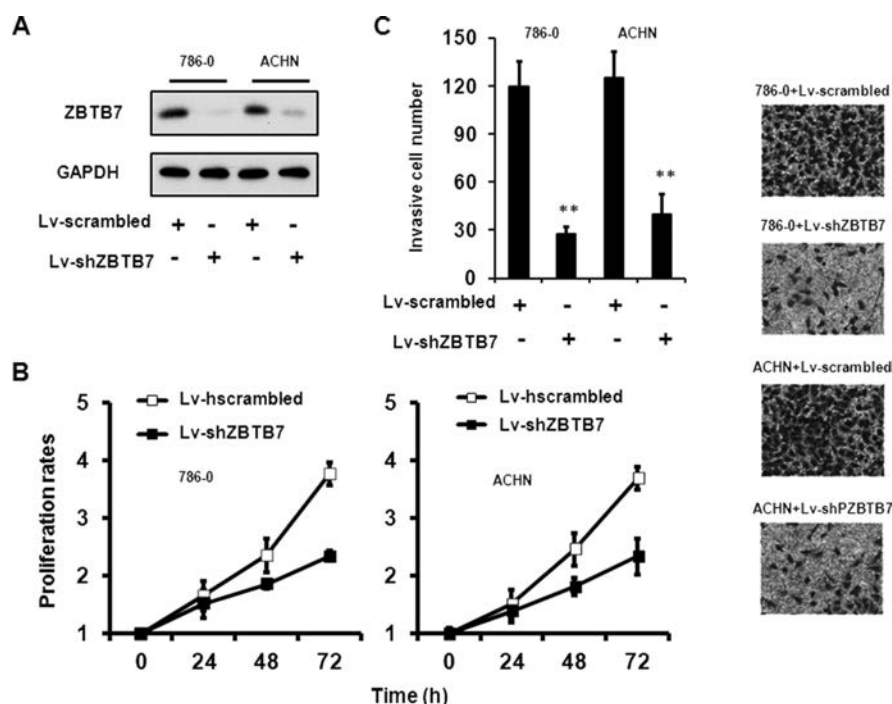
A lentiviral vector expressing a shRNA targeting ZBTB7 (Lv-shZBTB7) was used to suppress the expression of ZBTB7 in renal cancer cells (Fig. 2A). Lv-shZBTB7 infection reduced the proliferation rates of renal cancer cells (Fig. 2B). Furthermore, the ability of renal cancer cells to infiltrate through Matrigel was also suppressed by ZBTB7 suppression (Fig. 2C).

### ZBTB7 Overexpression Enhances the Proliferation and Invasiveness of Normal Cells

We were interested if the elevation in ZBTB7 expression could render normal kidney cells with higher proliferation rates and invasiveness. We employed a plasmid, pcDNA-ZBTB7, to increase the expression of ZBTB7 in HEK-293 cells (Fig. 3A). MTT assay revealed that pcDNA-ZBTB7 transfection enhances the proliferation of HEK-293 cells (Fig. 3B). Transwell assays showed that ZBTB7 overexpression was also able to promote the invasion of normal cells (Fig. 3C).



**Figure 1.** The expression profile of ZBTB7 in renal carcinoma. (A) Quantitative PCR was employed to detect ZBTB7 mRNA levels in a cohort of renal cancer specimens and their matched normal tissues ( $n=26$ ). (B) ZBTB7 expression was also evaluated by Western blotting in the above samples. (C) Quantitative PCR was employed to detect ZBTB7 mRNA levels in selected renal carcinoma cell lines. (D) ZBTB7 expression was also evaluated by Western blotting in the above cells.  $**p < 0.01$  versus normal tissue (A) and HEK-293 (C).



**Figure 2.** ZBTB7 downregulation impairs renal cancer cell proliferation and invasion. (A) ZBTB7 expression level was examined by Western blotting in renal cancer cells, 48 h after the infection of Lv-shZBTB7 (10 MOI) or Lv-scrambled (10 MOI). (B) The proliferation rates of kidney cancer cells were determined at the indicated time points, 24 h after the infection of Lv-shZBTB7 (10 MOI) or Lv-scrambled (10 MOI). (C) The invasiveness of renal cancer cells was evaluated by Transwell assays, 24 h after the infection of Lv-shZBTB7 (10 MOI) or Lv-scrambled (10 MOI). The stained migrate cells were counted under a microscope (200 $\times$ ). \*\* $p < 0.01$  versus Lv-scrambled.

#### *ZBTB7 Downregulation Reduced the Growth of Renal Cancer in Mice*

Subsequently, we investigated if ZBTB7 overexpression is important for the growth of renal cell cancers. Cells (786-0), which were infected with Lv-shZBTB7 or Lv-scrambled, were subcutaneously injected, followed by periodical measurement of tumor size at the indicated time points. ZBTB7 downregulation was shown to decrease the growth of 786-0 renal carcinoma xenografts (Fig. 4A). Also, the weight of tumors derived from Lv-shZBTB7-infected cells was lower than that of the control group (Fig. 4B).

#### *ZBTB7 Is an Authentic Target of miR-137 in Renal Cancer Cells*

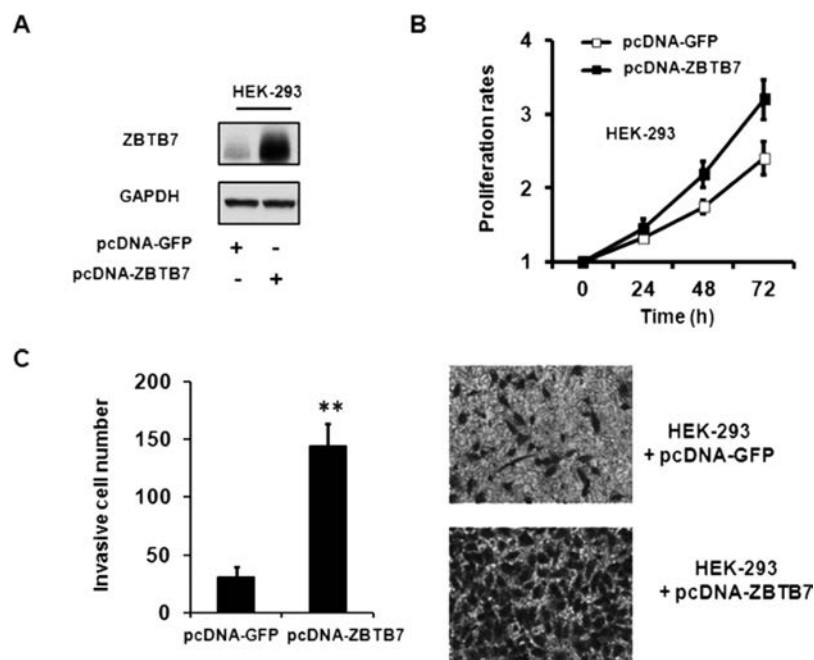
Given that ZBTB7 plays an important role for the progression of renal cancer cells, we subsequently studied what causes the increase in the expression of ZBTB7. A putative miR-137 MRE was found to be located within the 3'-UTR of ZBTB7 mRNA (Fig. 5A). To confirm the regulation of miR-137 on ZBTB7, we constructed psiCheck2-MRE-wt, which expresses luciferase in an miR-137's MRE-dependent mechanism, to test if miR-137 negatively regulates the expression of ZBTB7.

The data showed that miR-137 mimics suppresses luciferase expression by psiCheck2-MRE-wt, but not that by psiCheck2-MRE-mut, in 786-0 cells (Fig. 5B). Accordingly, miR-137 suppression was demonstrated to restore psiCheck2-MRE-wt-mediated luciferase (Fig. 5C). qPCR and Western blot revealed that miR-137 overexpression reduced the expression of ZBTB7 in renal cancer cells (Fig. 5D and E), while its suppression increased the abundance of ZBTB7 in normal cells (Fig. 5F and G).

#### *ZBTB7 Suppresses the Transcription of miR-137 by Binding to its Promoter*

Furthermore, we studied if ZBTB7 can conversely affect the expression of miR-137 in renal cancer cells. A putative ZBTB7 recognition site was found to be located within the miR-137 promoter (Fig. 6A). CHIP assay indicated that ZBTB7 can bind this predicted recognition site (Fig. 6B). Moreover, ZBTB7 can suppress the expression of luciferase by pGL3-pro-wt, which contains the recognition site, but not that by the mutant construct (pGL3-pro-mut) (Fig. 6C). qPCR also showed that ZBTB7 overexpression decreased miR-137 expression in normal cells (Fig. 6D), while ZBTB7 siRNA restored the expression of miR-137 in renal carcinoma cells (Fig. 6E).



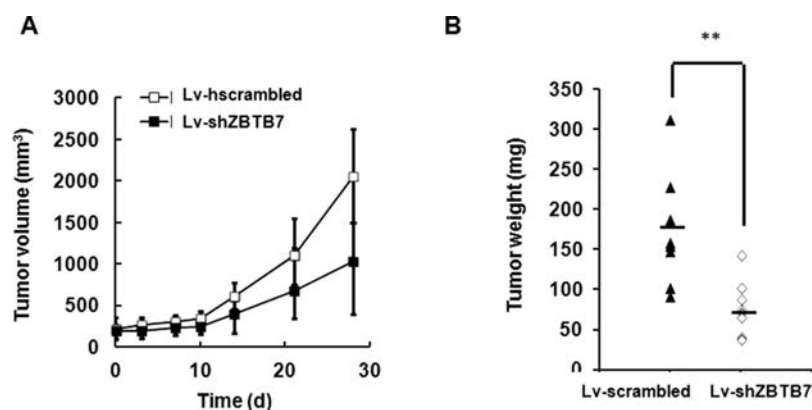


**Figure 3.** The effect of ZBTB7 overexpression on normal cells. (A) ZBTB7 expression level was examined by Western blotting in HEK-293 cells, 48 h after the transfection of pcDNA-ZBTB7 ( $1 \text{ ng}/1 \times 10^6$  cells) or pcDNA-GFP ( $1 \text{ ng}/1 \times 10^6$  cells). (B) The proliferation rates of normal cells were determined at the indicated time points, 24 h after the transfection of pcDNA-ZBTB7 ( $1 \text{ ng}/1 \times 10^6$  cells) or pcDNA-GFP ( $1 \text{ ng}/1 \times 10^6$  cells). (C) The invasiveness of normal cells was evaluated by Transwell assays, 24 h after the transfection of pcDNA-ZBTB7 ( $1 \text{ ng}/1 \times 10^6$  cells) or pcDNA-GFP ( $1 \text{ ng}/1 \times 10^6$  cells). The stained migrated cells were counted under a microscope (200 $\times$ ). \*\* $p < 0.01$  versus pcDNA-GFP.

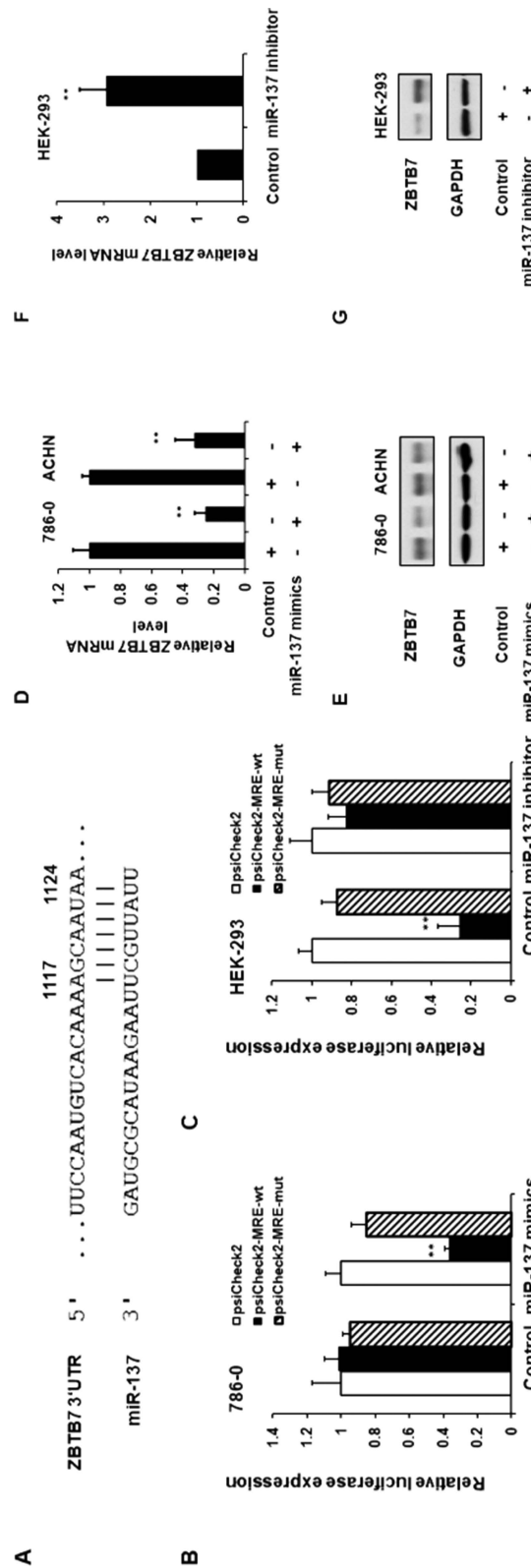
## DISCUSSION

ZBTB7 has been shown to be associated with various types of cancer, but its implication in renal cancer is still unknown<sup>15,16</sup>. Our study provided evidence showing that ZBTB7 contributes to the progression of renal cancer. This is the first time to demonstrate the link between ZBTB7 and kidney cancer.

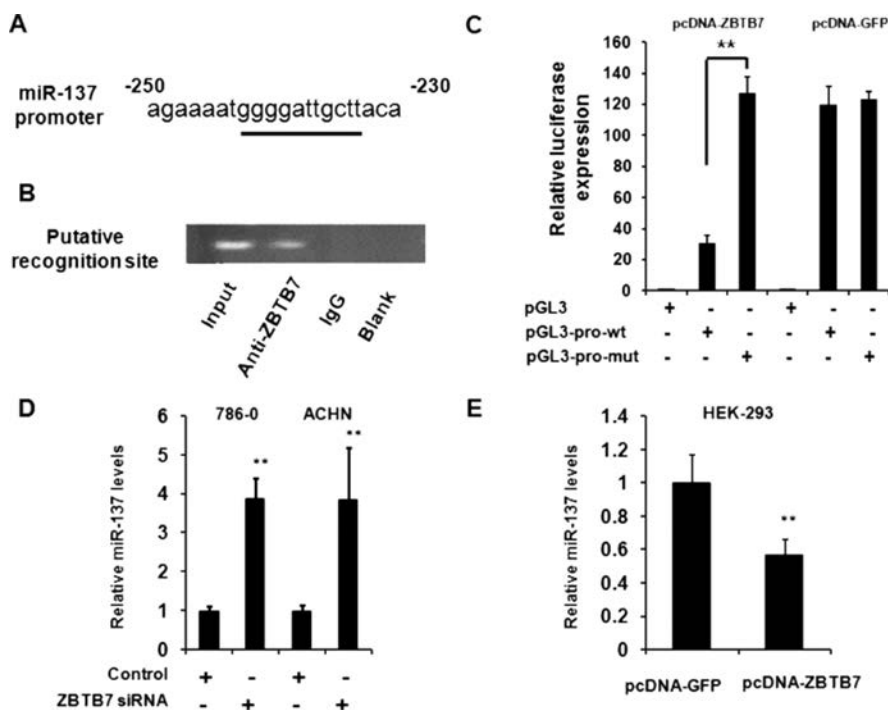
It is of interest what effectors mediate the oncogenic function of ZBTB7 on renal cancers. As a transcription factor, ZBTB7 exerts its biological activity mainly through binding the promoter region of target downstream targets and suppressing their transcription. In fact, some downstream genes have been well documented to be among the list of ZBTB7-regulated genes, such as survivin<sup>17</sup> and p38 MAPK<sup>18</sup>, which are also demonstrated to



**Figure 4.** The effect of ZBTB7 suppression on the growth of renal cancer in vivo. (A) 786-0 cells infected with Lv-shZBTB7 (10 MOI) or Lv-scrambled (10 MOI) was injected into the armpits of nude mice (both  $n = 8$ ). The diameters of tumors were measured, and the volumes are shown. (B) The tumors were also weighed after the mice were sacrificed. \*\* $p < 0.01$  versus Lv-scrambled.



**Figure 5.** ZBTB7 identified as a target of miR-137. (A) A predicted miR-137 MRE is located within ZBTB7 mRNA 3' UTR. (B) Renal cancer cells were transfected with psiCheck2-MRE-wt, psiCheck2-MRE-mut, or psiCheck2, followed by miR-137 mimics transfection (50 nM). After 48 h, luciferase expression was detected in the above cells. (C) Normal cells were transfected with psiCheck2-MRE-wt, psiCheck2-MRE-mut, or psiCheck2, followed by the transfection of miR-137 inhibitors (50 nM). After 48 h, luciferase expression was detected in the above cells. (D) The expression level of ZBTB7 mRNA was tested in kidney cancer cells, 48 h after transfection with miR-137 mimics (50 nM). (E) ZBTB7 protein was also examined by Western blotting under the above treatment. (F) The expression level of ZBTB7 mRNA was tested in normal cells, 48 h after transfection with miR-137 inhibitors (50 nM). (G) ZBTB7 protein was also examined by Western blotting under the above treatment.  $^{**}p < 0.01$  versus psiCheck2 (B and C) and Control (D and F).



**Figure 6.** The inhibitory effect of ZBTB7 on transcription of miR-137. (A) A putative ZBTB7 recognition site was found to be located within the promoter region of miR-137. (B) Renal cancer cells were transfected with the plasmid pcDNA-ZBTB7 ( $1 \text{ ng}/1 \times 10^6$  cells). Forty-eight hours later, cells were harvested for ChIP assays. The antibodies used are shown. (C) Normal cells were transfected with pcDNA-ZBTB7 ( $1 \text{ ng}/1 \times 10^6$  cells) or pcDNA-GFP ( $1 \text{ ng}/1 \times 10^6$  cells), and 24 h later, pGL3, pGL3-pro-wt, or pGL3-pro-mut was transfected. After another 48 h, luciferase expression was examined in these cells. (D) The expression level of miR-137 was tested in renal cancer cells, 48 h after transfection with ZBTB7 siRNA (50 nM). (E) The expression level of miR-137 was tested in normal cells, 48 h after transfection with pcDNA-ZBTB7 ( $1 \text{ ng}/1 \times 10^6$  cells) or pcDNA-GFP ( $1 \text{ ng}/1 \times 10^6$  cells).  $**p < 0.01$  versus pGL3-pro-wt (C), control, and pcDNA-GFP (E).

be effective therapeutic targets<sup>19</sup>. Therefore, it is required to further identify the downstream effectors of ZBTB7 in renal cancer in future studies.

miR-137, a well-established tumor suppressor for renal cancer, was found to target ZBTB7 in renal cancer cells. The downregulation of miR-137 may be the cause of the elevation in ZBTB7 expression. To our knowledge, this is the first time to reveal the molecular mechanism by which ZBTB7 expression is regulated in cancer cells. Our finding may facilitate the development of effective ZBTB7-targeting cancer therapeutic strategy.

It is worth noting that ZBTB7 also negatively regulates the expression of miR-137 in renal cancer cells, implying an autoregulatory loop existing in the molecular network of kidney cancer. In fact, similar circuits have been reported for other types of cancers, such as liver cancers<sup>20</sup> and glioma<sup>21</sup>. Our results reinforced the concept that such loops are a common regulatory mechanism of cancer initiation and progression.

Taken together, we conclude that the miR-137/ZBTB7 autoregulatory loop contributes to the proliferation and invasion of renal cancer cells. Targeting this network may be a promising strategy for kidney cancer therapy.

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