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ORIGINAL PAPER

Small interference RNA targeting Krüppel-like factor 8 inhibits the renal carcinoma 786-0 cells growth in vitro and in vivo

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Abstract
Purpose Krüppel-like factor 8 (KLF8) plays an important role in oncogenic transformation and is highly overexpressed in several types of human cancer. We investigated the expression of KLF8 in renal cell carcinoma (RCC) tissues and the role of small interference RNA targeting KLF8 on growth, cell cycle, and apoptosis of human renal carcinoma cell line 786-0 in vitro and in vivo.
Methods The expression of KLF8 protein and mRNA in human renal carcinoma samples was detected by immunochemistry and reverse transcription polymerase chain reaction (RT-PCR). The effects of small interference RNA (siRNA) targeting KLF8 on growth, invasiveness, cell cycle, and apoptosis of 786-0 cells were evaluated by MTT assay, Matrigel Invasion Assay, and flow cytometry in vitro. We also investigated effect of siRNA targeting KLF8 on growth of 786-0 cells in nude mice in vivo.
Results Immunohistochemistry and RT-PCR results showed the expression of KLF8 protein and mRNA in RCC specimens was significantly higher than that in the adjacent non-tumorous renal tissues ($P < 0.001$). KLF8-siRNA depressed the cellular growth and invasion of 786-0 cells in vitro. The flow cytometry results revealed that KLF8-siRNA could induce an increase in G0/G1 phase cells and induce cell apoptosis. Intratumor injection of siRNA targeting KLF8 inhibited the growth of 786-0 cells in vivo in nude mice tumor model.
Conclusions KLF8 possibly involved in regulating the cell growth, invasion, apoptosis, and proliferation of renal carcinoma cancer cells. Blocking the KLF8 channel might be a potential therapeutic strategy for RCC.

Keywords KLF8 · Renal cell carcinoma · Small inference RNA · Invasion · Growth

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Introduction

Renal cell carcinoma (RCC) is the most common malignant tumor arising from the kidney with high mortality and accounts for approximately 2% of adult cancers (Motzer et al. 1996). Clear cell RCC (ccRCC) is the most common histologic subtypes in RCC. An estimated 51,190 Americans will be diagnosed with RCC and 12,890 will die of the disease in the United States in 2007 (Jemal et al. 2007).

RCC remains the high mortality due to the lack of early detection methods and effective treatments for late-stage cancers. Twenty-five percent of patients present with locally invasive or metastatic RCC. Moreover, systemic therapeutic treatments of advanced RCC are largely ineffective and do not improve patient survival (Cohen and McGovern 2005; Linehan et al. 2004). Understanding the molecular mechanisms underlying RCC progression and metastasis is urgent for developing new strategies for early diagnosis and therapies required for improvement of patient survival.

Krüppel-like transcription factors (KLFs) are transcriptional regulators that contain the C_2H_2 zinc-finger motif and play diverse roles in the regulation of cell proliferation, cell differentiation, and development (Pearson et al. 2008). Krüppel-like factor 8 (KLF8) is one member of KLFs. Like other members of the KLFs, KLF8 shares the well-conserved DNA-binding zinc finger domains on its C-terminus. The N-terminal half of KLF8 is thought to determine its functional specificity through recruiting other proteins (Bieker 2001). KLF8 was initially identified as a transcription repressor (van Vliet et al. 2000). But this views have been changed. Over-expressed KLF8 has recently been found in some of human malignant tumors, and played an important role in oncogenic transformation (Wang and Zhao 2007; Wang et al. 2007). However, most studies of KLF8 have only focused on the cancer cell lines in vitro, not in tumorous tissues. Especially, whether and how KLF8 might play a role in RCC tumorigenesis, invasion, and progression is little known.

In the present study, we firstly investigated the expression of KLF8 in RCC samples by immunohistochemistry (IHC) and reverse transcription polymerase chain reaction (RT-PCR). Subsequently we constructed small interference RNA (siRNA) sequences targeting KLF8, transfected it into the human RCC cell line 786-0, and explored changes in growth, invasiveness, cell cycle, and cell apoptosis. The expression of cyclin D1 and FAK was also investigated when compared with scrambled siRNA transfected 786-0 cells. We also investigated the effects of KLF8 down-regulation on the growth of 786-0 cells in nude mice.

Materials and methods

Study population

Between January 2008 and May 2008, tumorous tissues and adjacent non-tumorous renal tissues (located approximately 3–5 cm from the tumor site) of the same patient were collected from 42 sporadic primary RCC patients underwent radical nephrectomy at the urology department of our hospital, and these patients have not received chemotherapy or radiation therapy previously. There were 22 men and 20 women with a mean age of 57.5 years (range 30–84 years). After the specimens were removed from the patients, each sample was cut into two parts, one part was promptly frozen at −80°C for RT-PCR and the other for immediate fixation with 10% neutral buffered formalin for IHC. The study was approved by the Ethics Committee of the hospital. The tumor were staged according to the 1997 International Union Against Cancer TNM classification of malignant tumors and graded according to the Fuhrman grading scheme (Storkel et al. 1997). All the specimens were diagnosed as ccRCC by pathology.

Immunohistochemistry

Paraffin-embedded tissue sections (5 μm thick) were dewaxed in xylene and rehydrated with a graded ethanol series. Consecutively, tissue sections were treated with 3% hydrogen peroxidase (15 min, room temperature) followed by incubation (overnight, 4°C) with 1:150 diluted rabbit polyclonal antibody KLF8 (Aviva System Biology, USA). Then they were incubated with 1:300 diluted peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, USA). Finally, the sections were developed in diaminobenzidine solution under microscopic observation and counterstained with hematoxylin. Experimental runs contained negative controls without application of the primary antibody. Immunostaining was assessed by two individual pathologists who were unaware of the clinicopathologic variables.

The intensity of KLF8 immunostaining (0, negative; 1, weak; 2, moderate and 3, intense) and the percentage of positive tumor cells (negative <5%, 5–25% = 1, 25–50% = 2, and >50% = 3) were assessed in at least four areas at 200× magnification, and minimum of 100 cells per specimen were counted. The tumors were finally classified as negative expression: score 0 (−); low expression: score 1–2 (+); intermediate expression: score 3–4 (++); high expression: score 5–6 (+++) (Tuna et al. 2004).

The construction and identification of KLF8-siRNA

Double stranded siRNA molecules with a two-base overhang at the 3-end of the antisense strand corresponding to three different KLF8 sites were designed and synthesized from GenePharma Co, Ltd (Shanghai, China). Three siRNA sequences targeting human KLF8 and one scrambled siRNA (used for a negative control) with the following sense and antisense sequences were used: KLF8-siRNA1, 5′-ACUUGGAGGUCCAACUUAATT-3′ (sense) and 5′-UUAAGUUACCUCCAAGGTG (antisense); KLF8-siRNA2, 5′-CGAUAUGGAUAACUCAUATT-3′ (sense)

and 5′-UAUGAGUUUAUCCAUAUCGAC-3′ (antisense); KLF8-siRNA3, 5′-CACUGGUUAAUGACAUCAATT-3′ (sense) and 5′-UUGAUGUCAUUAAACAGUGCTA-3′ (antisense); and scrambled siRNA (as negative control), 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense) and 5′-ACGUGACACGUUCGGAGAATT-3′.

Cell line and transfection

Human RCC cell line 786-0 (From the Cell Center of Shanghai Institute for Biological Sciences, China) was used in the study. 786-0 cells were maintained in RPMI 1640 medium (Gibco, USA) containing 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% fetal calf serum (Gibco, USA) and incubated at 37°C in a humidified atmosphere with 5% CO_2 (Katner et al. 2002).

3×10^5 786-0 cells were plated in a 6-well plate to reach a 30–50% confluency 24 h before transfection. Cells were transfected with siRNA1, siRNA2, siRNA3, and scrambled siRNA respectively by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For each well, 4 μg of the respective siRNA (20 pmol/μl) was incubated with 250 μl serum-free medium for 5 min. Subsequently, a mixture of 10 μl Lipofectamine 2000 and 250 μl serum-free medium which has been incubated for 5 min was slowly added. After incubation of 20 min at room temperature, 2 ml serum-free medium was added to the dilution, mixed again and transfection mixture was added to each well. After being incubated at 37°C for 8 h, the medium containing the transfection mixtures were replaced with the growth medium. The blank control was treated with PBS only. Each experiment was repeated three times.

Semi-quantitative RT-PCR analysis

All tissues and 786-0 cells total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA was synthesized from 2 μg total RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas, EU). Primers used in RT-PCR were as follows: KLF8 sense 5′-GCTCACCGCAGAATCCATACA-′3, antisense 5′-GTGCACCGAAAAGGCTTGAT-3; cyclin D1 sense 5′-GGGAGGAACAGAAGTGCGAGGAG-′3, antisense 5′-GGCACAGAGGGCAACGAAGGT-′3; focal adhesion kinase (FAK) sense 5′-GTATTGACAGGGAGGATGGAAG-′3, antisense 5′-AGGCCCGTCACATTCTGTA-′3; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense 5′-ACCACAGTCCATGCCATCAC-′3, antisense 5′-TCCACCACCCTGTTGCTGTA-′3. Cycling parameters (35 cycles) were as follows: denaturation (94°C, 30 s), annealing (KLF8 61°C, cyclin D1 and FAK 60°C, 40 s) and extension (72°C, 45 s). Relative KLF8, cyclin D1, FAK, E-cadherin mRNA expression is given as KLF8/GAPDH, cyclin D1/GAPDH, and FAK/GAPDH mRNA ratio. All experiments were performed in triplicate.

Western blot

48 h after transfection, the KLF8 protein expression of 786-0 cells were determined by western blot analysis. Cells were resuspended in CytoBuster protein extraction buffer. Suspensions were incubated at 48°C for 5 min and centrifuged for 30 min; then, identical amounts (50 μg of protein) of cell lysates were resolved by 10% SDS-PAGE. Gels were electroblotted onto a PVDF membrane (Millipore, Bedford, MA, USA). Subsequently PVDF membranes were incubated in the blocking solution (5% non-fat dry milk) for 1 h at room temperature. The PVDF membrane was respectively incubated with the rabbit polyclonal antibody KLF8 (diluted 1:1,000), mouse monoclonal antibody cyclin D1 (diluted 1:1,000, Santa Cruz Biotechnology, USA), mouse monoclonal antibody FAK (diluted 1:1,000, Santa Cruz Biotechnology, USA), mouse monoclonal antibody GAPDH (diluted 1:1,000, Santa Cruz Biotechnology, USA). After incubation with the secondary antibody (1:2,000 diluted, IgG-HRP) for 1 h at room temperature, immunoreactive bands were detected using the enhanced chemiluminescence Western blotting analysis system (GE Healthcare, Bucks, UK).

MTT assay

786-0 cells proliferation was measured by the MTT assay. Cells were seeded in 96-well plates at a density of 2×10^3 cells/well. After treating with KLF8 siRNA for 6 h, the medium was changed with 100 μl RPMI1640 with 10% FBS, Scrambled siRNA was used as control. At the indicated time points, 200 μl of 5 mg/ml solution of MTT (Sigma, USA) in PBS was added to each well. The plates were then incubated for 4 h at 37°C. The precipitate was then solubilized in 100% dimethylsulfoxide (Sigma, USA) 150 μl/well, and shaken for 10 min. Absorbance of each well was measured on a microplate reader at a wave length of 490 nm. All experiments were done in triplicate.

In vitro invasion assay

BD BioCoat invasion chambers were used in Matrigel Invasion Assays, 1×10^5 cells were added to the top chambers of 24-well transwell plates (BD Biosciences, 8-Ampore size), complete media were added to the bottom chambers, and serum in the complete medium as the chemo-attractant. Cells were transfected with siRNA 24 h

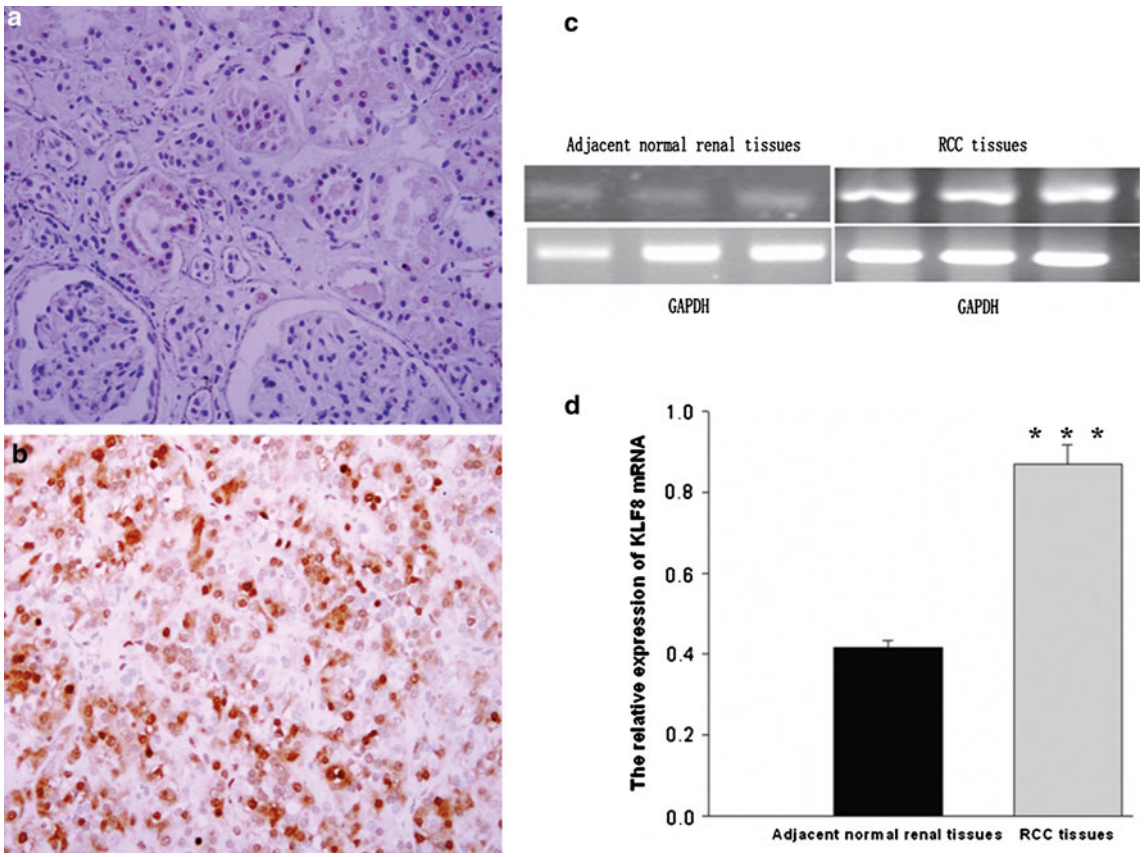

Fig. 1 Immunohistochemistry and RT-PCR analysis for KLF8 protein and mRNA expression in RCC tissues and adjacent normal renal tissues. **a**, **b** KLF8 protein expression in adjacent normal renal tissues and RCC tissues (200×). **c** KLF8 mRNA expression in adjacent normal renal tissues and RCC tissues. **d** RCC tissues had higher KLF8 mRNA level than adjacent normal renal tissues. $***P < 0.001$, as compared with adjacent normal renal tissues

before being processed for the invasion assays. 786-0 cells was cultured for 24 h. The medium in lower chamber was aspirated after culture and 5 ml of 4% paraformaldehyde in 1×PBS was added, incubating for 30 min at room temperature. The wells were washed by PBS after aspirating paraformaldehyde. The inner surface of the upper chamber was carefully wiped using a cotton swab and the membranes were stained with Giemsa. Cells that migrated to the lower surface of the filter were considered to have invaded through the matrix and were counted under a light microscopy (200×). Assays were performed three times using triplicate well.

Flow cytometry analysis

48 h after transfection, cell cycle and apoptotic cells were identified by flow cytometry analysis. Adherent cells were collected by trypsinization, washed in PBS and centrifugated. Cells were resuspended at 1×10^6 cells/ml in PBS and fixed in ice-cold ethanol overnight at 4°C. Fixed cells were centrifugated and washed once with PBS. Each sample was resuspended in propidium iodide (1 μg/ml, Sigma-Aldrich, USA). Samples were analyzed using a FACScan. The experiment was done in triplicate.

Tumor xenografts in nude mice

Male Balb/c nude mice of 5 weeks old were purchased from Shanghai Animal Center, China. All animals were kept under specific pathogen-free conditions and tended to in accordance with institutional guidelines. Tumor was implanted by subcutaneous injection of 3.5×10^6 cells/mouse in 200 μl of a 50/50 dilution of RPMI 1640 in PBS into the left flanks of the nude mice (O'Sullivan et al. 2007). When the tumor nodules reached 4 × 4 mm, the mice were randomly divided into three groups ($n = 6$). Equal volume of PBS was used for intratumor injection as blank control groups. Scrambled siRNA groups received intratumor injections of 50 μg scrambled siRNA and KLF8-siRNA2 groups received intratumor injection of 50 μg KLF8-siRNA2 every 3 day (Hu et al. 2006; Zhao

Table 1 Relationship between KLF8 expression and clinicopathological features of RCC

Clinicopathological features	Cases	KLF8 mRNA	*P* value	KLF8 protein				*P* value
				−	+	++	+++	
Tissue			**<0.001**					**<0.001**
RCC tissues	42	0.869 ± 0.049		6	16	11	9	
Adjacent normal renal tissues	42	0.416 ± 0.018		26	15	1	0	
Sex			0.160					0.127
Male	22	0.796 ± 0.068		1	9	5	7	
Female	20	0.936 ± 0.069		5	7	6	2	
Ages			0.471					0.670
<60	26	0.840 ± 0.054		5	9	7	5	
≥60	16	0.915 ± 0.096		1	4	4	4	
Tumor size (cm)			**<0.001**					**<0.01**
≤7 cm	31	0.751 ± 0.049		7	16	8	1	
>7 cm	11	1.201 ± 0.053		0	0	3	8	
Cell differentiate			0.527					0.150
High-mediate	30	0.849 ± 0.063		4	14	8	4	
Low	12	0.919 ± 0.072		2	2	3	5	
Clinical stage			**<0.001**					**<0.001**
T1–T2	27	0.680 ± 0.038		6	13	7	1	
T3–T4	15	1.209 ± 0.050		0	3	4	8	

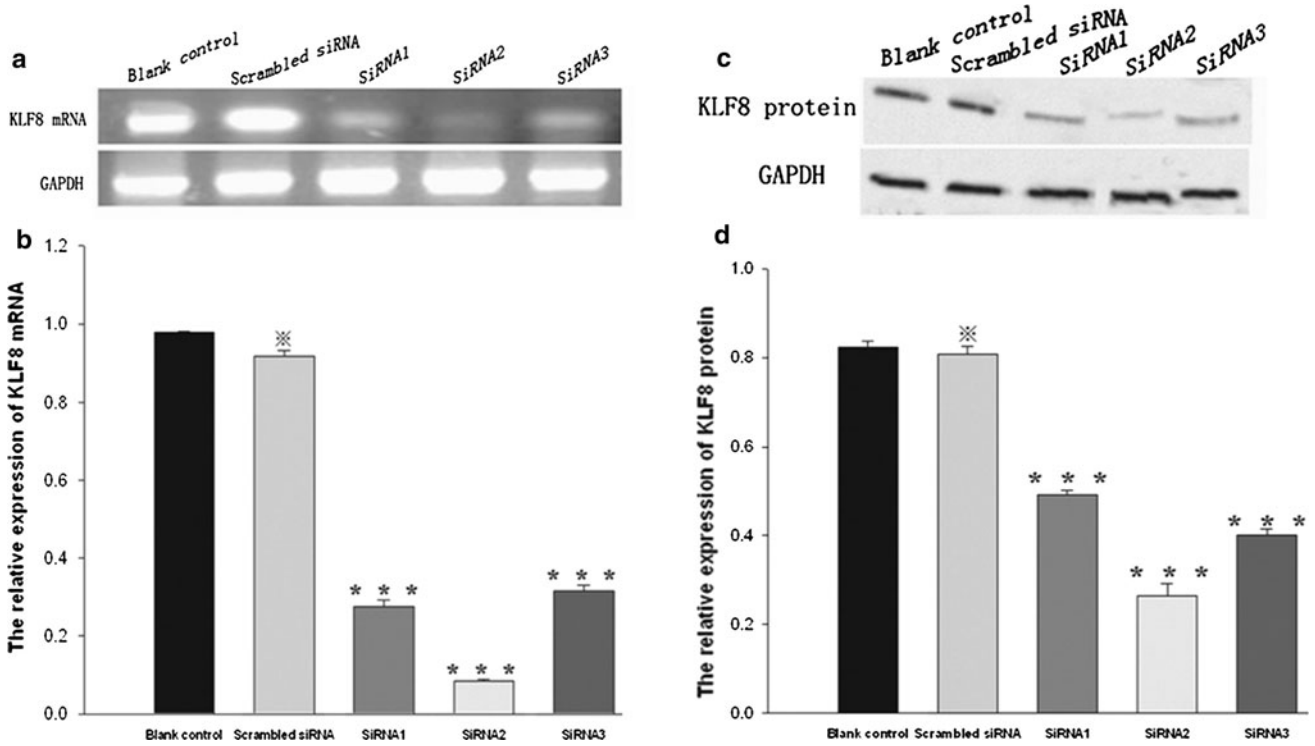

Fig. 2 The expression of KLF8 mRNA and protein in the renal cancer 786-0 cells after transfected with different KLF8-siRNA sequences. **a** RT-PCR analysis of KLF8 mRNA expression in different groups. GAPDH was shown as an internal control. **b** The relative expression of KLF8 mRNA in different groups. **c** Western blot analysis of KLF8 protein expression in different groups. GAPDH was shown as an internal control. **d** The relative expression of KLF8 protein in different groups. $^{※}P > 0.05$, $^{***}P < 0.001$, as compared with the blank control

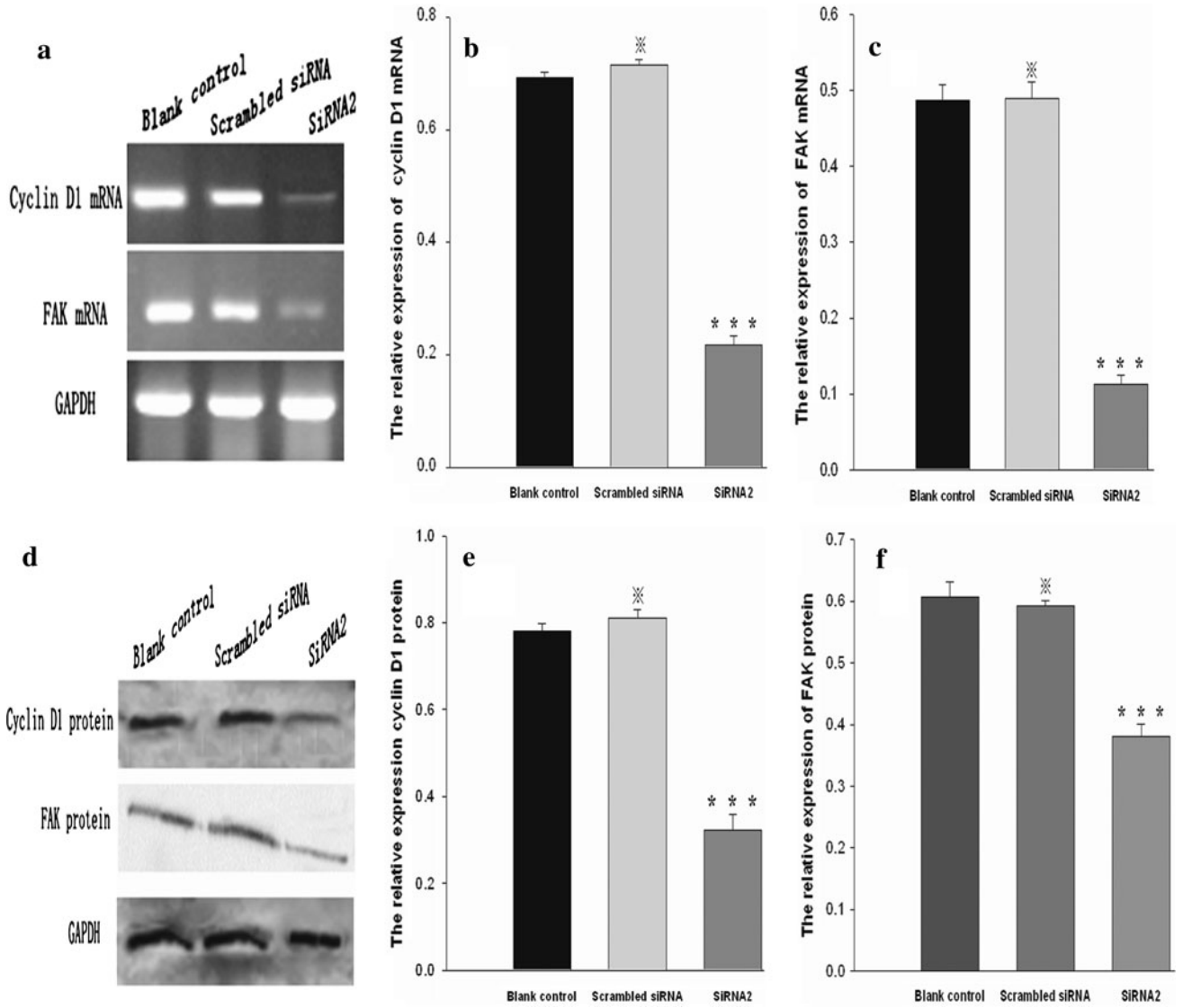

Fig. 3 The effects of KLF8-siRNA2 on mRNA and protein expression of cyclin D1 and FAK in 786-0 cells. **a** The mRNA expression of cyclin D1 and FAK was analyzed by RT-PCR. **b**, **c** The relative mRNA expression of cyclin D1 and FAK in different groups. **d** The protein expression of cyclin D1 and FAK was analyzed by Western blot. **e**, **f** The relative protein expression of cyclin D1 and FAK in different groups. $^{※}P > 0.05$, $^{***}P < 0.001$, as compared with the blank control

et al. 2008). The tumor size was measured twice every week with a caliper, and the diameters were recorded. Tumor volume (cm^3) was calculated by the formula: $W^2 \times L/2$, where L was the length and W was the width of the tumor (Wang et al. 2008a). The observations were terminated after 6 weeks from the day of siRNA injection.

Statistical analysis

Data were expressed as mean ± standard error (SE). The comparison between different tissues was assessed by Student's t test. The expression differences of IHC variables were assessed by rank sum test. Statistical significance of differences was determined by analysis of variance (ANOVA), followed by Dunnett's t test for individual group comparison. A value of $P < 0.05$ was regarded as statistically significant.

Results

Clinicopathological characteristics and KLF8 expression in RCC tissues

The expression of KLF8 protein in RCC and adjacent non-tumorous renal tissues was detected by IHC (Fig. 1a, b). KLF8 protein expression was positive in 36 cases of RCC tissues (36/42, 85.7%), higher than that in adjacent

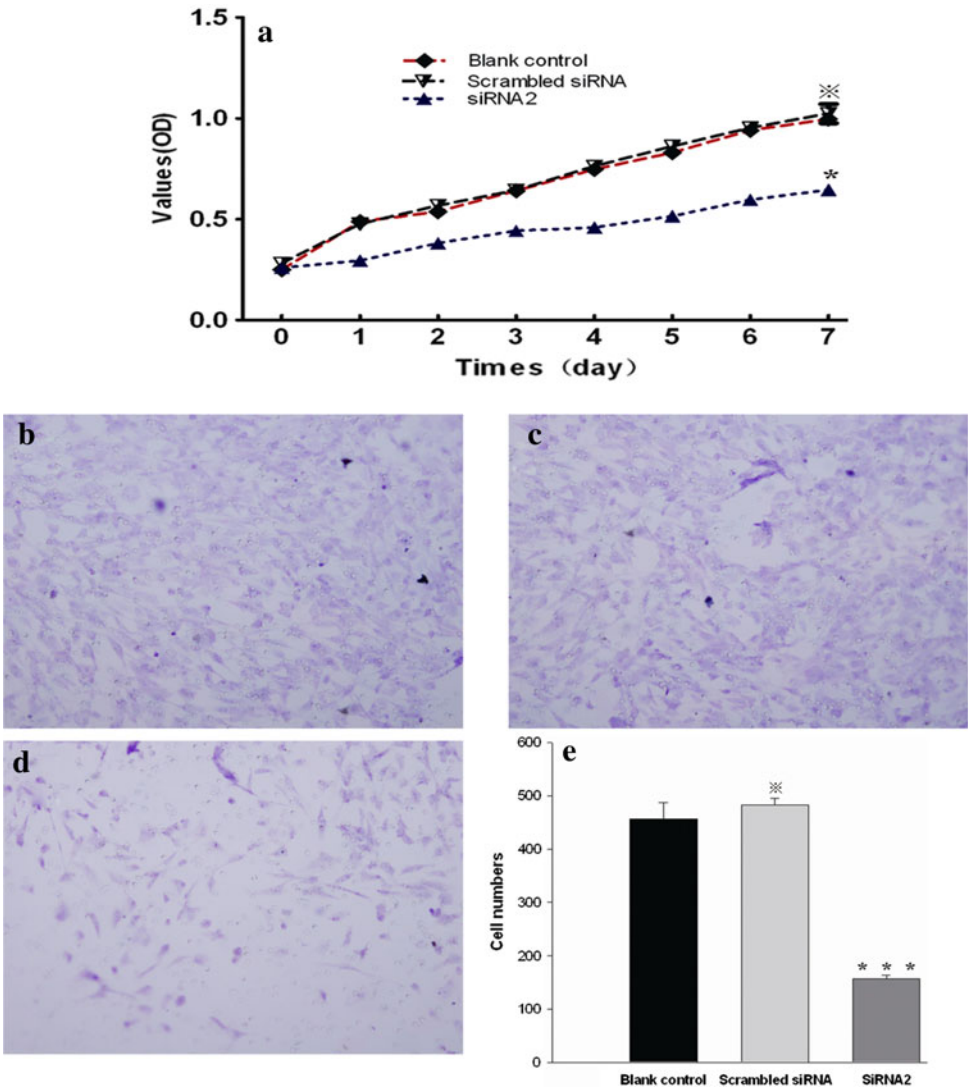

Fig. 4 The effects of KLF8-siRNA2 on growth, invasion of 786-0 cells in vitro. **a** 786-0 cells were incubated with KLF8-siRNA2 or scrambled siRNA. 786-0 cells were harvested at indicated time points and analyzed for viability using the MTT Assay. **b–d** The 786-0 cells number of blank control group, scrambled siRNA group, and KLF8-siRNA2 group was calculated in Matrigel Invasion Assay. **e** KLF8-siRNA2 significantly inhibited invasion ability of 786-0 cells. $^{※}P > 0.05$, $^{*}P < 0.05$, $^{***}P < 0.001$, as compared with the blank control

non-tumorous tissues (16/42, 38.1%, $P < 0.001$). RT-PCR results revealed that the relative expression of KLF8 in RCC and adjacent non-tumorous renal tissues was (0.869 ± 0.321) and (0.416 ± 0.117) respectively and statistically significant difference existed between them ($P < 0.001$) (Fig. 1c, d) (Table 1).

The relationship between the expression of KLF8 and clinicopathological features of RCC was summarized in Table 1. The high levels of KLF8 mRNA and protein were related with the larger tumor size ($P < 0.01$), high clinical stage ($P < 0.001$), but not related with sex, age and cell differentiate ($P > 0.05$).

KLF8 mRNA and protein expression after siRNA transfection

Three different KLF8-siRNA and scrambled siRNA were transfected with 786-0 cells to identify the siRNA sequence that most potently suppressed KLF8 mRNA level. As shown in Fig. 2a, KLF8-siRNA significantly inhibited the relative expression of KLF8 mRNA ($P < 0.001$): siRNA1 (0.276 ± 0.017), siRNA2 (0.084 ± 0.00), siRNA3 (0.316 ± 0.014), compared with blank control (0.977 ± 0.003) and scrambled siRNA (0.917 ± 0.014), whereas there was little difference between blank control and scrambled siRNA ($P > 0.05$) (Fig. 2b). SiRNA2 was identified as the most potent sequence which suppressed KLF8 mRNA expression.

In agreement with the RT-PCR analysis, Western blot analysis demonstrated a significant reduction in KLF8 protein level in three different KLF8-siRNA sequences ($P < 0.001$) (Fig. 2c): siRNA1 (0.491 ± 0.012), siRNA2 (0.264 ± 0.029), siRNA3 (0.401 ± 0.013) (Fig. 2d), when compared with that in blank control (0.822 ± 0.016) and that in scrambled siRNA (0.807 ± 0.019). The expression of KLF8 mRNA and protein was down-regulated effectively by KLF8-siRNA2. Subsequent experiments focused on the siRNA2 because it was the most effective at inhibiting KLF8 expression.

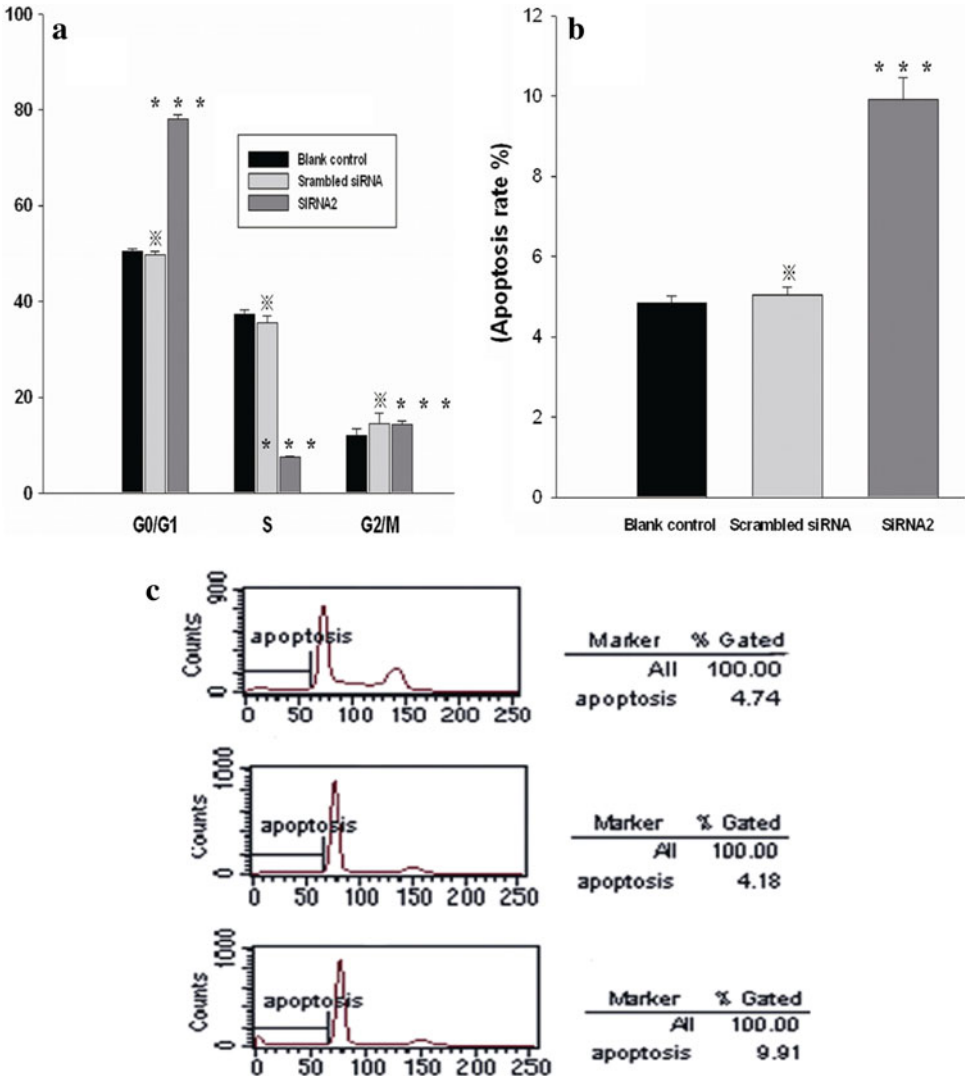

Fig. 5 The effects of KLF8-siRNA2 on cell cycle and cell apoptosis. **a–c** Flow cytometric analysis showed that knockdown of KLF8 expression induced G0/G1 arrest to disrupt cell cycle progression and increased cell apoptosis. $^{※}P > 0.05$, $^{***}P < 0.001$, as compared with the blank control

Cyclin D1 and FAK expression after siRNA transfection

We investigated the mRNA and protein expression of cyclin D1, FAK and E-cadherin in 786-0 after transfected with KLF8-siRNA2. Compared with blank control (0.693 ± 0.009), (0.487 ± 0.020), and scrambled siRNA group (0.715 ± 0.018), (0.490 ± 0.012), the relative mRNA expression of cyclin D1 (0.217 ± 0.018) and FAK (0.113 ± 0.012) was down-regulated significantly in KLF8-siRNA2 group ($P < 0.001$) (Fig. 3a–c). The relative protein expression of cyclin D1 (0.217 ± 0.018) and FAK (0.113 ± 0.012) was decreased significantly in KLF8-siRNA2 group ($P < 0.001$) (Fig. 3d–f), when compared with blank control group (0.780 ± 0.017), (0.810 ± 0.021), and scrambled siRNA group (0.607 ± 0.024), (0.593 ± 0.008) (see Fig. 3).

Effect of KLF8-siRNA2 on renal cancer cells growth

To determine whether the reduction of KLF8 expression affects the growth of 786-0 cells in vitro, the growth rate of cells after transfected with KLF8-siRNA2 or scrambled siRNA were examined using MTT assay for 7 days. KLF8-siRNA2 reduced growth of 786-0 cells significantly ($P < 0.05$) (Fig. 4a), as compared to blank control and scrambled siRNA.

Effect of KLF8-siRNA2 on invasion ability of renal cancer cells

To investigate the effects of KLF8-siRNA2 on tumor cell invasion, the number of migrated cells was counted in each 200× field under the microscope. Our results showed that the migrated cells in siRNA2 treated group (156 ± 7) (Fig. 4d) decreased significantly, when compared with

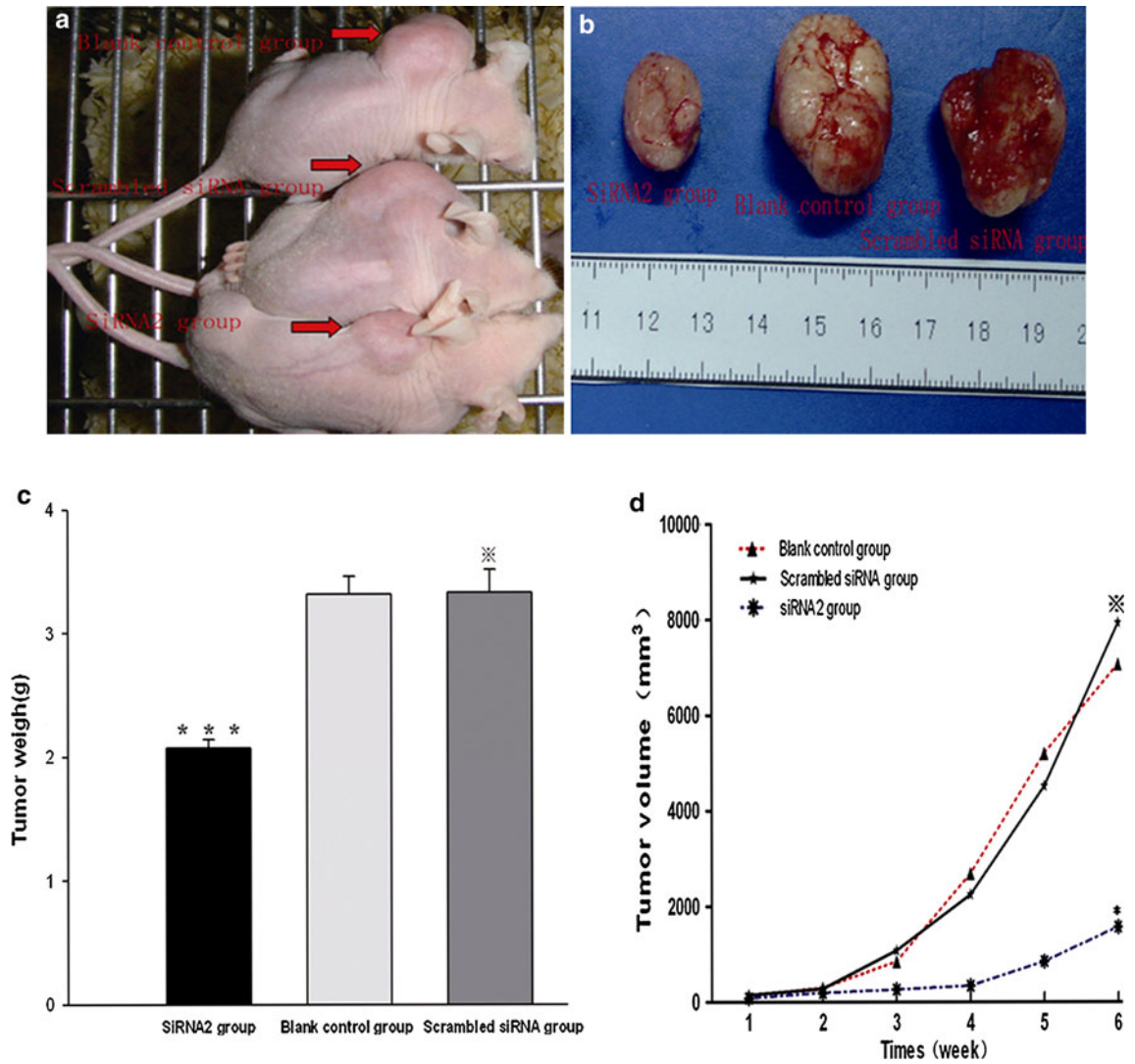

Fig. 6 Effects of KLF8-siRNA on tumor growth in nude mice. **a–c** The tumor size and weight of KLF8-siRNA2 group was significantly decreased than that of blank control group and scrambled siRNA group. **d** Tumor growth curve showed a significant growth tendency in blank control group and in scrambled siRNA group, while the tumor growth in KLF8-siRNA2 group was obviously inhibited. ※$P > 0.05$, *$P < 0.05$, ***$P < 0.001$, as compared with the blank control group

those in black control groups (456 ± 32) (Fig. 4b) and those in scrambled siRNA groups (483 ± 13) (Fig. 4c) ($P < 0.001$) (Fig. 4e).

Effect of KLF8-siRNA2 on cell cycle and cell apoptosis

The KLF8-siRNA2 was found to have an effect on the cell cycle. KLF8-siRNA2 group induced an increase in G0/G1 phase cells (78.1 ± 0.91%, $P < 0.001$) (Fig. 5a) as compared with blank control (50.4 ± 0.6)% and scrambled siRNA group (49.8 ± 0.71)%, and decrease in S phase (7.46 ± 0.27%, $P < 0.001$) and G2/M phase (14.4 ± 0.64%, $P > 0.05$) cells as compared with blank control (37.4 ± 0.87 and 12.1 ± 1.37%) and scrambled siRNA group (35.6 ± 1.44 and 14.5 ± 2.07%, respectively).

The percentage of apoptotic cells in KLF8-siRNA2 group was much higher (9.92 ± 0.55%, $P < 0.001$) (Fig. 5b, c) than that in blank control group (4.85 ± 0.18)% and that in scrambled siRNA group (5.04 ± 0.21)%, while there was no statistical significances between that in blank control and that in scrambled siRNA group ($P > 0.05$).

Inhibitory effect of KLF8-siRNA2 in vivo

We also examined the effect of KLF8-siRNA2 on growth of 786-0 cells in nude mice. After 6 weeks, tumor size and weight from the KLF8-siRNA2 group (2.073 ± 0.067 g, $P < 0.001$) (Fig. 6a–c) was also significantly decreased than that from blank control group and from scrambled siRNA group (3.317 ± 0.144 and 3.333 ± 0.185 g,

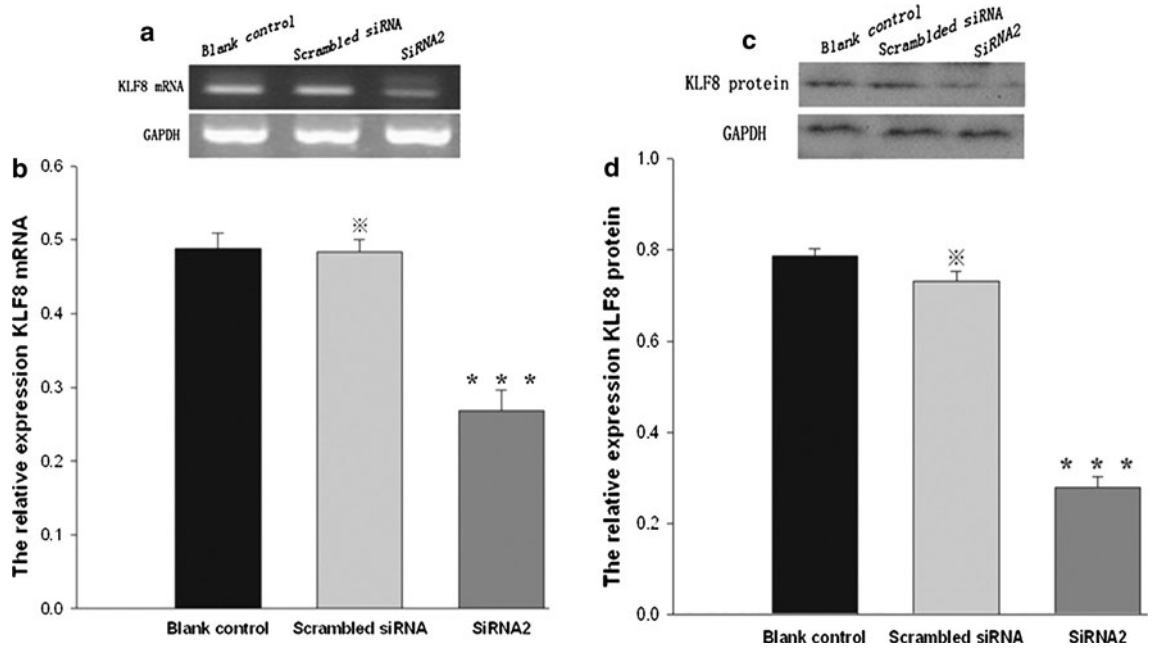

Fig. 7 Effects of KLF8-siRNA2 on KLF8 expression in vivo as determined by RT-PCR and Western blot. **a** KLF8 mRNA expression in different groups. **b** The relative mRNA expression of KLF8 in different groups. **c** KLF8 protein expression in different groups. **d** The relative protein expression of KLF8 in different groups. $^{※}P > 0.05$, $^{***}P < 0.001$, as compared with the blank control group

respectively), while there was no statistically significance between blank control group and scrambled siRNA group ($P > 0.05$).

The tumor growth curves indicated the significant growth inhibition in KLF8-siRNA2 group ($P < 0.05$) (Fig. 6d), but no differences of blank control group and scrambled siRNA group ($P > 0.05$).

Compared with blank control group (0.488 ± 0.021) and scrambled siRNA group (0.483 ± 0.017), the relative mRNA expression of KLF8 (0.269 ± 0.028) in KLF8-siRNA2 group was down-regulated significantly ($P < 0.001$) (Fig. 7a, b). The relative protein expression of KLF8 in KLF8-siRNA2 group (0.278 ± 0.044) was also decreased significantly ($P < 0.001$) (Fig. 7c, d), when compared with blank control (0.785 ± 0.016) and scrambled siRNA group (0.731 ± 0.023). There was no statistically significance between the expression of KLF8 mRNA and protein in blank control group and that in scrambled siRNA group ($P > 0.05$).

Discussion

Despite numerous studies with different treatment modalities, advanced RCC remains highly resistant to systemic therapy (Clark et al. 2003; Messing et al. 2003). It is important to find a new way to effectively inhibit renal cancer cells growth and avoid the side effects of drugs. Gene target therapies have proved to be a promising way to achieve this goal (Imyanitov and Moiseyenko 2007; Masiero et al. 2007; Tschoep et al. 2007).

Recently KLF8 has been thought as a key transcription factor downstream of FAK in the regulation of the cell cycle and also plays a critical role in oncogenic transformation and epithelial to mesenchymal transition (EMT). Aberrant overexpression of KLF8 has been found in several human cancer cell types including ovarian, and breast cancer (Wang et al. 2008b). But the possible role of KLF8 in RCC that we know is very little.

In the present study, we firstly indicated that KLF8 mRNA and protein expression was significantly increased in RCC tissues compared to adjacent non-tumorous renal tissues ($P < 0.001$). Meanwhile, the high levels of KLF8 protein were related with the large tumor size and the high clinical stage ($P < 0.05$). These observations suggested that KLF8 overexpression might involve in malignant transformation, pathogenesis, and progression of RCC.

SiRNA emerged over 8 years as a promising new approach for gene silencing in cells (Elbashir et al. 2001; McManus and Sharp 2002). This inhibits gene expression in a sequence-specific manner by degradation of the corresponding mRNA and has been proven to be a powerful tool for suppressing gene expression (Mandriota et al. 2001; Padera et al. 2002; Rocchi et al. 2006). SiRNA is now widely used in cancer studies, and may provide a promising way towards RCC therapy.

In the present study, we constructed KLF8-siRNA which can efficiently knock down the expression of KLF8

in both mRNA and protein levels on ccRCC derived cell line 786-0 cells and inhibit the growth of tumor cells. Flow cytometric analysis revealed that knockdown of KLF8 expression induced G0/G1 arrest to disrupt cell cycle progression in 786-0 cells. And cyclin D1 expression was efficiently suppressed in 786-0 cells after transfecting with specific KLF8-siRNA. Our results were consistent with the findings of Zhao et al. KLF8 may regulate cell cycle progression of renal cancer cells by inducing the expression of cyclin D1. We also found that silencing KLF8 expression was accompanied by a decrease in the cell invasiveness. Depressed KLF8 expression can induce cell apoptosis. Furthermore, a strong anti-tumor effect of KLF8-siRNA in vivo was observed, as tumor growth in nude mice with xenograft was significantly suppressed when KLF8 mRNA and protein was silenced by intra-tumor injection of KLF8-sRNA.

These findings demonstrate that KLF8 plays a critical role in renal cancer cell proliferation, invasion, and cell apoptosis. The underlying mechanism that results in KLF8 over-activation is currently unknown. A recent study showed that KLF8 mediated cell cycle progression downstream of FAK by up-regulating cyclin D1 (Zhao et al. 2003). FAK can regulate many types of cellular events including cell proliferation, survival, migration, invasion, and EMT (Avizienyte and Frame 2005; Nakamura et al. 2001; Schlaepfer et al. 1999), and is implicated to play a crucial role in metastatic progression of human carcinoma given its overexpression in many types of invasive human cancer (McLean et al. 2005). Our findings also suggested that suppressed expression of KLF8 mRNA can correspondly decrease the mRNA and protein expression of FAK. Nevertheless related studies have suggested that KLF8 expression was regulated by FAK signaling (Wang et al. 2008b; Zhao et al. 2003). Our results suggested the mutual regulation may be existed between KLF8 and FAK. Accordingly the phenomenon may occur not only in RCC but in many other types of human cancer cells. The detail need to be further studied.

These findings demonstrate that KLF8 plays a critical role in renal cancer cell proliferation, invasion, and cell apoptosis. The mutual regulation may be existed between KLF8 and FAK. And KLF8-siRNA may be used as a potent tool for RCC and some other tumors with overexpression of the KLF8 gene. Nevertheless, improving RNAi specificity, prolonging its effects, and increasing the transfection efficiency and biological safety of this method are key issues that must be resolved.

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Conflict of interest statement We declare that we have no conflict of interest.

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