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*Original Research*

# TRIM59 is a key regulator of growth and migration inrenal cell carcinoma

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**Abstract:** Renal cell carcinoma (RCC) is the most common renal neoplasms and metastatic is common. Previous data have shown that the tripartite motif (TRIM) family proteinswere implicated in human tumoriogenesis. In this study, we aimed to investigate the role of TRIM59 in the cell growth and migration in RCC. The expression of TRIM59 in human RCC tissues was initially examined by qRT-PCR. Alentivirus-based shRNA against TRIM59 (Lv-shTRIM59) was constructed. The effects of TRIM59 knockdown on cell proliferation were examined by *in vitro* MTT assay, colony formation assay and *in vivo* a mouse xenograft model of RCC. Cell migration and invasion after knockdown of TRIM59 were also examined by transwell assay. Our data showed that the mRNA level of TRIM59 in cancerous tissues was 2-fold increased as compared with non-cancerous tissues. Knockdown of TRIM59 in a RCC cell line 786-O significantly slowed down cell proliferative rate and decreased both the colony number and sizes. In the mouse model, knockdown of TRIM59 consistently inhibited tumor growth *in vivo*. Moreover, it was shown that cell migration and invasion were suppressed by 68% and 50%, respectively in TRIM59-depleted 786-O cells. Our data suggest that TRIM59 may serve as a pro-oncogenic protein in promoting the progression of RCC. Knockdown of TRIM59 may be a promising strategy concerning the early detection and treatment of RCC.

***Key words:*** TRIM59; Growth; Migration; Invasion; Renal cell carcinoma.

## Introduction

Renal cell carcinoma (RCC) is a renal neoplasims that originates in the lining of the proximal convoluted tubule, it is the most common type of renal neoplasms and account for 90% of cases, the most common RCC type is clear-cell RCC, and account for 70% of cases. Metastasis is very common, and about 30% patients were diagnosed metastasis (1). For the last decades, the development of biological and clinical prognostic factors has led to the risk directed therapy which has improved the outcome dramatically and made the treatment of this malignancy as one of the great success stories. However, even with the current multimodality treatment, statistics showed that after radical nephrectomy and inferior vena cava (IVC) thrombectomy, 5-year survival is about 64%, and it declines rapidly to approximately 9% if metastasis (2).

The tripartite motif (TRIM) family proteins are revolutionarily a class of highly conservative proteins that have been implicated in a number of critical processes including human tumoriogenesis(3).Proteins from this family are structurally conservative in the N-terminal really interesting new gene (RING) domain which are E3 ubiquitin ligases and frequently involved in the ubiquitin-proteasome system and proteolysis (3).Currently, 77 members have been discovered in this family. The functionof most TRIM family members is poorly understood andwas surmised only based on computational analysis fromtheir RING finger (4). However, one critical role which is shared by some known members and widely reported is the involvement into the development and progression of human tumors (5,6). TRIM59, one of the members from this family, is a surface molecule which has not aroused great interest to researchers since its discovery. Its oncogenic activity has not been identified until the year 2011. Using a transgenic mouse, TRIM59 has been innovatively shown to mediate the progression of prostate cancer through interacting with theRas signaling pathway (5).In another study with the immunohistochemicaltechnique, TRIM59 was deciphered to be a multiple cancer biomarker (6). Interestingly, TRIM59 was remarkably up-regulated in gastric tumors and promoted the degradation of p53in anubiquitinationpathway, by which it promotes tumor growth, cell proliferation and migration in gastric tumors (7).All these studies have indicated that TRIM59 may be critically involved in human tumoriogenesis. However, whether TRIM59 exhibits oncogenic activities in other tumor types remains largely unknown.

This study aimed to investigate the role of TRIM59 in the cell growth and migration in human RCC. Expression of TRIM59 was initially determined in clinical RCC tissues. Furthermore, a lentivirus-delivered

shRNA against TRIM59 (Lv-shTRIM59) was constructed to deplete the expression of TRIM59 in RCC cell 786-O. The effects of TRIM59 knockdown on cell proliferation, migration and invasion would be explored in 786-O cells in this study. This report represents the first report to our knowledge to investigate the critical roles of TRIM59 in RCC.

## Materials and Methods

### Human samples

A total of 20 patients suffering from RCC were collected during their admission to The Fourth Hospital of Hebei Medical University in year 2013.01.01 to 2015.01.01. The matched adjacent non-cancerous tissues were also obtained for each case. All the patients and their parents showed their full consent to participate in our study and written consent forms were obtained from each patient. All of the experimental protocols in this research were in compliance with the official polices and approved by the Ethics Committee in The Fifth People's Hospital of Shanghai.

### Cell culture

Human RCC cell line 786-O and a normal embryonic kidney cell line CCC-HEK-1 were obtained from the cell bank of the Chinese Academy of Sciences (CAS) (Shanghai, China). Both cell lines were maintained in Dulbecco's modified eagle medium (DMEM) (Gibco, Los Angeles, CA, USA) supplied with 10% fetal bovine serum (FBS) (Gibco)at37°C in a 5% $CO_2$ humidity-controlled incubator.

### Total RNAs extraction and cDNA synthesis

The total RNAs of human tissues and cultured cells were extracted usingTrizol Reagent (TakaRa, Shiga, Japan) according to manufacturer's instruction. Isolated RNAs were initially determined of their quality and concentrations usingNanodrop 2000 (Thermo Scientific, U.S.A.), and then reversely transcribed tocDNAsusing the PrimeScript RT Master Mix Perfect Real Time (TaKaRa, Shiga, Japan).

### Quantitative real-time polymerase chain reactions (qRT-PCR)

All qRT-PCRs were performed in an ABI PRISM 7900 Real-Time System with the SYBR Premix Ex Taq Kit (TaKaRa, Shiga, Japan). Briefly, the protocol was as follows: initial denaturation step at 95℃ for 2min, 35 repetition of the three-step cycling program consisting of 30sec at 95℃ (denaturation), 1min at 55℃ (primers annealing), and 30sec at 72℃ (elongation), and final extension step for 10min at 72℃. Amplification productions were determined by 1% (w/v) agarose gels (Bio-Rad, California, USA). *β-actin* wasincluded as the internal control. All quantitative data were normalized to the internal control gene. Primers used were as follows: TRIM59:forward,5'-TACGAGAGCAGCAGCTTGAA-3'reverse,5'-ACGGGTTGAACCTCAGGAAG-3' β-actin:forward, 5'-GTGGACATCCGCAAAGAC-3' reverse, 5'-AAAGGGTGTAACGCAACTA-3'

### Western blot analysis

For cultured cells, when cell confluence reached 90%, cells were washed twice with PBS, followed by lysing with a general lysis buffer (PH=7.5) to generate the whole protein lysate. An equal amount of 50μg protein was loaded to each lanein a 12% SDS-PAGE gel. Proteins were then transferred to polyvinylidenedifluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). Thereafter, the membranes wereblocked with TBS/0.1%Tween-20 (TBST) supplemented with 5% skim milk for 1h,before proceeding to primary antibody incubations over night.The membrane was then incubated with primary antibodies against TRIM59 (1:1000), vimentin (1:1000), N-cadherin (1:1000), E-cadherin (1:1000), cyclin B1 (1:1000), Cdc2 (1:1000) and GAPDH (1:5000) for 4 °C overnight..Immunoreactivity was determined with enhanced chemoluminescent autoradiography (Thermo Scientific, PA, USA) and each experiment was repeated at least three times.

### Recombination lentivirus construct and shRNA interference

In order to create TRIM59-knockdown cell lines, siRNA targeting TRIM59 (5'- ACATTACAGGCAACCATTAAA-3') was inserted into the pFH-L plasmid (Hollybio, Shanghai, China). The non-silencing small interference RNA (siRNA) (siCon, 5'-TTCTCCGAACGTGTCACGT-3') was also used as control. The lentivirus-based short hairpinRNA-expressing vectors were constructed, confirmed by DNA sequencing. For the transfection, $1\times10^7$ of 786-O cells were seeded in 10-cm dishes and cultured for 36h to reach90% confluence. At 2h before transfection, the medium was replaced with FBS-free DMEM. The plasmidmixture containing pFH-L-shTRIM59 (or pFH-L-shCon) and pVSVG-I/pCMVΔR8.92 packaging vectors, as well as Lipofectamine 2000 (Invitrogen,Carlsbad, CA) were added to the cells. At 5h after incubation, themedium was replaced with DMEM containing 10% FBS. Lentiviral particles (Lv-shTRIM59 or Lv-shCon) wereharvested at 48h after transfection and purified by ultra-centrifugation according to previous reports[9,10].As the lentivirus carries greenfluorescence protein (GFP), the viral titer was determined by countingGFP-expressing cells under a fluorescence microscopy asdescribed in previous reports(8).

### Cell viability determination

MTT assay was conducted to determine the cell viability under distinct treatments. Briefly, cells from each group were cultured for a consecutive 5 days. For each monitored day, 2mg/ml of MTT solution was added to each well. After incubated for another 4h at 37°C, media was discarded and 200μl DMSO was added into each well. The plate was shaked for 5 min and the optical density was obtained at 570 nm.

### Wound-healing assay and Transwell assay

786-O cells under distinct treatments were plated on 6-well plates to form a confluent monolayer. Wounds were made with sterile pipette tips and the wound-recovery was observed per 12h. For migration assay, 786-O cells were cultured in 24-well plates and harvested in serum-free DMEM media as single cell suspension. A total of 150ul of cell suspension was then seeded in the

upper chamber (Corning, New York, USA); meanwhile, the lower chamber was filled with 600μl DMEM medium with 10% FBS. For the invasion assay, the upper surface of chamber was pre-coated with Matrigel 6h before seeding cells. After incubated at 37°C for 12h, the cells were fixed with ice-cold methanol for 30min and stained with 0.1% crystal violet for 15min. Cells under the lower surface of chamber were imaged under a microscope at a 200× magnification. Each assay was repeated at least three times with each time in duplication.

### Xenograft model of human RCC

Six-week-old female athymic nude mice (BALB/c$^{nu/nu}$) were used for the experiment. Twelve mice were randomly assigned to the Lv-TRIM59 group or Lv-shNC group (n=6 for each group). 1 786-O cells infected with Lv-shTRIM59 or Lv-shNC($1 \times 10^6$) were injected subcutaneously into the mammary fat pad of the mice. Tumor dimensions were measured each dayfor a whole 7 days and tumor volumes (TV) were calculated as described previously (9). On the 7$^{th}$ day, all mice were sacrificed and the tumors were dissected for weighing.

### Statistical analysis

The results were all exhibited as means ± standard deviation (SD). Statistical analysis was carried out with the Student's *t*-test. Difference was considered significant when $P<0.05$.

## Results

### TRIM59 is highly expressed in RCC.

Initially, the expression of TRIM59 in RCC tissues was examined by qRT-PCR. A total of 20 clinical tissues were collected. As shown in Fig.1A, the mRNA level of TRIM59 in the 20 cancerous tissues was approximately two times of that in the non-cancerous tissues. Moreover, when the proteins from the RCC cell line 786-O and normal embryonic kidney cell line CCC-HEK-1 were synchronously loaded, it was observed that the protein level of TRIM59 in 786-O cells was remarkable; however, TRIM59 was barely detected in the normal CCC-HEK-1 cells (Fig.1B). These data suggest that TRIM59 is highly expressed in RCC.

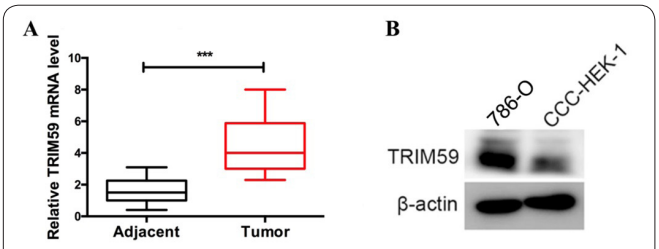


**Figure 1. TRIM59 is highly expressed in Renal cell carcinoma.** (**A**) The mRNA level of TRIM59 was examined in 20 cases of clinical RCC. The paired adjacent non-cancerous tissues were also included as control. (B) A RCC cell line 786-O and a normal embryonic kidney cell line CCC-HEK-1 were synchronously cultured. The protein level of TRIM59 in the two cell lines were detected using western blot analysis.

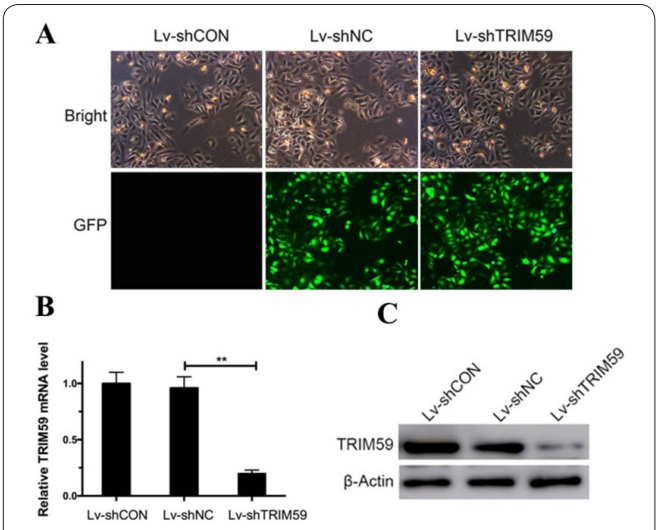


**Figure 2. A lentivirus-based shRNA against TRIM59 is effective to deplete the expression of TRIM59.** (**A**) A lentivirus-delivered shRNA against TRIM59 (Lv-shTRIM59) was constructed to infect the 786-O cells. A lentivirus without any shRNA (Lv-shCon) or with a scramble negative control shRNA (Lv-shNC) was also synchronously infected with 786-O cells. All cells that were infected with a shRNA (specific or scramble) will express the green fluorescence protein (GFP) which exhibits green fluorescence under a fluorescence microscope. Infection efficiency could be confirmed by observing the GFP-expressing cells. (**B, C**) the total RNAs and proteins from each group were further collected. RNAs were subject to qRT-PCR analysis, while total proteins were preceded to western blot analysis. Both the mRNA and protein levels of TRIM59 were depleted by Lv-shTRIM59 in 786-O cells.

### Alentivirus-based shRNA against TRIM59 is effective to deplete the expression of TRIM59.

To further investigate the function of TRIM59 in RCC, we constructed a lentivirus-delivered shRNA against TRIM59 (Lv-shTRIM59) to deplete the expression of TRIM59 in the 786-O cells. Alentivirus without any shRNA (Lv-shCon) or with a scramble negative control shRNA (Lv-shNC) was also synchronously infected with 786-O cells. All cells that were infected with a shRNA (specific or scramble) will express the green fluorescence protein which exhibits green fluorescence under a fluorescence microscope. Observations under a fluorescence microscope suggested that cell infection efficiency was as high as 85% (Fig.2A). Based on the successful infection, we further collected the total RNAs and proteins from each group. It was shown that the cells infected with Lv-shCon or Lv-shNC exhibited a similar mRNA level of TRIM59; however, the relative mRNA level of TRIM59 in cells infected with Lv-shTRIM59 was decreased by up to 60% when compared with control groups (Fig.2B). The TRIM59 protein was barely detected after cells infected with Lv-shTRIM59, while it was remarkably detected in the control groups (Fig. 2C). All these data suggest the successful infection of cells with lentivirus and also the high efficiency of Lv-shTRIM59 in depleting the expression of TRIM59.

### Knockdown of TRIM59 inhibits cell proliferation in 786-O cells.

The effects of TRIM59 knockdown on cell proliferation were then investigated *in vitro*. In the MTT assay, cell proliferation under distinct treatments was consecutively monitored for 6 days. For all these days, cells with control treatments (infected with Lv-shCon or Lv-shNC) exhibited no significant differences in prolifera-

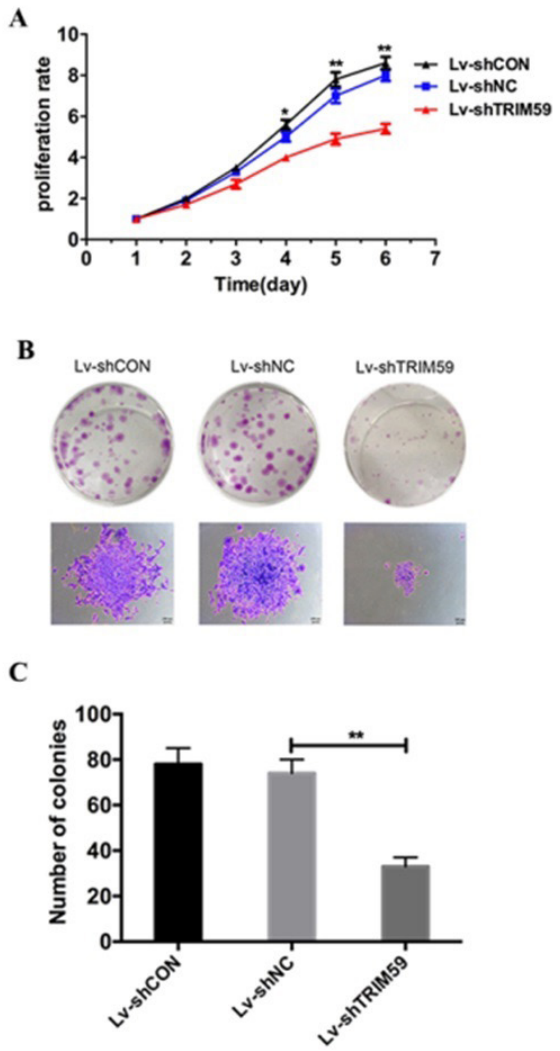


**Figure 3. Knockdown of TRIM59 inhibits cell proliferation in 786-O cells.** (**A**) In the MTT assay, cell proliferation under distinct treatments was consecutively monitored for 6 days. For all these days, cells with control treatments (infected with Lv-shCon or Lv-shNC) exhibited similar proliferative rates. However, 786-O cells infected with Lv-shTRIM59 exhibited a significantly slower proliferative rate since the 4th day onward. By the 6th day, cell numbers in Lv-shTRIM59 groups was approximately 62.5% of those in control groups. (**B**) Colony formation assay was conducted with 786-O cells infected with control (Lv-shCon or Lv-shNC) or specific shRNA (Lv-shTRIM59). Colonies were stained with crystal violet and the representative size of each colony was shown in each group. (**C**) Counting of the colony numbers showed that only an average of 38 colonies was formed in Lv-shTRIM59 group, accounting for 50% of those in the control groups.

tion. However, 786-O cells infected with Lv-shTRIM59 exhibited a significantly slower proliferative rate since the 4th day onward. By the last day, cell numbers in Lv-shTRIM59 groups was approximately 62.5% of those in control groups (Fig. 3A). In the colony formation assay, the colonies stained with crystal violet were visually less observed in Lv-shTRIM59 group (Fig. 3B, upper panel). Magnification of the colonies showed that the size of cell colony was also smaller by inhibiting TRIM59 expression (Fig. 3B, lower panel). Counting of the colony numbers further showed that only an average of 38 colonies was formed in Lv-shTRIM59 group, making it an approximately 50% decrease as compared with control groups (Fig. 3C). These data suggests that knockdown of TRIM59 significantly inhibits cell proliferation in RCC cell line 786-O.

**Knockdown of TRIM59 inhibits tumor growth in a mouse xenograft model of RCC.**

A mouse model of human RCC was also established by using 786-O cells. 786-O cells were infected with Lv-shNC or Lv-shTRIM59 prior to injection into mice. It was observed that the tumor volume in the Lv-shTRIM59 group mice was increasing gradually, while it was increasing dramatically in the Lv-shNC group. On the 7th day, the average tumor volume in Lv-shTRIM59 group was approximately 25% of that in the Lv-shNC group. One mouse from the Lv-shTRIM59 group even did not exhibit any remarkable neoplasm (Fig. 4A). Dissection of all these tumors further showed that the size of each tumor mass was smaller in the Lv-shTRIM59 group as compared with the control group (Fig. 4B). Furthermore, all tumors were weighed. It was shown that the average weight of tumors was 0.7g in

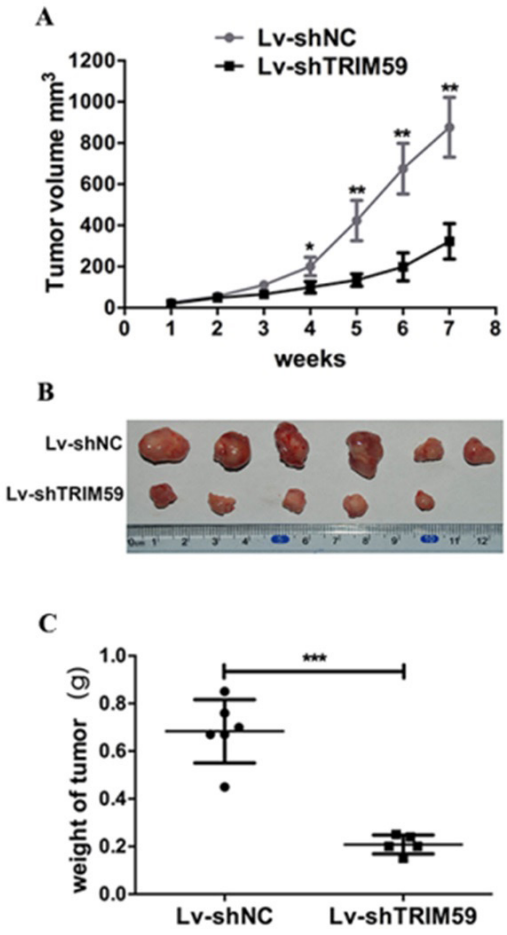


**Figure 4. Knockdown of TRIM59 inhibits tumor growth in a mouse xenograft model of RCC.** (**A**) A mouse model of human RCC was established by using 786-O cells with control or specific shRNA against TRIM59. The tumor volumes from two groups of mouse were measured for 7 days. On the 7th day, the average tumor volume in Lv-shTRIM59 group was approximately 25% of that in the Lv-shNC group. (**B**) Tumor size was visually smaller in the Lv-shTRIM59 group as compared with the Lv-shNC group. One mouse from the Lv-shTRIM59 group even did not exhibit any remarkable neoplasm. (**C**) Weighing of each dissected tumor showed that the average weight of tumors was 0.7g in the control group; however, it was only 0.2g for tumors from the Lv-shTRIM59 group.

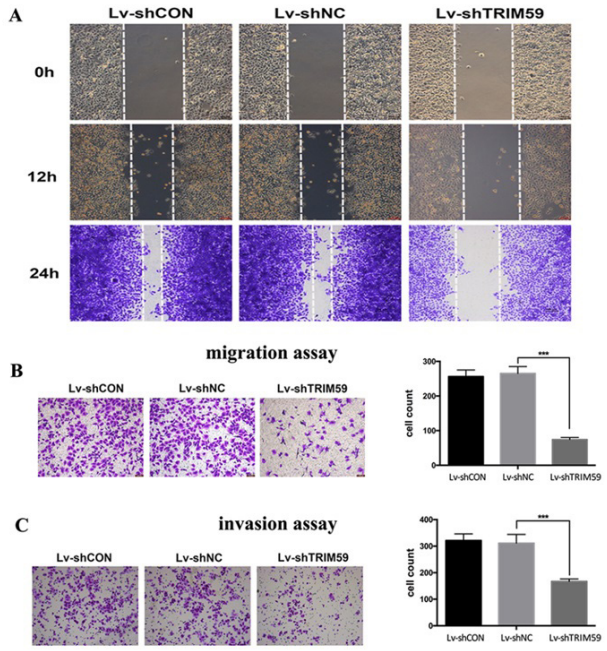


**Figure 5. Knockdown of TRIM59 inhibits cell migration and invasion in 786-O cells.** (**A**) wound-healing assay was conducted to assess cell migration in distinct groups of cells. The would-recovery in each group was observed at 12h and 24h after scratch. (**B, C**) in the transwell migration assay and invasion assay, after 12h free growth, cells that migrated to the lower chamber were stained with crystal violet. A total of 5 fields were randomly selected and photographed for each group. Cells from the selected 5 fields were counted and averaged. For the invasion assay, an extra Matrigel was coated on the upper surface of the chamber.

the control group; however, it was only 0.2g for tumors from the Lv-shTRIM59 group (Fig. 4C). These observations suggest that knockdown of TRIM59 inhibits tumor size and weight in the xenograft model of human RCC.

### Knockdown of TRIM59 inhibits cell migration and invasion in 786-O cells.

Further, cell migration and invasion was assessed after cells were depleted of TRIM59. In the wound-healing assay, it could be observed that 12h free growth had allowed cells to partially recover the wound in the control groups but not in the Lv-shTRIM59 group. Twenty-four hours after scratch, the wound was remarkably closed by up to 50% in the control groups. However, remarkable wounds were still readily observed in the Lv-shTRIM59 group (Fig. 5A). Consistently, when 786-O cells were depleted of TRIM59, cells that migrated to the lower chamber were visually decreased and accounted only for 30% of that in control groups (Fig. 5B). Likewise, in the invasion assay, 786-O cells that transmigrated through the Matrigel were less observed. In fact, only an average of 140 cells, in contrast with the over 300 cells in control groups, invaded into the lower chamber (Fig. 5C). These data suggest that knockdown of TRIM59 decreases cell migration and invasion abilities as well.

### Knockdown of TRIM59 affected EMT-associated proteins and decreased key cell cycle regulators

To explore the molecular mechanism of TRIM59 regulation of tumor growth and migration, the expression levels of tumor-correlated proteins in infected RCC cells were measured by western blot analysis using GAPDH as a control (Figure 6). We found that the expression of Vimentin, N-cadherin, cyclin B1, Cdc2, and Cdc25A were down-regulated under TRIM59 depletion, while E-cadherin expression was increased.

## Discussion

RCC is the most common type of renal neoplasms, and Clear cell RCC is the most frequent renal cancer histologic subtype, accounting for about 70% of cases. Survival rates are poor, with estimated five-year survival rates at only approximately 64% if not metastasis. Vascular endothelial growth factor receptor tyrosine kinase inhibitors (VEGFR TKi) were used to treat advanced RCC, despite recent advances, the prognosis for these patients remains poor. Further insights into activatedsignaling pathways in RCC will help guide development of improved therapies (9-11).

TRIM family has been implicated in multiple critical processes including tumoriogenesis (5,6). TRIM59, a member from this family, has been recently achieved great attention from researchers. The present study showed that TRIM59 was highly expressed in clinical RCC tissues as it was in the gastric cancer and prostate cancer (4,7). Moreover, using a lentivirus system, the TRIM59 was significantly depleted in the RCC cell line 786-O. Knockdown of TRIM59 significantly slowed down cell proliferation and colony formation *in vitro*. Consistently, tumor growth was also inhibited by knockdown of TRIM59 in a mouse model of human RCC. These data as well as the previous reports have

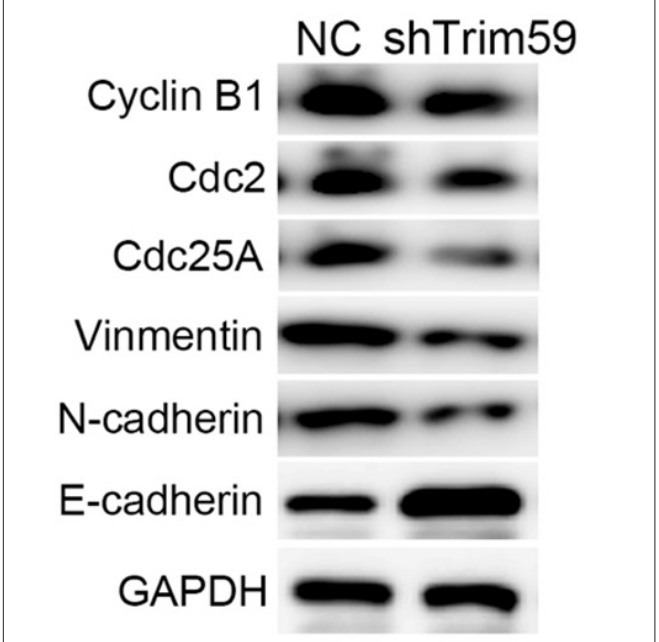


**Figure 6. Knockdown of TRIM59 affected EMT-associated proteins and decreased key cell cycle regulators.** Expression of mesenchymal markers, including N-cadherin and vimentin, were significantly suppressed when TRIM59 was depleted from RCC cells. Nevertheless, expression of the epithelial marker E-cadherin was increased after depletion of TRIM59. In addition, key cell cycle regulators, such as Cdc25A, cyclin B1, and Cdc2, were also detected in control and linc-TRIM59-depleted RCC cells.

collectively suggested that TRIM59 might be a critical biomarker indicating tumor growth. In fact, TRIM59 has been revealed to be a multiple cancer biomarker (7). Hence, TRIM59 may serve as a biomarker with applications in routine screening for RCC. Moreover, it was observed that knockdown of TRIM59 significantly inhibited cell migration and invasion abilities in 786-O cells. Our observations indicated that aside from tumor development, TRIM59 might also exert its biological activity in the progression of RCC.

On the mechanism, Vimentin is an intermediate filament protein expressed in mesenchymal cells, is an indispensable component of the cytoskeleton, and is responsible for maintaining cell integrity (12-14). Based on its function, vimentin can be used as a biomarker of malignant tumors (15).N-cadherin is another common cancer molecular maker which is commonly found in cancer cells and facilitates cancer migration (16).Cyclin B1 has been reported to be a cell regulator implicated in mitosis; cyclin B1 degradationcan inhibit tumor progression without affecting normal cells(17,18). Cdc2 is also a pivotal cell cycle regulator and its dysfunction can result in abnormalities in tumor cells (19).Cdc25A is known to promote the G1-to-S phase and G2-to-M phase transition and is always overexpressed in several cancer types (20).The expression levels of vimentin, N-cadherin, cyclin B1, Cdc2, and Cdc25A were all reduced in RCC cells lacking TRIM59. Based on the functions of these proteins, silencingof TRIM59may have affected RCC cell growth by downregulating their expression. The only protein that was upregulated was E-cadherin, a member of the cadherin superfamily that is involved in a variety of cancers. Lack of E-cadherin or its dysfunction are involved in cancer progression and metastasis; expression of E-cadherin is notably reduced in cancer cells (21). In agreement with the results of previous studies, we found that knockdown of TRIM59recovered E-cadherin expression, enabling inhibition of RCC growth.

Structurally, TRIM59 contains the RING domain which acts as E3 ubiquitin ligases and by which it regulates numerous cellular processes including cell cycle progression, gene transcription and signal transduction (22). Furthermore,TRIM59 is involved in NF-κB and IRF3/IRF7-mediated signal pathway (23). DNA promoter hypermethylation and c-Myc overexpression repress the expression of TRIM59 in cancers (24). In addition, it is suggested that Ras pathway is also involved in TRIM59 function. The initial functional targets of TRIM59 was on the Ras signal pathway as an early and rapid signal transmitter (25). Therefore, the expression of TRIM59 may be strictly controlled and its function in cancers may be achieved through complex signal pathways. Further work concerning exactly which pathway contributes to TRIM59-mediated biological activities in RCC is still needed.

In all, the present study identified TRIM59 as a novel mediator of tumor growth and migration in human RCC. Knockdown of TRIM59 significantly decreased cell proliferation and colony formation *in vitro* and also tumor growth *in vivo*. Cell migration and invasion were also suppressed after knockdown of TRIM59 in 786-O cells. Our data provide novel evidence that therapeutics targeting against TRIM59 may be a potential strategy for early detection and treatment of RCC in clinic.

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