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Targeted Silencing of *Kim-1* Inhibits the Growth of Clear Cell Renal Cell Carcinoma Cell Line 786-0 In Vitro and In Vivo

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To investigate the effect of *Kim-1* on 786-0 cells in vivo and in vitro, several experiments such as quantitative real-time PCR, Western blot, MTT, colony formation, and flow cytometry were performed to evaluate the biological behavior of 786-0 cells treated with *Kim-1* siRNA. Furthermore, the tumor xenograft model was applied to BALB/c nude mice to assess the effect of *Kim-1* silencing. Lentivirus-mediated RNAi effectively silenced *Kim-1* in 786-0 cells. *Kim-1* knockdown significantly inhibited the proliferation and colony formation ability of 786-0 cells ($p < 0.01$). The cell cycle of 786-0 cells was arrested in the G_0/G_1 phase ($p < 0.01$). Early and late apoptosis were significantly increased in the *Kim-1* siRNA cells ($p < 0.01$). In addition, growth of 786-0 cells was significantly inhibited in the *Kim-1*-silenced mice. In conclusion, knockdown of *Kim-1* inhibits the growth of 786-0 cells in vitro and in vivo, indicating that *Kim-1* could be used as a potential target for clear cell renal cell carcinoma therapy.

Key words: Oxaliplatin; Kidney injury molecule-1 (Kim-1); Clear cell renal cell carcinoma; RNA interference; Proliferation

INTRODUCTION

Renal cell carcinoma (RCC) has been reported to be the second most common cancer in the urological system. It accounts for approximately 3% of malignant neoplasms worldwide[1] with an estimated 338,000 new cases and 102,000 deaths annually[2]. Clear cell renal cell carcinoma (ccRCC) is the most common (75%) and the main lethal histologic subtype of RCC[3]. To date, approximately 40% of patients are first diagnosed with advanced metastasis. Surgery remains the only possible curative therapy for patients with localized RCC; conventional DNA-damaging therapies are useless due to the development of multidrug resistance[4,5]. The major obstacle for ccRCC management is to identify new therapeutic targets, as well as to develop novel single or combined treatment strategies.

Kidney injury molecule-1 (Kim-1), a 38.7-kDa type I transmembrane glycoprotein[6], is now recognized as a potential biomarker for the early detection of kidney tubular injury. *Kim-1* expresses rarely in the normal kidney and other organs, while being dramatically upregulated in kidney injury or diseases such as repercussion injury, drug-induced acute kidney injury, and RCC[7,8]. Kim-1 plays an important role in kidney recovery and tubular regeneration because it mediates the phagocytosis of apoptotic cells. Besides, *Kim-1* expression is associated with the degree of interstitial fibrosis and inflammation[9]. Bailly et al. demonstrated that *Kim-1* was significantly upregulated in kidney tissues, and diffused *Kim-1* predicted a poor prognosis in RCC[10]. The messenger RNA (mRNA) expression of *Kim-1* in RCC was shown to be positively connected with TNM and pathology classification, with a bigger and more malignant tumor, the higher the expression of *Kim-1*, according to Trpkov et al[11]. Han et al. found that *Kim-1* is significantly upregulated in 769-P cells, RCC tissues, and urine, suggesting that *Kim-1* may be a therapeutic target[12].

These studies suggest that *Kim-1* is highly correlated with RCC, while research on *Kim-1* in ccRCC is rare. Therefore, in this study, we analyzed the expression of *Kim-1* in ccRCC 786-0 cells both in vivo and in vitro. We examined the effect of lentivirus-mediated knockdown of the *Kim-1* gene in 786-0 cells. Our results showed that *Kim-1* small interfering RNA (siRNA) suppressed the gene and protein expression to a large extent in vitro, which resulted in reduced cell viability, cell apoptosis, and the cell cycle being arrested in the G_0/G_1 phase. Furthermore, silencing of *Kim-1* inhibited the growth of

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ccRCC 786-0 cells in vivo. These results indicated that *Kim-1* could be a potential target for gene therapy in the treatment of ccRCC.

MATERIALS AND METHODS

Reagents and Cell Culture

The ccRCC cell line 786-0 was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and propagated in RPMI-1640 (Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Excell Bio, Shanghai, P.R. China), 100 U/ml penicillin (Excell Bio), and 100 U/ml streptomycin (Excell Bio) in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed every 2 days, and the cell line was passaged every 4–5 days.

Transfection and Infection

pGCSIL-*KIM-1*-RNAi-LV and nontargeted control pGCSIL-neg-RNAi-LV were purchased from GeneChem Biomedical Co., Ltd. (Shanghai, P.R. China). The 786-0 cells in the logarithmic growth phase were trypsinized and diluted to the appropriate concentration, and then were seeded in six-well microplates and cultured overnight. Lentiviral transfection was performed with Lipofectamine RNAiMAX reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. Six hours after transfection, the medium containing virus was changed with fresh culture medium. The 786-0 cells were divided into three groups dependent on their treatment in subsequent assays: the CON group (blank control group, cells received no infection), the NC group (negative control group, cells were infected with pGCSIL-neg-RNAi-LV), and the KD group (*KIM-1* RNAi group, cells were infected with pGCSIL-*KIM-1*-RNAi-LV).

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from the three groups of 786-0 cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol, 48 h after transfection. A cDNA was synthesized using 1 μg of total RNA at 42°C for 1 h using a PrimeScript reverse transcription (RT) reagent kit (Invitrogen). This cDNA was then subjected to PCR amplification with specific primers in 25-μl mixtures using Platinum SYBR Green qPCR SuperMix DUG (Invitrogen). The purity and integrity of total RNA were assessed by spectrophotometry (260 nm). The expression of the target gene was evaluated using a relative quantification approach ($2^{-\Delta\Delta}$Ct method) with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as the internal reference.

The specific primer pairs were as follows: *K*im-1, 5′-TGAAGGGTCGGAGTCAACGG-3′ (forward) and 5′-CCTGGAAGATGGTGATGGG-3′ (reverse); *GAPDH*, 5′-CTCAGACACCATGGGGAAGGTGA-3′ (forward) and 5′-ATGATCTTGAGGCTGTTGTCATA-3′ (reverse).

Western Blot Analysis

Protein was harvested from cells with RIPA Lysis Buffer (Beyotime, Shanghai, P.R. China) according to the manufacturer's instructions at 48 h after transfection. BCA Protein Assay kit (Beyotime) was used to determine protein concentration. Equal amounts of protein samples (10 μg per condition) were prepared in loading buffer and boiled at 100°C for 10 min, and then the boiled samples were separated on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Billerica, MA, USA) at 100 V for 1 h. The PVDF membranes were blocked with 5% nonfat milk in phosphate-buffered saline (PBS) for 1 h at room temperature and subjected to anti-Kim-1 primary antibody (Sigma-Aldrich, St. Louis, MO, USA) incubation at 4°C overnight. After washing, membranes were probed with goat anti-mouse secondary antibodies. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) Western blot detection reagents (Thermo Fisher Scientific, Waltham, MA, USA). Before each step, the PVDF membranes were washed three times by PBST. Protein quantification was conducted by ImageJ software [National Institutes of Health (NIH), Bethesda, MD, USA]. The gray values of protein were achieved as gray level of protein band/gray level of loading control.

Methylthiazoltetrazolium (MTT) Assays

To observe cell proliferation, lentivirus-infected 786-0 cells were seeded in 96-well plates (Corning Costar, Cambridge, USA) at a cell density of 3,000 cells/well. The untreated cells served as the CON group. The plates were incubated in a 37°C humidified incubator. After 24, 48, or 72 h, cells were treated with MTT solution (Sigma-Aldrich) at a concentration of 5 mg/ml for 4 h at 37°C, followed by the addition of 200 μl of dimethyl sulfoxide (DMSO; Sigma-Aldrich) to stop the reaction. Complete dissolution was achieved with gentle shaking for 5 min. The absorbance at 450 nm was measured using the microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

To detect colony formation ability, lentivirus-infected cells were trypsinized and seeded into 6-cm dishes at a density of 800 cells/dish. The untreated cells served as the CON group. Cells were fed with fresh RPMI-1640 supplemented with 10% FBS every 4 days. After 2 weeks, the cell colonies were fixed using 4% paraformaldehyde (PFA; Sigma-Aldrich) for 15 min. Then we stained the

fixed colonies with Giemsa (Sigma-Aldrich) for 20 min and washed twice with ddH_2O. Most of the single 786-0 cells had formed colonies with more than 50 cells, and the colonies were determined quantitatively by counting the number of positively stained cells in five fields at 200× magnification.

Cell Cycle Analysis

The cell cycle phase distribution was determined by flow cytometry. Lentivirus-infected cells were seeded into 6-cm dishes and harvested 48 h after transfection. Cell cycle distribution was determined using the following procedure. The cells were treated with 0.25% trypsin (Sigma-Aldrich) to detach them from the plate and then fixed with 70% ice-cold ethanol followed by 0.1% sodium citrate (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 20 mg/ml propidium iodide (PI; BD, Franklin Lakes, NJ, USA). At least 1×10^6 events were collected in each histogram. Flow cytometer (FACS Calibur™; BD) was applied to detect DNA content.

Apoptosis Assay

We stained the cells with annexin V-APC (BD), which could differentiate intact cells from apoptotic cells. A total of 1×10^6 cells were collected using trypsin, washed twice with ice-cold PBS, and put into binding buffer (1 mg/ml annexin V-APC) in the dark for a 30-min incubation following the manufacturer's instructions. Finally, flow cytometer was used to analyze annexin V-APC staining.

Tumor Xenograft Model

All animal experiment protocols in this study were approved by the institutional ethical committee. Six female nude mice between 4 and 5 weeks old were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, P.R. China). Mice were injected subcutaneously with 786-0 cells (5×10^6 cells in 200 µl PBS) into the left axilla to establish tumors. After the tumors reached an average volume of approximately 50 mm^3 [V (cm^3) = $a\times b^2/2$, where a represents long diameter, and b represents short diameter], the mice were randomly split into two groups: the CON group and the KD group. After 2 weeks, the mice were euthanized, and the tumors were excised and weighed.

Statistical Analysis

Each experiment was repeated three times, and data were presented as the mean ± standard deviation (SD). Statistical analysis was done by GraphPad software, version 5. The differences between each group were tested for significance using the Student's two-sided t-test. A value of $p<0.05$ was considered statistically significant.

RESULTS

Kim-1 *siRNA Downregulated mRNA and Protein Level*

To examine the specific effect of *Kim-1* siRNA in 786-0 cells, the expression levels of *Kim-1* mRNA and protein were determined quantitatively using qRT-PCR and Western blot analyses, respectively. Compared with the NC group, relative mRNA and protein expression of *Kim-1* are significantly decreased ($p<0.01$, $n=3$) (Fig. 1) in 786-0 cells of the KD group, whereas the NC group has no significant difference compared with the CON group. These results demonstrate that *Kim-1*

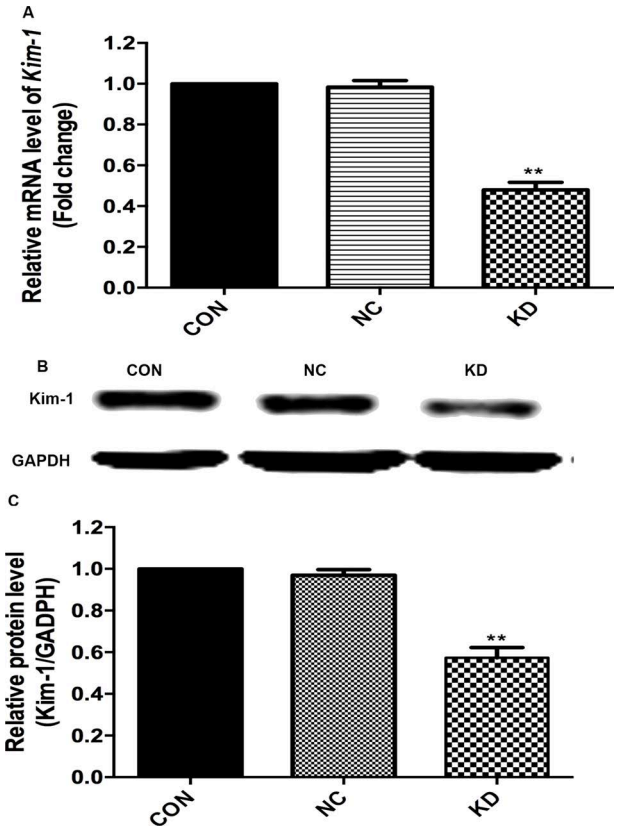

Figure 1. Kidney injury molecule-1 (*Kim-1*) small interfering RNA (siRNA) downregulated mRNA and protein levels. (A) *Kim-1*-RNAi-LV downregulated *Kim-1* mRNA expression in 786-0 cells [**$p<0.01$ compared with the NC group (negative control group, cells were infected with pGCSIL-neg-RNAi-LV)]. Cells were harvested 48 h after transfection with siRNA. Total RNA was extracted, and quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed. The error bars represent the standard errors of three independent experiments. (B) *Kim-1*-RNAi-LV downregulated Kim-1 protein expression in 786-0 cells. Western blot analysis was performed 48 h after infection with *Kim-1*-RNAi-LV. (C) Densitometry analysis of Western blots from three independent experiments, respectively (**$p<0.01$ compared with the NC group). *GAPDH* was used as internal control.

siRNA suppresses *Kim-1* expression at both the mRNA and protein levels.

Kim-1 *Promotes 786-0 Cell Proliferation and Colony Formation*

To determine whether inhibition of *Kim-1* affects the proliferation of 786-0 cells, cell growth was determined after 24, 48, and 72 h using the MTT assay. As shown in Figure 2A, the viability of 786-0 cells was significantly decreased after *Kim-1* siRNA transfection ($p<0.01$, $n=3$). Furthermore, the colony formation assay showed that *Kim-1* knockdown resulted in much smaller and fewer colonies, compared to the CON group and the NC group ($p<0.01$) (Fig. 2B and C). However, the NC group remained unchanged compared with the CON group ($p>0.05$). These data indicate that *Kim-1* depletion inhibits the proliferation of 786-0 cells in vitro.

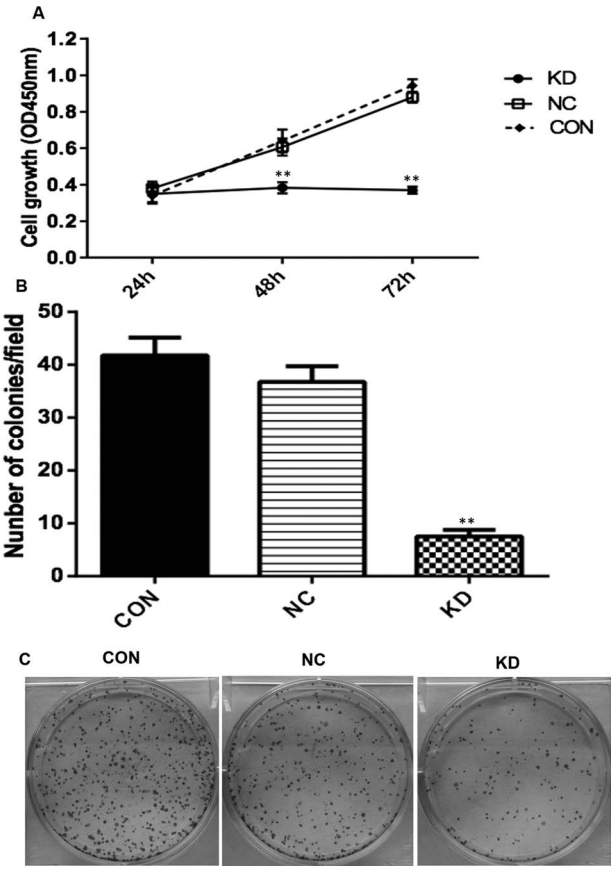

Figure 2. *Kim-1* promotes 786-0 cell proliferation and colony formation. (A) Cell proliferation was determined by MTT assay (**$p<0.01$ compared to the control and NC groups). (B) Cell proliferation inhibitory effect of *Kim-1* RNAi was confirmed after 2 weeks by colony formation assay (**$p<0.01$ compared to the control and NC groups). (C) Representative pictures of the whole plates are shown. The data are representative of three independent experiments. Data are presented as the mean ± standard deviation (SD).

Kim-1 *RNAi Arrested 786-0 Cell Cycle in the G_0/G_1 Phase*

To understand how *Kim-1* promotes 786-0 cell proliferation, we determined the effect of *Kim-1* depletion on the cell cycle progression of 786-0 cells through flow cytometry analysis. There was a significant increase in cell number in the G_0/G_1 phase and a corresponding reduction in the G_2/M phase after *Kim-1* silencing ($p<0.01$, $n=3$) (Fig. 3). What the results demonstrate is that *Kim-1* suppression can delay 786-0 cell proliferation in vitro.

Kim-1 *RNAi Induced 786-0 Cell Apoptosis*

In order to determine the effects of *Kim-1* depletion on apoptosis of 786-0 cells, annexin V/PI double staining was performed. As the results showed, the percentage of apoptosis in the KD group was significantly increased, compared with the CON and the NC groups ($p<0.01$, $n=3$) (Fig. 4). Statistical analysis revealed that approximately a twofold increase in late apoptotic populations was detected in the KD group compared with the CON and NC groups, respectively. Through these data, we can see that downregulation of *Kim-1* induced more apoptosis of 786-0 cells, suggesting that suppression of cell proliferation may be caused by apoptotic cell death resulting from *Kim-1* RNAi.

Kim-1 *RNAi Inhibited 786-0 Cell Tumorigenesis In Vivo*

To further investigate the potential effects of reduced *Kim-1* expression on the tumorigenic phenotype, 786-0 cells infected with pGCSIL-*Kim-1*-RNAi-LV (the KD group) or untreated (the CON group) were injected into nude mice to determine in vivo tumor growth. Tumors derived from cells treated with *Kim-1*-RNAi were significantly decreased in size compared with the CON group mice ($p<0.001$) (Fig. 5) after 2 weeks. These results demonstrated that the downregulation of *Kim-1* inhibited 786-0 cell growth in vivo.

DISCUSSION

ccRCC, the most common form of RCC, was designated a malignant renal epithelial neoplasm in the 2004 World Health Organization (WHO) classification[13]. Major clinical challenges for ccRCC are the lack of efficacious therapy and the frequent development of metastatic disease because of its native resistance to chemotherapies and radiotherapy. Currently, there is a lack of effective treatment for ccRCC; 10%–20% of patients have tumors that are initially refractory to therapy, and a large majority relapse after a median of 10–12 months[14]. Molecular

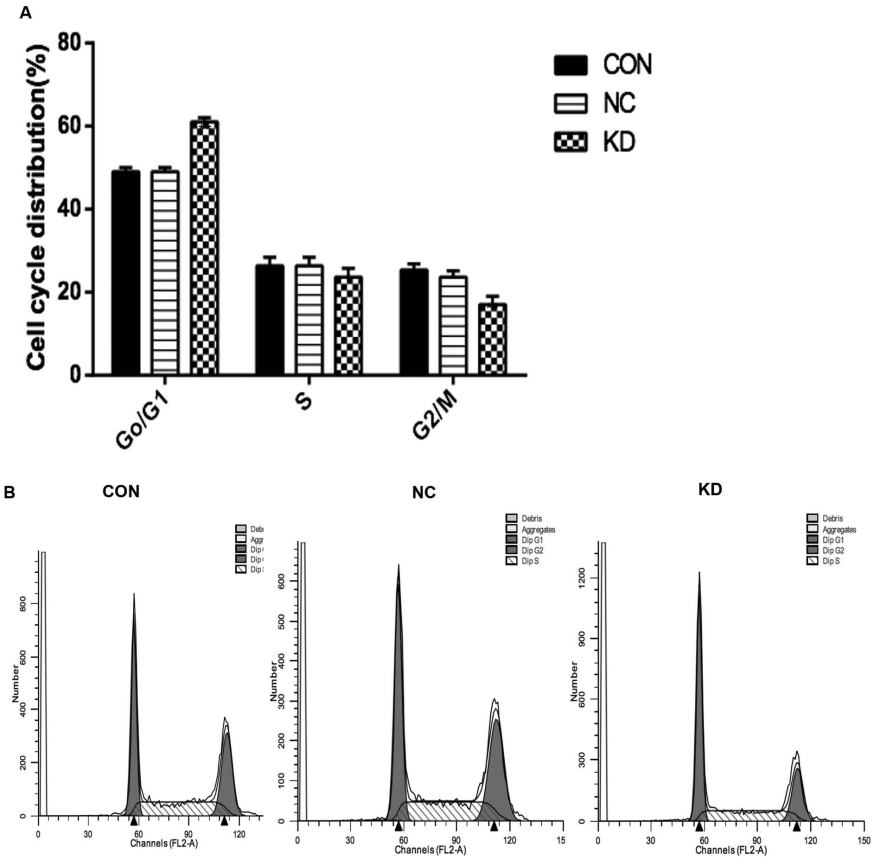

Figure 3. *Kim-1* RNAi arrested 786-0 cell cycle in the G_0/G_1 phase. (A) The proportion of cells in the G_0/G_1 phase significantly increased, while the proportion in the G_2/M phase decreased in the KD group (*KIM-1* RNAi group, cells were infected with pGCSIL-*KIM-1*-RNAi-LV) after treatment with *Kim-1* RNAi for 48 h. (B) Flow cytometry (FCM) analysis showed that *Kim-1* RNAi arrests 786-0 cells in the G_0/G_1 phase of the cell cycle. The data are representative of three independent experiments.

genetic profiles reflect the biological characteristic of a specific renal tumor and could be used to predict the response to therapy and have great potential to detect therapeutic targets. Several studies have sought to identify the genetic basis of ccRCC, and some unique genetic alterations have been identified[15]. Mutation or silencing of Von Hippel–Lindau (*VHL*) tumor suppressor gene occurs in a majority of inherited and sporadic ccRCC, resulting in hypoxia inducible factor-α (HIF-α) stabilization, which causes abnormal expression of a series of growth factors[16]. New targeted medicine based on *VHL* has been developed, which is the most important treatment progress in recent years[17]. While the therapeutic effect is currently not adequate in ccRCC, many more studies are being performed to evaluate means of improving efficacy.

Kim-1 expression in humans was first explored in kidney biopsy specimens from patients with acute tubular necrosis (ATN), suggesting that *Kim-1* was a predictor for the risk of developing ATN[18]. Later, a number of studies have subsequently shown the reliability of using urinary *Kim-1* as a sensitive and reproducible biomarker for the early detection and prediction of kidney disease. *Kim-1* is expressed at high levels in human RCC and shows positive correlation with tumor stage and grade. Now *Kim-1* has been qualified by the US Food and Drug Administration (FDA) and European medicines agency as a high-sensitive and specific urinary biomarker to monitor drug-induced kidney injury. However, the possibility of using *Kim-1* as a new therapy target in ccRCC still remains unclear. We therefore sought to explore the biological activity of *Kim-1* in 786-0 cells in vitro and in vivo.

RNAi has proven to be a powerful tool to suppress gene expression for molecular biological research. It is considered that RNAi could be a tool for cancer gene therapy when combined with lentivirus, especially for some multidrug resistance genes or chemoresistance targets. Sabbisetti et al. downmodulated *KIM-1* in RCC cell lines using an siRNA lentiviral approach, which led to G_1 cell cycle arrest phase and senescence. They demonstrated

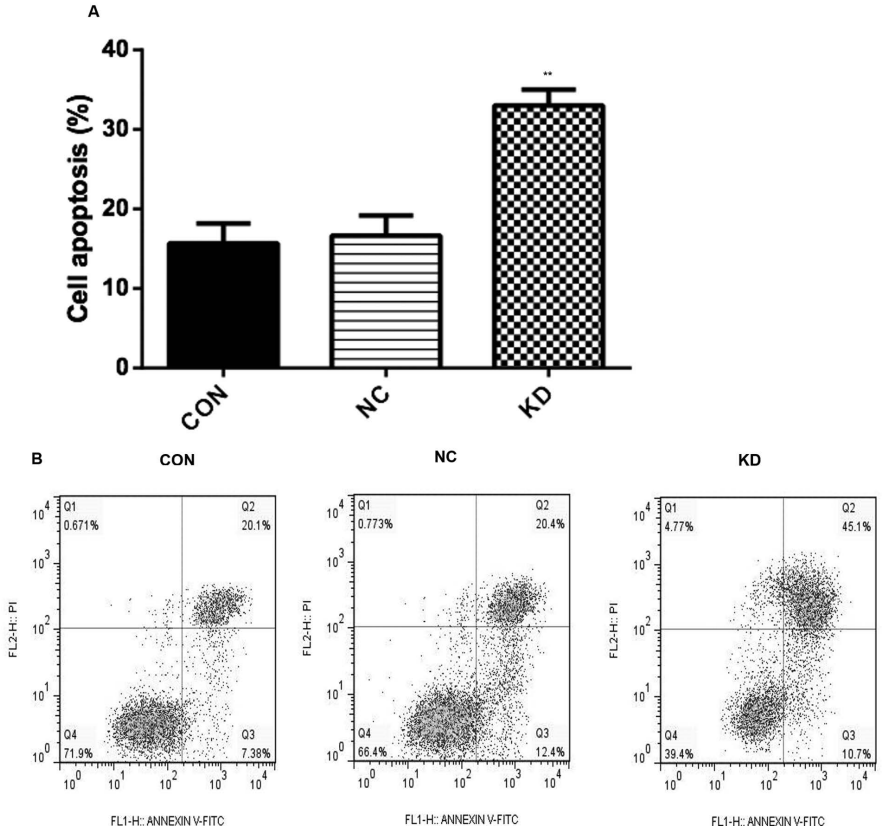

Figure 4. *Kim-1* RNAi induced 786-0 cell apoptosis. (A) 786-0 cells were transfected with *Kim-1*-RNAi-LV for 48 h, and apoptosis was detected by FCM. *Kim-1* RNAi significantly promoted cell apoptosis in the KD group [$^{**}p<0.01$ compared to the CON (blank control group, cells received no infection) and NC groups]. (B) The lower right area shows early apoptotic cells, and the upper area shows late apoptotic cells. The data are representative of three independent experiments.

that the expression of *KIM-1* converts a normal epithelial cell into a "semiprofessional phagocyte" and facilitates the removal of apoptotic and necrotic cells[19]. We therefore sought to downregulate the *Kim-1* gene by devising a vector-borne siRNA that, by binding to a target sequence on the mRNA, would lead to the degradation of *Kim-1* transcripts via endonucleolytic cleavage. We successfully constructed a highly efficient and stable lentivirus vector system, which could efficiently knock down *Kim-1* gene in infected 786-0 cells.

In this study, we investigated the correlated effects of *Kim-1* silencing on the biological effects in ccRCC 786-0 cells in vitro and in vivo. Some studies demonstrated that expression of *Kim-1* in ccRCC was able to provide advantages to EBV-mediated cell growth and transformation and enhance the malignant potential in vivo. Our data indicated that when *Kim-1* was downregulated, the proliferation of 786-0 cells was significantly decreased in vitro, while cell apoptosis was obviously increased, and more 786-0 cells were arrested in the G_0/G_1 phase of the cell cycle. Based on previous reports and our results, we suggest that silencing of *Kim-1* weakens cell proliferation of ccRCC cells via increasing cell cycle arrest and inducing late apoptosis. The tumor xenograft mouse model provides a tool for preclinical studies of cancer. BALB/c nude mice have been widely utilized for siRNA in vivo studies targeting human genes because of their immunocompromised characteristics. With continued intramural injection of *Kim-1* siRNA, the inhibitory effects of downregulated *Kim-1* on xenograft tumor growth are observed. We have clearly demonstrated that the siRNA-mediated *Kim-1* gene silencing led to suppression of 786-0 cell growth in vitro and in vivo, which highlights the application potential of *Kim-1* gene as a therapeutic target for ccRCC. However, the detailed mechanism of cell growth inhibition by downregulating *Kim-1* expression has not been clarified. For further investigation, we would try to clarify that mechanism, which may explore new ways of novel therapies against ccRCC.

In summary, as our results show, downregulation of *Kim-1* by lentivirus-mediated RNAi can significantly suppress 786-0 cell proliferation in vitro and in vivo, which provides a potential attractive new anticancer therapeutic strategy for human ccRCC.

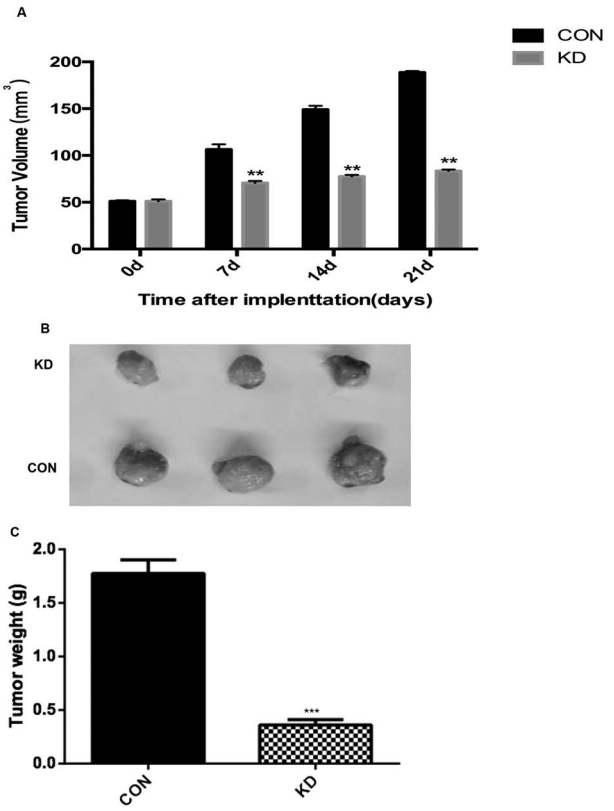

Figure 5. *Kim-1* RNAi inhibited 786-0 cell tumorigenesis in vivo. (A) *Kim-1* knockdown in 786-0 cells or control cells was injected subcutaneously into the left flank of nude mice ($n=3$). At 2 weeks after implantation, *Kim-1* knockdown cells produced smaller tumors than control cells. (B) The tumor from each group was weighed immediately after the dissection. Tumor weights were significantly decreased after *Kim-1* siRNA (***$p<0.001$ compared to CON group, $n=3$).

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