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ORIGINAL ARTICLE

Silencing of HIF-1α suppresses tumorigenicity of renal cell carcinoma through induction of apoptosis

K Xu, Q Ding, Z Fang, J Zheng, P Gao, Y Lu and Y Zhang

Department of Urology, Huashan Hospital, Fudan University, Shanghai, China

Hypoxia-inducible factor-1α (HIF-1α) is a main responder to intracellular hypoxia and is overexpressed in many human cancers, including renal cell carcinoma (RCC). To better understanding of the role of HIF-1α in the tumorigenicity of RCC, we used short-hairpin RNA (shRNA) interference to inhibit HIF-1α expression in the human renal cancer cell line, Caki-1 and OS-RC-2. Silencing of HIF-1α significantly reduced the expression of HIF-1α in both of renal cancer cell lines. *In vitro* downregulation of HIF-1α inhibited Caki-1 and OS-RC-2 cell growth, migration and invasion. The results further showed that HIF-1α silencing resulted in caspase-dependent apoptosis of Caki-1 and OS-RC-2 through regulation of PI3K/Akt pathway and Bcl-2-related proteins expression. *In vivo* animal studies showed that tumor growth was significantly inhibited in HIF-1α shRNA-transfected RCC. Intratumor gene therapy with polyethylenimine-loaded HIF-1α shRNA also resulted in tumor growth suppression. Thus, this study demonstrates that downregulation of HIF-1α could suppress tumorigenicity of RCC through induction of apoptosis, and HIF-1α shRNA may be a promising strategy for the treatment of RCC.
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Keywords: renal cell carcinoma; HIF-1α; RNA interference; apoptosis; tumor therapy

Introduction

Renal cell carcinoma (RCC) is the third most common urological cancer with an incidence of approximately 5–10 per 100 000 and comprises 2–3% of all malignancies.[1,2] Despite the use of multimodal therapy (chemotherapy, radiation therapy and surgery), the long-term disease-free survival rate of RCC patients is still disappointingly low.[3–6] Therefore, the identification of new therapeutic targets is needed.

Hypoxia-inducible factor-1 (HIF-1) is the major transcription factor involved in the adaptive response to hypoxia and consists of HIF-1α and HIF-1β subunits.[7–8] Overexpression of HIF-1α has been detected in several human cancers.[9] Significant associations between HIF-1α overexpression and patient mortality have been shown in cancers of the brain (oligodendroglioma), breast, cervix, oropharynx, ovary and uterus (endometrial).[10–13] Activation of HIF-1α in cancer has been shown to regulate angiogenesis, tumor growth, progression, metastatic spread and glucose metabolism by acting as a transcription factor for crucial proteins (that is, vascular endothelial growth factor (VEGF), erythropoietin 1 (EPO1), PDGF, epidermal growth factor receptor (EGFR), Glut1).[14,15] In addition, HIF-1α is also important in regulating the cell cycle and apoptosis.[16,17] Recently, suppression of HIF-1α expression by various technologies, including RNA interference (RNAi), has been used as the tools for cancer therapy.[18–20]

Several lines of evidence point to a role for HIF-1α in the pathogenesis of RCC. High levels of HIF-1α have been noted in conventional RCC and the HIF-1α level is a favorable independent prognostic factor in RCC.[21–23] Under hypoxic conditions, HIF-1α is stabilized and accumulates due to the inactivation or absence of the von Hippel–Lindau tumor suppressor gene, which is the most frequent genetic alternation observed in RCC.[24] Despite its unquestioned role as a important regulator of tumoral pathophysiology, the lack of mechanistic or functional data has led to an incomplete understanding of how HIF-1α mediates RCC cancer progression. To address this issue, we used RNAi to produce specific and long-term silencing of HIF-1α in RCC cancer cell lines Caki-1 and OS-RC-2. For the first time, our findings demonstrated that silencing of HIF-1α suppresses tumorigenicity of RCC through induction of apoptosis. Furthermore, the possible HIF downstream mediators and HIF-dependent apoptosis signaling pathways were explored. In addition, we used polyethylenimine (PEI) as our gene delivery system, to efficiency inhibit Caki-1 and OS-RC-2 tumor growth in a nude mouse model.

Correspondence: Professor Q Ding, Department of Urology, Huashan Hospital, Fudan University, 12 Wu Lu Mu Qi Road, Shanghai 200040, China.
E-mail: huashandingqiang@126.com
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Materials and methods

RNAi design

Using GenBank sequence NM001530 for human HIF-1α mRNA and computer analysis software developed by Ambion (Austin, TX), we selected four candidate sequences in the HIF-1α cDNA sequence for RNAi. The targeted HIF-1α sequences were HIF-A: CTGATGACCAGGAACTTGA; HIF-B: CTGGACACAGTGTGTT TGA; HIF-C: ATCCAGAGTCACTGGAACT; HIF-D: TGACTCAGCTATTCACCAA. Human mismatch or scrambled siRNA sequences possessing limited homology to human genes served as a negative control. For the construction of hairpin-type siRNA expression plasmids, we synthesized oligonucleotides with the hairpin sequence, the terminator sequence and overhanging sequences. Then, we annealed the fragments and were ligated between the *Bam*HI and *Hin*dIII sites on the pSilencer 2.1-U6 hygro vector (Ambion).

Real-time quantitative RT-PCR analysis

Gene expression was quantified by real-time quantitative RT-PCR using QuantiTect SYRB Green dye (Qiagen, Valencia, CA). DNA amplification was carried out using iCycler (Bio-Rad, Hercules, CA) and the detection was performed by measuring the binding of the fluorescence dye SYBR Green I to double-stranded DNA. Nucleotide sequences of specific primers for the selected cytokines were as follows—VEGF forward primer: 5′-ATGAACT ITCTGCTGTCTTGG-3′, reverse primer: 5′-TCACCGC CTCGGCITGTCACA-3′; Glut1 forward primer: 5′-CA ACTGTGTGGTCCCTACGTCTTC-3′, reverse primer: 5′-TCACACTTGGGAATCAGCCCC-3′; EGFR forward primer: 5′-CTTCTTGCAGCGATACAGCTC-3′, reverse primer 5′-ATGCTCCAATAAATTCACTGC-3′; aldolase A forward primer: 5′-CACTGGGATCACCTTCCTGT-3′, reverse primer: 5′-AAGACACCACACACCACTGT-3′; MMP-2 forward primer: 5′-TGACGGTAAGGACGG ACTC-3′, reverse primer: 5′-TGGAAGGGAATGGAA AC-3′; uPA forward primer: 5′-ACTACTACGGCTCTG AAGTCACCA-3′, reverse primer: 5′-GAAGTGTGAGA CTCTCGTGTAGAC-3′; β-actin forward primer: 5′-GAT TGGAATCTGGCTACT-3′, reverse primer: 5′-TAGGGC TGAAGCACAG GG-3′.

Cell culture and transfection

The human renal cancer cell lines Caki-1 and OS-RC-2 were purchased from the Institute of Cell Biology (Shanghai, China). The cell lines were maintained in Dulbecco's modified Eagle's Medium (DMEM)/F12 medium with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2. When cells reached 80–90% confluence, they were transfected with plasmids in the following manner using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Incubation was continued in 20% O_2 (normoxia) or 1% O_2 (hypoxia) conditions. For *in vivo* experiment, stable transfected cell clones were selected in hygromycin B (Invitrogen) containing medium at 400 μg ml^{-1}.

In vitro *cell growth assay*

The *in vitro* growth rates of plasmid-transfected cells were assessed at 72 h. Cells were grown in monolayer culture to 60% confluence, harvested using Trypsin and plated at a density of 5×10^3 cells per well into separate wells of a 96-well plate (Costar; Corning Inc., Corning, NY). Incubation was continued in 20% O_2 (normoxia) or 1% O_2 (hypoxia) conditions. The cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg ml^{-1}) after 72 h. The color intensity was measured at 490 nm using an enzyme-linked immunosorbent assay reader (Dynatech, Chantilly, VA). The absorbance units obtained for plasmid-transfected cells were each divided by the absorbance units obtained for untreated wild-type cells and expressed as a growth index. By definition, untreated wild-type cells were assigned an index of 1. The experiments were performed in triplicate.

In vitro *migration and invasion assays*

The motility and invasiveness of plasmid-transfected cells were evaluated in 24-well transwell chambers with upper and lower culture compartments separated by polycarbonate membranes with 8-μm-sized pores (Costar 3422; Corning Inc.). Before plating cells into the transwells, DMEM–0.1% bovine serum albumin (BSA) was incubated in the top chamber of each transwell at 37 °C for 1 h to saturate nonspecific binding sites and was then subsequently removed. A total of 5×10^4 cells suspended in 100 μl of DMEM–0.1% BSA were plated into the top chamber. DMEM–10% FBS was placed into the bottom chamber to exert its effect as a chemoattractant. Incubation was continued in 20% O_2 (normoxia) or 1% O_2 (hypoxia) conditions for 48 h. The cells that migrated through the 8-μm-sized pores and adhered to the lower surface of the membrane were fixed with 3.7% paraformaldehyde, stained with 0.2% crystal violet and washed with 1× phosphate-buffered saline (PBS) three times. The dye was eluted using 30% acetic acid and quantification of cell number was performed using colorimetric analysis with a microplate reader (absorbance at 590 nm). The absorbance units obtained for plasmid-transfected cells were each divided by the absorbance units obtained for untreated wild-type cells and expressed as a migration index. By definition, untreated wild-type cells were assigned an index of 1.

In a similar manner, the invasiveness of plasmid-transfected cells was evaluated in Matrigel (Collaborative Biomedical Products, Bedford, MA)-coated 24-well transwell chambers. Matrigel was used at a concentration of 0.4 mg ml^{-1}. Cells, media, experimental conditions and analysis performed were similar to those of migration assays. Triplicate assays were performed for each group of cells in both migration and invasion assays and results are expressed as mean ± s.d.

Apoptosis analysis

Fluorescein FragEL DNA Fragmentation Detection kit (Calbiochem, San Diego, CA) was used for the TUNEL assay. The fluorescein of cells carrying DNA labeled with

FITC-dUTP (TUNEL-positive cells) was analyzed by FACScan (Becton Dickinson, San Diego, CA).

Western blot analysis

Subcellular fractionation was performed by centrifugation technique as described previously. Nuclear extracts were generated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL). Total cell lysate was prepared in $1\times$SDS buffer. Proteins at the same amount were separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After probing with individual antibodies, antigen/antibody complex was visualized by enhanced chemiluminescence's reagent Supersignal (Pierce Biotechnology). The antibodies used were anti-HIF-1α (R&D Systems, Minneapolis, MN), and anti-PTEN (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PDK1, anti-Akt and anti-p-Akt (Boster Biotechnology, Wuhan, China), anti-Bax, anti-Bim anti-Bcl-xL, and anti-Bcl-2 (Beyotime, Haimen, China). Anti-GAPDH (Ambion) was used as an internal control.

Animal studies

Six- to eight-week-old female Nude mice were obtained from the Planned Parenthood Research Institute (Shanghai, China). All animals in this study were housed under pathogen-free conditions and were maintained in accordance with guidelines of the Committee on Animals of the Second Military Medical University, Shanghai, China. Caki-1/HIFsi-B2, OS-RC-2/HIFsi-B3, shRNA control renal cells and wild-type renal cells in mid-log-growth phase were harvested by trypsinization. Single-cell suspensions (2×10^6 cells in 0.1 ml Hank's balanced salt solution) were injected s.c. into the nude mice. The tumors were measured every 4 days with a caliper, and the diameters were recorded. Tumor volume was calculated by the formula: $a^2b/2$, where a and b are the two maximum diameters.

For the HIF-1αsi gene therapy experiments, 2×10^6 wild-type Caki-1 and OS-RC-2 cells were injected s.c. into nude mice. After 5 days, when the tumors were palpable, the mice were divided into four groups. Group 1 mice were used as untreated controls. Group 2 mice were received intratumor injections with 100 μl $1\times$PBS. Group 3 received intratumor injections (20 μg per mouse) of PEI/control vector twice weekly for various times. Group 4 received intratumor injections with 20 μg PEI/HIF-1αsi per mouse twice weekly as described for group 3. Tumor size was measured as described above.

Statistical analysis

GraphPad Prism 4 (GraphPad Software, San Diego, CA) was used to visualize and perform data analysis by t-test, one-way and repeated-measures analysis of variance, Tukey's multiple comparison test, Mann–Whitney U-test and Wilcoxon signed-rank test.

Results

RNAi decreases HIF-1α expression in renal cancer cell lines Caki-1 and OS-RC-2

Under normoxic conditions, the HIF-1α protein was expressed at low level in Caki-1 and OS-RC-2 cells. However, hypoxia strongly induced HIF-1α protein expression in both cell lines (Figure 1a). Short-hairpin RNA (shRNA) expression vectors were designed to target four different HIF-1α mRNA sequences. Transfection efficiency in Caki-1 and OS-RC-2 cells was about 88 and 82%, respectively. After 48 h of culture, in Caki-1 cells, HIF-1α mRNA and protein expression was significantly inhibited by HIF-1α shRNA vector transient-transfection, particularly pSilencer/HIF-B and pSilencer/HIF-D. In pSilencer/HIF-A, -B, -C and -D transient-transfected cells, HIF-1α levels were reduced by ∼65, ∼14, ∼38 and ∼19%, respectively, compared with control and untreated Caki-1 cells. Similarly, the reduction of HIF-1α levels in OS-RC-2 cell was observed by HIF-1α shRNA vector transient transfection. No significant difference was observed in HIF-1α protein levels between wild-type cells and control shRNA vector-transfected cells (Figure 1b). In addition, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels did not differ significantly among the treated cells and controls, suggesting that this RNAi-mediated effect was specific.

Effect of HIF-1α silencing on renal cancer cell growth, migration and invasion in vitro

It has been reported that HIF-1α is important in cell growth in several tumor cells,[7,8] therefore we first checked the effect of HIF-1α silencing on Caki-1 and OS-RC-2 cell growth *in vitro*. Under normoxia or hypoxia condition, HIF-1α shRNA significantly inhibited the growth of Caki-1 cells, especially pSilencer/HIF-B-transfected cells compared with control shRNA. Similar results were observed in OS-RC-2 cells. Moreover, the growth suppression of both HIF-1α shRNA vector-transfected cell lines was more efficient under hypoxic conditions compared with normoxic ones (Figure 2a).

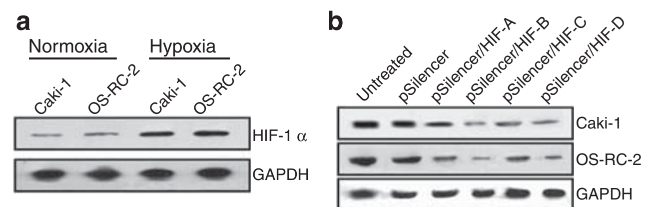

Figure 1 Downregulation of hypoxia-inducible factor-1 (HIF-1α) expression in renal cell carcinoma (RCC) cell lines by RNAi. (**a**) Immunoblottings of HIF-1α protein. Expression of HIF-1α protein was detected in two RCC cell lines (Caki-1 and OS-RC-2) under normoxic and hypoxic conditions for 24 h. (**b**) Effect of HIF-1α shRNA expression vectors on HIF-1α protein expression in RCC cell lines under hypoxic condition. HIF-1α protein expression levels were analyzed using western blots of RCC cell lines performed 48 h after transfection with the pSilencer/HIF vectors. Level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was served as an internal control.

We further evaluated whether the downregulation of HIF-1α expression would alter the migration ability of Caki-1 and OS-RC-2 cells. Under normoxia or hypoxia condition, HIF-1α shRNA significantly reduced the motility of Caki-1 and OS-RC-2 cell. In particularly, under hypoxia condition, pSilencer/HIF-B-transfected Caki-1 and OS-RC-2 showed 63 and 58% reduction of motility, respectively, compared with control and untreated Caki-1 cells (Figure 2b). No significant differences were detected between untreated cells and the control shRNA insert cells.

We also evaluated whether downregulation of HIF-1α expression would alter the ability of renal cancer cell line to invade Matrigel. Under hypoxia condition, pSilencer/HIF-B and -D effectively suppressed the invasiveness of Caki-1 and OS-RC-2 cells. However, under normoxia condition, the decreased invasiveness was not observed in pSilencer/HIF-transfected cells (Figure 2c). Taken

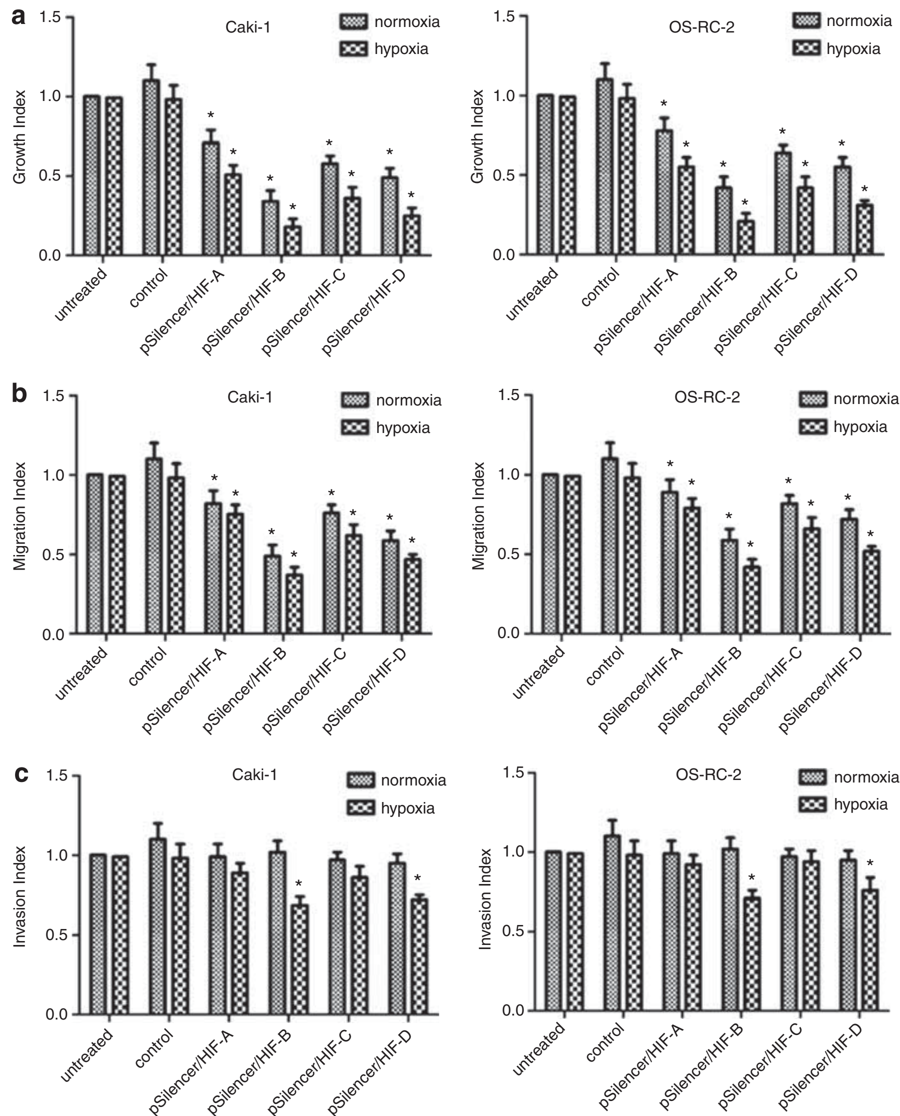

Figure 2 Effect of hypoxia-inducible factor-1 (HIF-1α) silencing on renal cancer cell lines growth, migration and invasion *in vitro*. (**a**) Effect of HIF-1α shRNA on renal cell carcinoma (RCC) cell lines proliferation. At 72 h after transfection, RCC cell lines were seeded in 96-well plates with complete medium for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (**b**) The impact of HIF-1α silencing on RCC cell migration. The migration of untreated, HIFsh-A, HIFsh-B, HIFsh-C and HIFsh-D, shRNA control cells was assessed by incubating cells in transwell chambers for 48 h. Migrated cells were stained with 0.2% crystal violet, subjected to elution by acetic acid and quantified in a microplate reader (A590) by definition. (**c**) The impact of HIF-1α silencing on RCC cell invasiveness. The invasiveness of untreated, HIFsh-A, HIFsh-B, HIFsh-C and HIFsh-D, shRNA control cells was assessed by incubating cells in Matrigel-coated transwell chambers for 48 h. The data represent the mean ± s.d. of triplicate assays. $^{*}P<0.05$ versus control.

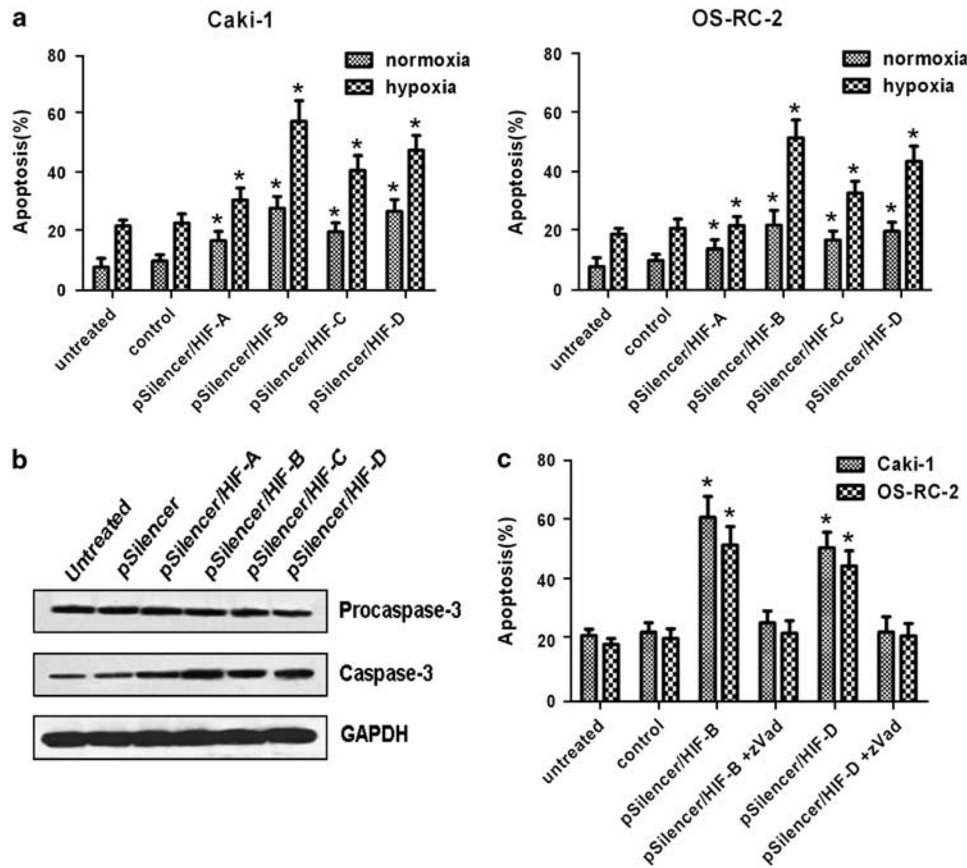

Figure 3 Hypoxia-inducible factor-1 (HIF-1α) silencing induces caspase-dependent apoptosis. (**a**) Renal cell carcinoma (RCC) cell lines were transfected with shRNA plasmids for 24 h, and were then exposed to serum starvation under normoxic and hypoxic conditions for 48 h. Apoptotic cells were detected by fluorescence flow cytometry with terminal transferase dUTP nick-end labeling (TUNEL) assay. (**b**) Levels of procaspase-3 and caspase-3 expression in Caki-1 cells 48 h after transfection with different vectors. Cytosolic subcellular fractions were isolated and subjected to western blotting with indicated antibodies. Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control. (**c**) RCC cell lines were treated with the caspase inhibitor zVAD-fmk (80 μM) 2 h before being subjected to serum starvation under normoxic and hypoxic conditions for 48 h. Data are present as means ± s.d. from three independent experiments. $^{*}P<0.05$ versus control.

together, these data suggest that downregulation of HIF-1α expression in renal cancer cell line through shRNA resulted in decreased proliferation, cell motility and invasiveness, especially under hypoxia condition.

HIF-1α silencing induces caspase-dependent apoptosis

Tumor cells are often deficient in apoptotic regulation, which leads to the expansion of tumor cells, metastasis and invasion.[25,26] Previous studies have shown that HIF-1α may promote resistance to apoptosis of tumor under hypoxia condition.[16,17] Thus, we further investigated that the effect of HIF-1α downregulation on the survival of renal cancer cell line *in vitro*. Hypoxia increased the proportion of the apoptosis of renal cancer cell line compared to normoxic conditions. Under normoxic conditions, the apoptosis of in HIF-1α shRNA-treated cells was not significantly enhanced compared with that of control shRNA-treated groups. However, under hypoxia conditions, transfection with these HIF-1α shRNA vectors dramatically increased the percentage of terminal transferase dUTP nick-end labeling (TUNEL)-positive apoptotic cells, especially pSilencer/HIF-B and -D-transfected groups compared to controls (Figure 3a).

To further analyze the apoptosis observed in HIF shRNA-transfected cells under hypoxia conditions, we examined the level of caspase-3, a critical parameter for apoptosis. In Caki-1 cells, procaspase-3 was not significantly changed at 48 h post-transfection with HIF shRNA. However, the level of caspase-3 was greatly increased after HIF shRNA transfection, especially pSilencer/HIF-B and -D-transfected groups (Figure 3b). Similar results were also observed in OS-RC-2 cells (data not sown). Furthermore, the broad-spectrum caspase inhibitor zVAD-fmk significantly inhibited HIF shRNA-mediated cell death (Figure 3c). Thus, downregulation of HIF-1α-induced apoptosis is caspase dependent in renal cancer cell line.

HIF-1α silencing suppresses VEGF, Glut1, aldolase A and MMP-2 expression

It has been shown that several proteins expression associated with HIF-1α, such as VEGF, Glut1 and aldolase A expression in tumor angiogenesis, tumor

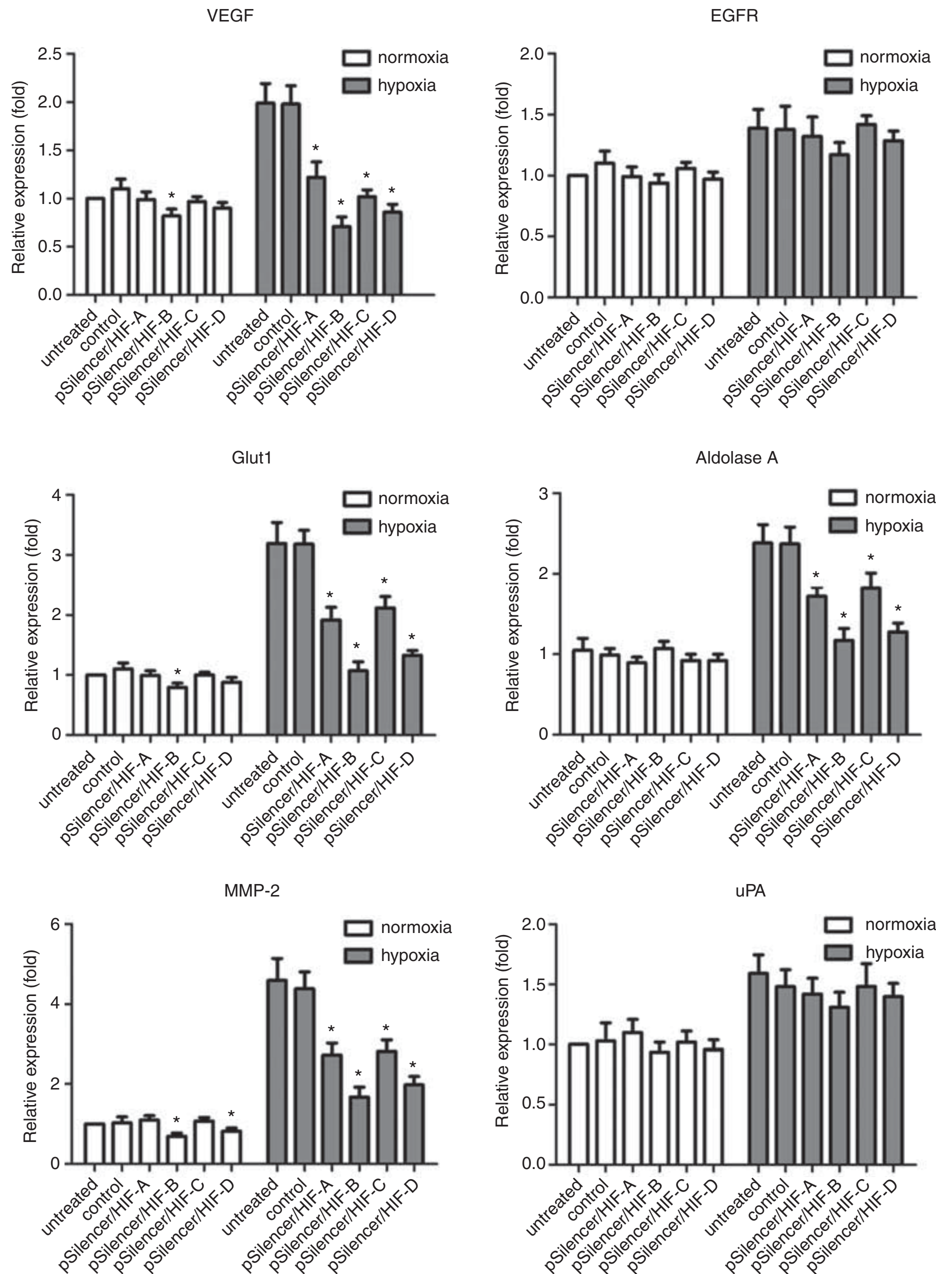

Figure 4 Vascular endothelial growth factor (VEGF), Glut1, aldolase A, epidermal growth factor receptor (EGFR), matrix metalloproteinase 2 (MMP-2) and urokinase plasminogen activator (uPA) mRNA levels in renal cell carcinoma (RCC) Caki-1 cells 48 h after transfection with different vectors. mRNA levels were quantified using real-time RT-PCR. Data were normalized using β-actin as an internal control. Data are present as means ± s.d. from three independent experiments. $^*P<0.05$ versus control.

growth, progression, metastatic spread and glucose metabolism.[14,15] Therefore, we checked whether HIF-1α shRNA affected the mRNA levels of these proteins in RCC cells. In Caki-1 cells, hypoxia induced mRNA expression of VEGF, Glut1, EGFR and aldolase A. The mRNA expression of VEGF, Glut1 and aldolase A in pSilencer/HIF-B- and -D-transfected cells was significantly decreased in hypoxia condition. However, no significant changes of EGFR mRNA expression were detected after pSilencer/HIF transfection (Figure 4). Similar results were observed in OS-RC-2 cells (data not shown).

Matrix metalloproteinase 2 (MMP-2) and urokinase plasminogen activator (uPA) have also been shown to be important in tumor metastatic spread and invasion. As shown in Figure 4, in Caki-1 cells, hypoxia induced mRNA expression of MMP-2 and uPA. After transfection with HIF-1α shRNA expression vectors, the levels of uPA expression were unchanged under normoxic or

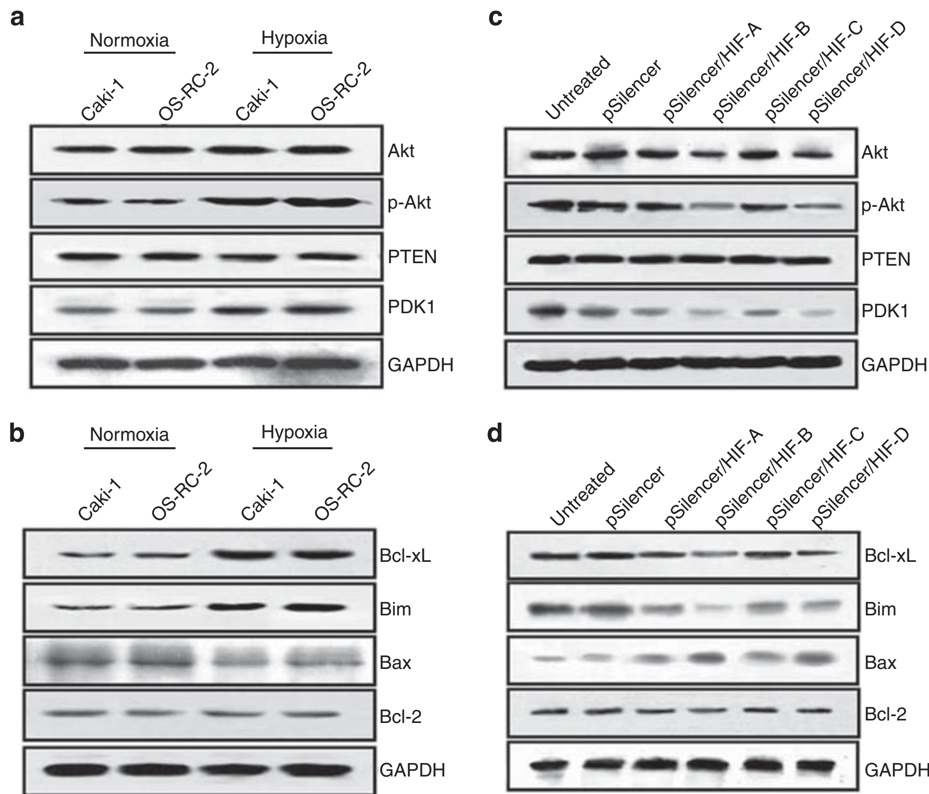

Figure 5 Hypoxia-inducible factor-1 (HIF-1α) silencing regulated the apoptosis signaling in renal cancer cell lines. (**a**, **b**) Expression of PI3K/Akt signaling proteins and Bcl-2-related proteins were detected in two renal cell carcinoma (RCC) cell lines (Caki-1 and OS-RC-2) under normoxic and hypoxic conditions for 48 h. (**c**, **d**) Effect of HIF-1α silencing on the expression of PI3K/Akt signaling proteins and Bcl-2-related proteins in Caki-1 cells. Cytoplasmic protein extracts were harvested 48 h later and subjected to western blotting with anti-Akt, anti-p-Akt, anti-PTEN, anti-PDK1, anti-Bcl-xL, anti-Bcl-2, anti-Bax and anti-Bim. Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control.

hypoxic condition. However, under hypoxia conditions, transfection with pSilencer/HIF, especially pSilencer/HIF-B and -D significantly suppressed the expression of MMP-2, compared with controls. Similar results were also observed in OS-RC-2 cells (data not shown).

HIF-1α silencing regulates the apoptosis signaling in renal cancer cells

To further investigate the molecular mechanism underlying HIF-1α-mediated survival of renal cancer cell line, we examined whether HIF-1α silencing affects the activity of proteins involved in the signal transduction of renal cancer cell survival. The activity of phosphatidylinositol 3-OH kinase (PI3K) and phosphorylation of the serine-threonine kinase Akt (also called 'protein kinase B') have important functions in tumor cell survival.[27] As shown in Figure 5a, hypoxia increased phosphorylation of Akt and PDK1, which activates Akt, in wild-type Caki-1 and OS-RC-2 cells. However, the level of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a negative regulator of PI3K/Akt signaling was unchanged in both of RCC cell lines under hypoxia. We further examined the effect of HIF-1α silencing on PI3K/Akt signaling. In pSilencer/HIF-transfected Caki-1 cells, we found that the levels of Akt phosphorylation and PDK1 were effectively suppressed under hypoxia condition, especially pSilencer/HIF-B- and -D-transfected groups. The level of PTEN was not affected by HIF-1α silencing (Figure 5c). Similar results were observed in OS-RC-2 cells (data not shown). Thus, HIF-1α silencing might induce apoptosis through regulation of the PI3K/Akt pathway.

A group of proteins designated 'BH3-only Bcl-2 family proteins', including Bim, and another group of proteins designated 'multi-BH domain proteins', including Bak and Bax, are also critical in cell survival.[28] As shown in Figure 5b, in wild-type Caki-1 and OS-RC-2 cells, hypoxia increased the expression of Bim and Bcl-xL, and decreased the expression of Bax. The expression of Bcl-2 was unchanged under normoxic or hypoxia condition in Caki-1 and OS-RC-2 cells. The effect of HIF-1α silencing on Bcl-2-related proteins was determined in Caki-1 cells under hypoxia condition. As expected, HIF-1α silencing, especially pSilencer/HIF-B and -D transfection resulted in an obvious decrease of Bim and Bcl-xL, and a dramatic increase of Bax expression (Figure 5d). Similar results were also observed in OS-RC-2 cells (data not shown). Therefore, downregulation of HIF-

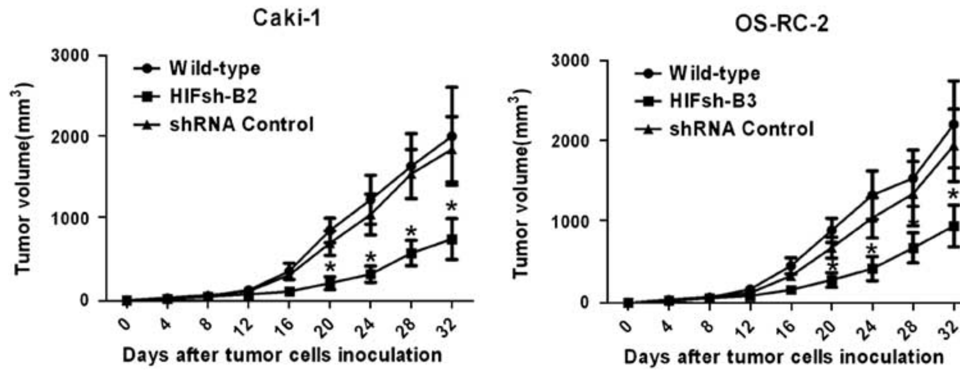

Figure 6 Effect of hypoxia-inducible factor-1 (HIF-1α) shRNA on renal tumor growth in mice. Caki-1/HIFsh-B2 or OS-RC-2/HIFsh-B3 cells, wild-type Caki-1 or OS-RC-2, control shRNA-transfected cells were inoculated s.c. into nude mice ($n = 10$ each group). Tumor growth was monitored and tumor volume was calculated. *$P < 0.05$ versus control.

1α-mediated cell apoptosis is probably regulated by the quantitative balance between proapoptotic and antiapoptotic Bcl-2 family proteins.

Effect of HIF silencing on renal tumor growth in vivo

To investigate the inhibitory effects on tumor growth by HIF-1α RNAi, we directed our attention to generating stable, HIF-1α-downregulated clonal cell lines. On the basis of higher efficiency of pSilencer/HIF-B compared with other groups, we selected target sequence B for stable transfectants. At 1 month after transfection and selection, western blot analysis demonstrated that HIF-1α protein expression was significantly decreased, compared with wild-type Caki-1 and OS-RC-2. Our most significant clones, Caki-1/HIFsh-B2 and OS-RC-2/HIFsh-B3, showed 89 and 83% reduction of HIF-1α expression in Caki-1 and OS-RC-2, respectively (data not shown). Stable HIF-1α-downregulated clonal cell lines Caki-1/HIFsh-B2 and OS-RC-2/HIFsh-B3 were inoculated s.c. into nude mice. Wild-type Caki-1 and OS-RC-2 grew rapidly, resulting in palpable tumors 4–5 days after injection (Figure 6). By contrast, tumor formation was significantly slower after inoculation of Caki-1/HIFsh-B2 and OS-RC-2/HIFsh-B3 cells (Figure 6). No significant difference in either tumor growth or survival time was detected between the tumors induced by the shRNA control cells and the wild-type cells.

Effect of HIF-1α shRNA gene therapy on renal tumor growth

We next investigated whether HIF-1α shRNA can be used as a gene therapy. We elected to use PEI as our gene delivery system because of previous successful report with this nonviral vector.[29] Caki-1 and OS-RC-2 cells were injected s.c. into nude mice. After 5 days, the palpable tumors were injected with PEI/HIFsh-B or PEI/sh control. As shown in Figure 7, PEI/HIFsh-B gene therapy significantly inhibited Caki-1 and OS-RC-2 tumor growth in mice compared with the PEI/shRNA control (Figure 7). These results indicate that using PEI as HIF-1α RNAi delivery system, the renal tumor growth could be effectively inhibited *in vivo*.

Discussion

HIF-1α expression and subsequent HIF-1 activation in cancer cells have important functions in cancer progression by controlling the gene expression related to cancer cell proliferation, apoptosis and metastasis.[30] Here, we showed that gene therapy targeting HIF-1α may have therapeutic benefit in the growth of RCC. This was carried out by selectively inhibiting HIF-1α expression using shRNA. To deliver HIF-1α shRNA, we constructed several vector-based expression systems in which sense and antisense strands of short HIF-1α sequences were transcribed into hairpin structures under the control of a U6 promoter. We designed different shRNA expression plasmids that specifically target degradation of mRNAs encoding HIF-1α. After treatment with shRNA, the expression of HIF-1α protein was greatly reduced under hypoxic conditions. The effects of these siRNAs were investigated in two human renal carcinoma cell lines, Caki-1 and OS-RC-2 *in vitro* and *in vivo*.

Recent evidence suggests that HIF-1α has a significant function in regulating RCC development, invasive phenotypes and survival. Thus, we hypothesize that downregulation of HIF-1α expression will lead to a reduction of RCC growth. Our data showed that HIF silencing effectively suppressed the proliferation of Caki-1 and OS-RC-2 cells under normoxic or hypoxia conditions. Except the reduced growth of renal cancer cell lines, the migration and invasion were also suppressed by HIF silencing, which was not reported in previous studies. HIF-1 transactivates a repertoire of genes, including *VEGF*, glucose transporters (*Glut1*), glycolytic pathway enzymes, EPO and inducible nitric oxide synthase, which mediate angiogenesis, motility, cell proliferation/survival and glucose/iron metabolism in hypoxia.[14–17] Here, we found that RNAi targeting HIF-1α not only down-

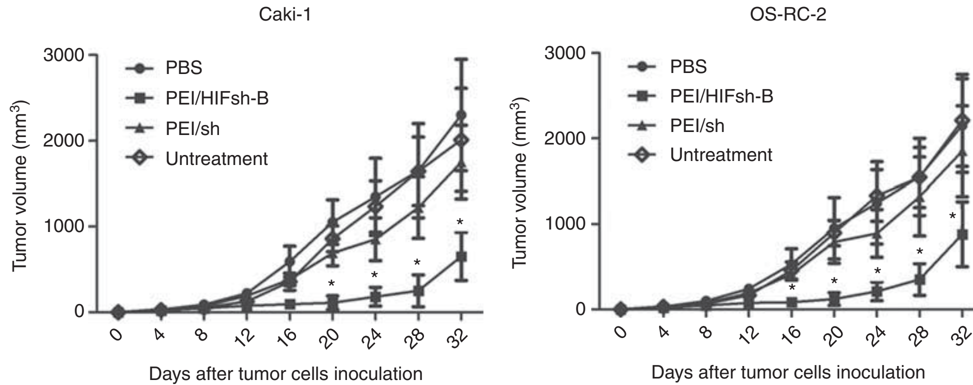

Figure 7 Effect of hypoxia-inducible factor-1 (HIF-1α) RNAi gene therapy on renal tumor growth in mice. Mice received s.c. injection of Caki-1 and OS-RC-2 cell lines. After 5 days, mice were divided into four groups ($n=10$ each group) and given intratumoral injections of polyethylenimine/sh, polyethylenimine/HIFsh-B or phosphate-buffered saline (PBS). Tumor size was measured at various time points.

regulates the HIF-1α expression, but also reduced HIF-1α downstream targets and mediators, including VEGF, Glut1, aldolase A and MMP-2 expression. Owning to the reduced expression of these proteins, HIF-1α shRNA-transfected RCC cells are motility and invasion impaired, and tumor growth was slower *in vitro*.

Apoptosis is important in regulation of tumor cells growth, metastasis and invasion.[25,26] Several studies reported that HIF-1α regulated cancer cell cycle and apoptosis.[16,17] Here, for the first time to the best of our knowledge, we show that depletion of HIF-1α by RNA-mediated interference promotes RCC cell lines apoptosis *in vitro* through caspase-dependent manner under hypoxic condition. Accumulated evidence indicates that regulation of proapoptotic and antiapoptotic factors has a central function in cell survival signal-mediated antiapoptotic mechanism. In RCC cell lines, we found that HIF-1α silencing downregulated the phosphorylation of Akt, which is a common mediator of cell survival signals, has been shown to inhibit apoptosis by attenuating activity of proapoptotic factors Bad and caspase-9, and enhance survival by increasing the phosphorylation of IKKβ, which led to activation of nuclear factor-κB.[31,32] In addition, previous study reported that Akt could inhibit apoptosis of tumor through negative regulating of FKHR, a member of the forkhead family of transcription factors, which could promote apoptosis by inducing the expression of Bim and FasL.[33] Further study showed that HIF-1α silencing appears to reduce Akt activation indirectly through inhibition of PDK1, which activates Akt. The mitochondria are central relaying station for caspase-dependent death pathways.[34] Here, we also found that HIF-1α silencing mediated the apoptosis in a caspase-dependent manner. Mitochondria respond to multiple death stimuli including those in which proapoptotic Bcl-2 family proteins such as Bak/Bax induce mitochondrial membrane permeabilization and cause the release of apoptotic molecules, such as cytochrome *c* and AIF. Upon release into the cytoplasm, cytochrome *c* activates Apaf-1 and triggers the caspase cascade, leading to cell death.[35–37] Thus, here, we suggested that HIF-1α silencing regulated the balance between proapoptotic protein Bax and antiapoptotic proteins Bcl-xL and Bim, inducing the dysfunction of mitochondria, which led to caspase-dependent death of RCC cell lines. The postulated mechanism involved in which depletion of HIF-1α promotes RCC cells to apoptosis is shown in Figure 8. Of course, although the changes of several proteins of apoptosis-related signaling were determined, the precise mechanism needs more extensive study.

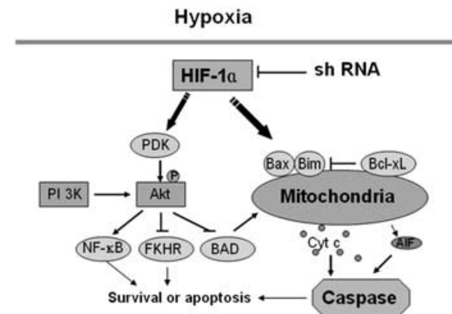

Figure 8 Schematic representation of the postulated mechanism involved in hypoxia-inducible factor-1 (HIF-1α) silencing inducing apoptosis of renal cell carcinoma (RCC) cell lines.

In the present study, the synthesis of GAPDH was reported as unaffected and, in the case of apoptosis, increased expression of some proteins and activities such as caspase-9 and BIM and BAX were observed upon treatment with the HIF-1α shRNA. However, the issue of nonspecific inhibition is still confounded because the increased proteins are in the apoptosis pathway, which is a response pathway for many cellular insults. To further verify whether the inhibition of many cellular properties that could be attributed to the effects of inhibited HIF-1α or could be attributed to nonspecific damaging effects (off target) effects of the shRNA sequences, we selected a control RCC cell line, 786-0, which did not express HIF-1α, and did not show decreased growth, migration and apoptosis when treated with the shRNA sequences *in vitro*. The results of western blot analysis showed that

expression of some proteins and activities such as caspase-9 and BIM and BAX was unaffected, suggesting that the expression of many cellular properties could be attributed to the effects of specific inhibition of HIF-1α (data not shown). However, change in some cellular properties might be resulted from shRNA sequences indirectly, and intermediated by other proteins directly regulated by HIF-1α.

Our *in vitro* and *in vivo* data represent the first report describing mechanistic evidence for HIF-1α as a mediator in growth, migration, invasion and survival of renal cancer, using Caki-1 and OS-RC-2 cell lines, and suggest that HIF-1α is a potential therapeutic target for renal tumor. PEI, a readily available synthetic polycation introduced for transfection a few years ago,[38] is an ideal candidate for gene delivery owing to its relatively high transfection efficiency and low toxicity. Thus, we elected to use PEI as the shRNA vector delivery system to exert gene therapy because the success with PEI has been reported.[29] Our data showed that administration of PEI/HIFshRNA into effectively suppressed tumor growth in mice loaded with two different renal cancer cell lines. Importantly, no adverse effects were observed in the mice treated with PEI/HIFshRNA intratumor injection. However, in the present study, systemic i.v. administration of PEI/HIFshRNA at the same dose only slightly suppressed renal tumor growth (data not shown). Furthermore, as PEIs with higher concentration tend to show toxicity in the lung,[39] it is limited to increase the injected dose of PEI/shRNA complex. Therefore, further studies are needed to find more effective and tissue-targeted shRNA delivery system for systemic application, and investigate the treatment effect of HIF-1α silencing on tumor in animals or humans.

In the present study, we merely demonstrated that HIF1 downregulation suppresses tumorigenicity of renal cancer cell lines. In the nude mice experiments demonstrated, we exclusively used permanent renal cancer cell lines. Thus, the effect of HIF-1α shRNA on the human renal cancer specimens or freshly isolated primary human renal cancer cells needs further investigation in the future experiments. In conclusion, we have shown that shRNA technology can be used to specifically inhibit HIF-1α expression in RCC cell lines. Silencing of HIF-1α suppressed tumorigenicity of RCC through induction of apoptosis, and decreased tumor growth *in vivo*. Therefore, targeting HIF-1α with RNAi may have therapeutic benefit for patients with renal tumor, even other solid tumor.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

RNAi, RNA interference; shRNA, short-hairpin RNA; RCC, renal cell carcinoma.

References

1 McLaughlin JK, Lipworth L. Epidemiologic aspects of renal cell cancer. *Semin Oncol* 2000; **27**: 115–123.
2 Murai M, Oya M. Renal cell carcinoma: etiology, incidence and epidemiology. *Curr Opin Urol* 2004; **14**: 229–233.
3 Yagoda A, Petrylak D, Thompson S. Cytotoxic chemotherapy for advanced renal cell carcinoma. *Urol Clin North Am* 1993; **20**: 303–321.
4 Motzer RJ, Bacik J, Murphy BA, Russo P, Mazumdar M. Interferon-α as a comparative treatment for clinical trials of new therapies against advanced renal cell carcinoma. *J Clin Oncol* 2002; **20**: 289–296.
5 Bukowski RM. Natural history and therapy of metastatic renal cell carcinoma: the role of interleukin-2. *Cancer* 1997; **80**: 1198–1220.
6 Yagoda A. Phase II cytotoxic chemotherapy trials in renal cell carcinoma: 1983–1988. *Prog Clin Biol Res* 1990; **350**: 227–241.
7 Ke QD, Max C. Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol* 2006; **70**: 1469–1480.
8 Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003; **3**: 721–732.
9 Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ *et al*. The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. *Am J Pathol* 2000; **157**: 411–421.
10 Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D *et al*. Overexpression of hypoxia-inducible factor 1α in common human cancers and their metastases. *Cancer Res* 1999; **59**: 5830–5835.
11 Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ *et al*. The expression and distribution of the hypoxia-inducible factors HIF-1α and HIF-2α in normal human tissues, cancers, and tumor-associated macrophages. *Am J Pathol* 2000; **157**: 411–421.
12 Beasley NJ, Leek R, Alam M, Turley H, Cox GJ, Gatter K *et al*. Hypoxia-inducible factors HIF-1α and HIF-2α in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients. *Cancer Res* 2002; **62**: 2493–2497.
13 Volm M, Koomagi R. Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer. *Anticancer Res* 2002; **20**: 1527–1533.
14 Harris AL. Hypoxia-α key regulatory factor in tumour growth. *Nat Rev Cancer* 2002; **2**: 38–47.
15 Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med* 2003; **9**: 677–684.
16 Thomas R, Kim MH. HIF-1 alpha: a key survival factor for serum-deprived prostate cancer cells. *Prostate* 2008; **68**: 1405–1415.
17 Wang Y, Pakunlu RI, Tsao W, Pozharov V, Minko T. Bimodal effect of hypoxia in cancer: role of hypoxia inducible factor in apoptosis. *Mol Pharmacol* 2004; **1**: 156–165.
18 Sun X, Kanwar JR, Leung E, Lehnert K, Wang D, Krissansen GW. Gene transfer of antisense hypoxia inducible factor-1 alpha enhances the therapeutic efficacy of cancer immunotherapy. *Gene Therapy* 2001; **8**: 638–645.
19 Maemura K, Hsieh CM, Jain MK, Fukumoto S, Layne MD, Liu Y *et al*. Generation of a dominant-negative mutant of endothelial PAS domain protein 1 by deletion of a potent C-terminal transactivation domain. *J Biol Chem* 1999; **274**: 31565–31570.

20 Mizuno T, Nagao M, Yamada Y, Narikiyo M, Ueno M, Miyagishi M *et al*. Small interfering RNA expression vector targeting hypoxia-inducible factor 1 alpha inhibits tumor growth in hepatobiliary and pancreatic cancers. *Cancer Gene Ther* 2006; **13**: 131–140.
21 Wiesener MS, Munchenhagen PM, Berger I, Morgan NV, Roigas J, Schwiertz A *et al*. Constitutive activation of hypoxia-inducible genes related to overexpression of hypoxia-inducible factor-1a in clear cell renal carcinoma. *Cancer Res* 2001; **61**: 5215–5222.
22 Klatte T, Seligson DB, Riggs SB, Leppert JT, Berkman MK, Kleid MD *et al*. Hypoxia-inducible factor 1 alpha in clear cell renal cell carcinoma. *Clin Cancer Res* 2007; **13**: 7388–7393.
23 Lidgren A, Hedberg Y, Grankvist K, Rasmuson T, Vasko J, Ljungberg B. The expression of hypoxia-inducible factor 1alpha is a favorable independent prognostic factor in renal cell carcinoma. *Clin Cancer Res* 2005; **11**: 1129–1135.
24 Yao M, Yoshida M, Kishida T, Nakaigawa N, Baba M, Kobayashi K *et al*. VHL tumor suppressor gene alterations associated with good prognosis in sporadic clear-cell renal carcinoma. *J Natl Cancer Inst* 2002; **20**: 1569–1575.
25 Mehlen P, Puisieux A. Metastasis: a question of life or death. *Nat Rev Cancer* 2006; **6**: 449–458.
26 Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer* 2002; **2**: 277–288.
27 Wang J, Yang J, Kuropatwinski K, Wang W, Liu XQ, Hauser J *et al*. Transforming growth factor beta induces apoptosis through repressing the phosphoinositide 3-kinase/AKT/survivin pathway in colon cancer cells. *Cancer Res* 2008; **68**: 3152–3160.
28 Fan KX, Dai JX, Wang H, Wei HF, Cao ZG, Hou S *et al*. Treatment of collagen-induced arthritis with an anti-osteopontin monoclonal antibody through promotion of apoptosis of both murine and human activated T cells. *Arthritis Rheum* 2008; **58**: 2041–2052.
29 Jia SF, Worth LL, Densmore CL, Xu B, Zhou Z, Kleinerman ES. Eradication of osteosarcoma lung metastases following intranasal interleukin-12 gene therapy using a nonviral polyethylenimine vector. *Cancer Gene Ther* 2002; **9**: 260–266.
30 Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003; **3**: 721–732.
31 Qiao J, Kang J, Ishola TA, Rychahou PG, Evers BM, Chung DH. Gastrin-releasing peptide receptor silencing suppresses the tumorigenesis and metastatic potential of neuroblastoma. *Proc Natl Acad Sci USA* 2008; **105**: 12891–12896.
32 Jeong SJ, Pise-Masison CA, Radonovich MF, Park HU, Brady JN. Activated AKT regulates NF-kappaB activation, p53 inhibition and cell survival in HTLV-1-transformed cells. *Oncogene* 2005; **24**: 6719–6728.
33 Tang ED, Nuñez G, Barr FG, Guan KL. Negative regulation of the forkhead transcription factor FKHR by Akt. *J Biol Chem* 1999; **274**: 16741–16746.
34 Tsujimoto Y. Cell death regulation by the Bcl-2 protein family in the mitochondria. *J Cell Physiol* 2003; **195**: 158–167.
35 Gross A, Jockel J, Wei MC, Korsmeyer SJ. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *EMBO J* 1998; **17**: 3878–3885.
36 Ruffolo SC, Breckenridge DG, Nguyen M, Goping IS, Gross A, Korsmeyer SJ *et al*. BID-dependent and BID-independent pathways for BAX insertion into mitochondria. *Cell Death Differ* 2000; **7**: 1101–1108.
37 Weiss JN, Korge P, Honda HM, Ping P. Role of the mitochondrial permeability transition in myocardial disease. *Circ Res* 2003; **93**: 292–301.
38 Boussif O, Lezoualc'h F, Zanta A, Mergny MD, Scherman D, Demeneix B *et al*. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc Natl Acad Sci USA* 1995; **92**: 7297–7301.
39 Urban-Klein B, Werth S, Abuharbeid S, Czubayko F, Aigner A. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA *in vivo*. *Gene Therapy* 2005; **12**: 461–466.