

# RNAa and Vector-Mediated Overexpression of DIRAS1 Suppresses Tumor Growth and Migration in Renal Cell Carcinoma

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**The downregulation of DIRAS1 has been suggested to potentially contribute to tumor development and progression in several human cancers. However, the role of DIRAS1 in renal cell carcinoma (RCC) remains elusive. In this study, we examined the DIRAS1 expression level in RCC cell lines and tissues. Both RNA activation (RNAa) and vector transfection methods were used to upregulate the expression of DIRAS1 in RCC cells. Expression analysis revealed that DIRAS1 was significantly downregulated in RCC cell lines and tissues compared with nontumorigenic renal cells and adjacent nontumor tissues individually. Promoter methylation analysis indicated that the reduced DIRAS1 expression might be partly mediated by epigenetic modulation. The RNAa-mediated overexpression of DIRAS1 inhibited cell proliferation and tumorigenicity *in vitro* and *in vivo*. The re-activation of DIRAS1 also promoted apoptosis and suppressed migration and invasion in RCC cells. The ectopic expression of DIRAS1 via an expression vector recapitulated the RNAa results. These results reveal that DIRAS1, functioning as a putative tumor suppressor in RCC cells, could potentially be a therapeutic target and RNAa could be a therapeutic strategy for RCC.**

## INTRODUCTION

Renal cell carcinoma (RCC) is the most common type of kidney cancers in adults, accounting for approximately 3% of all adult malignancy cases worldwide. The incidence and mortality rates of RCC have increased considerably in recent years, with more than 62,000 new cases estimated in 2016 in the United States.<sup>1</sup> It is estimated that approximately 25% of patients with RCC have metastases at the time of initial diagnosis and another 25% have locally advanced disease.<sup>2</sup> Considering the resistance of RCC to chemotherapy, hormone therapy, and radiation therapy,<sup>3,4</sup> it is of fundamental importance to explore novel therapeutic targets and more effective treatment options for this fatal disease.

DIRAS1 is located in chromosome 19p13.3 and consists of 2 exons that encode a protein of 198 amino acids.<sup>5</sup> DIRAS1 belongs to the small guanosine triphosphatase (GTPase) Ras superfamily.<sup>6</sup> Unlike the oncogenic properties more common to small GTPases such as K-Ras and RhoA,<sup>7,8</sup> DIRAS1 is frequently downregulated in human cancers due to DNA copy number loss, loss of heterozygosity

(LOH), and hypermethylation of the promoter.<sup>5,6,9</sup> The restoration of DIRAS1 has been reported to suppress tumor growth and metastasis in human neural tumors,<sup>6</sup> esophageal cancer,<sup>5</sup> colorectal cancer,<sup>9</sup> and ovarian cancer.<sup>10</sup> To our knowledge, the expression pattern and biological function of DIRAS1 in RCC remain unclear.

It has been proposed that double-stranded RNA (dsRNA) can activate endogenous gene expression by targeting promoter sequences in a process termed RNA activation (RNAa).<sup>11–13</sup> The RNAa technique has been widely used to upregulate tumor suppressor genes, including p21,<sup>11</sup> NKX3-1,<sup>14</sup> PAWR,<sup>15</sup> and E-cadherin,<sup>16</sup> in various human cancers. RNAa is a promising discovery and represents a novel approach to gene overexpression, in addition to traditional vector-based systems. In this study, we used both RNAa and vector-mediated overexpression to investigate the molecular function of DIRAS1 in RCC.

## RESULTS

### DIRAS1 Is Downregulated in RCC Cell Lines and Tissue Samples

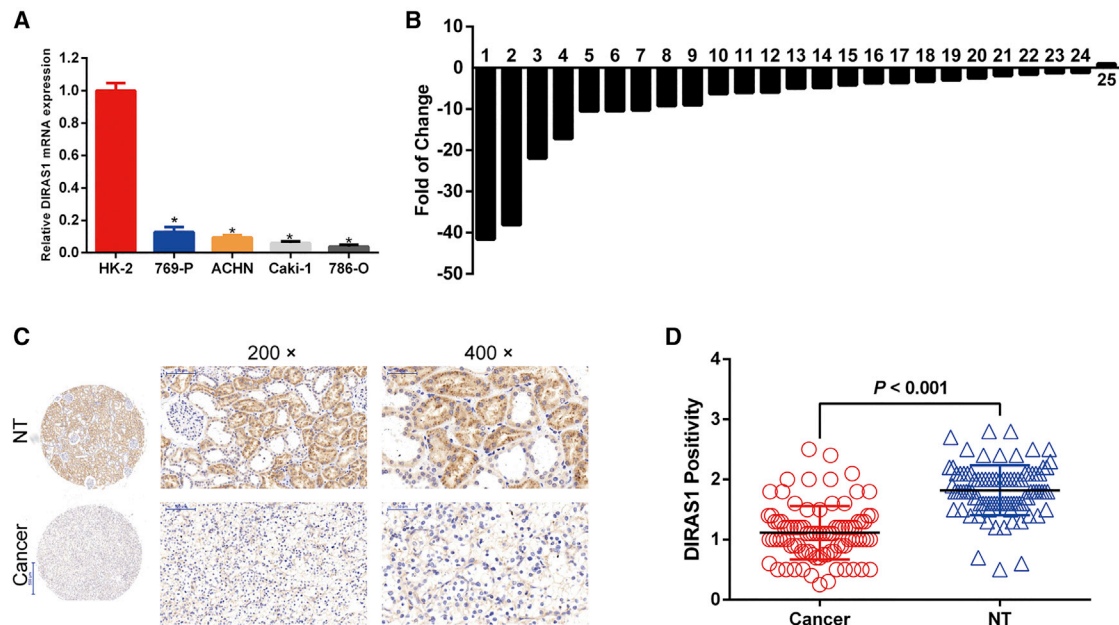
We evaluated DIRAS1 expression level in a panel of five renal cell lines: four cancerous (769-P, ACHN, Caki-1, and 786-O) cell lines and one nontumorigenic or benign (HK-2) cell line. Cellular mRNA expression, as measured by quantitative real-time PCR, revealed that DIRAS1 transcript levels in all four cancerous cell lines were lower than those in the nontumorigenic or benign control cells. DIRAS1 mRNA expression was significantly downregulated by 80% or more in each RCC cell line compared with the expression level in HK-2 cells (Figure 1A). To validate the expression pattern of DIRAS1 in RCC, we quantified the mRNA expression level of DIRAS1 in 25 pairs of human RCC tissues and adjacent nontumorous

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**Figure 1. DIRAS1 Expression Was Downregulated in RCC Cell Lines and Tissue Samples**

(A) Results of quantitative real-time PCR revealed that DIRAS1 transcript levels in all four cancerous cell lines were lower than those in HK-2 cells. Error bars represent the SD from three independent experiments. (B) Transcript level of DIRAS1 was generally lower in tumor tissues than in the adjacent nontumor tissues (24 of 25 presented a downregulated pattern). (C) Typical IHC images of the TMA. DIRAS1 localized to the membrane and cytoplasm. (D) Statistical analysis showed that the expression level of DIRAS1 protein was aberrantly lower in RCC tissues than in adjacent nontumor tissues. Error bars represent the SD from 90 pairs of RCC and nontumor adjacent tissues. \* $p < 0.05$ .

tissues by quantitative real-time PCR. The results indicated that the transcript level of DIRAS1 was generally lower in tumor tissues than that in the matched nontumor tissues (24 of 25 presented with a downregulated pattern) (Figure 1B).

We then investigated the expression pattern and subcellular localization of the DIRAS1 protein in a tissue microarray (TMA) containing 90 pairs of RCC and nontumor adjacent tissues using immunohistochemical (IHC) analysis. DIRAS1 was located in the membrane and cytoplasm (Figure 1C). Statistical analysis indicated that the protein expression level of DIRAS1 in RCC tissues was significantly lower than that in adjacent nontumor tissues (Figure 1D,  $p < 0.001$ ).

#### Downregulation of DIRAS1 in RCC Cells Is Associated with Promoter Hypermethylation

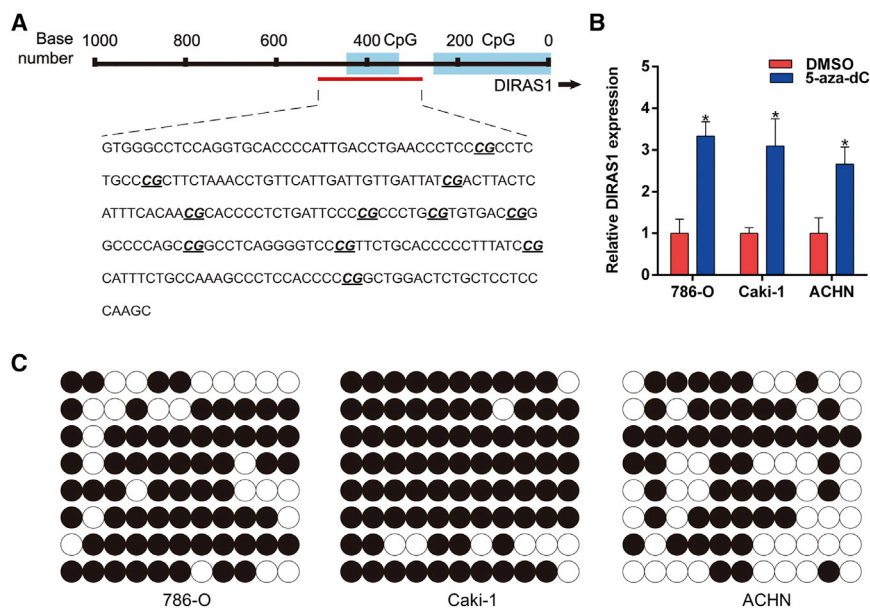
We used a CpG island searcher program (<http://www.urogene.org/methprimer/>) to identify the CpG islands in the 1,000 bp region upstream of DIRAS1 (Figure 2A). The expression of DIRAS1 in ACHN, 786-O, and Caki-1 cells was significantly increased after treatment with 5-Aza-CdR, a DNA methyltransferase inhibitor (Figure 2B). We then used bisulfite sequencing PCR (BSP) to identify the methylation status of the predicted CpG islands. The results revealed that the CpG islands of the DIRAS1 promoter region were aberrantly hypermethylated in ACHN, 786-O, and Caki-1 cells (Figure 2C).

#### RNAa-Based Overexpression of DIRAS1 in RCC Cells

To explore the biological function of DIRAS1 in RCC cells, we introduced the RNAa technique to activate endogenous DIRAS1 expression. Three candidate dsRNAs (dsDIRAS1-473, dsDIRAS1-755, and dsDIRAS1-824) were designed according to rules derived from previous studies.<sup>11,12</sup> These dsRNAs targeted the DIRAS1 promoter at sites ranging from -824 to -473 relative to the transcription start site (Figure 3A). DIRAS1 expression was evaluated by quantitative real-time PCR 3 days after the dsRNAs were transfected into RCC cells. The results suggested that compared with the controls, dsDIRAS1-755 induced DIRAS1 expression by approximately 3.0-fold, whereas dsKLF4-473 and dsKLF4-824 did not significantly alter DIRAS1 expression levels (Figure 3B). Consistent with mRNA induction, western blot analysis revealed that the DIRAS1 protein expression level was upregulated by dsDIRAS1-755 in 786-O and Caki-1 cells (Figure 3C).

#### DIRAS1 Inhibits the Proliferation and Tumorigenicity of RCC Cells *In Vitro* and *In Vivo*

We used the Cell Counting Kit-8 (CCK-8) assay to quantitatively analyze the impact of DIRAS1 on the growth of RCC cells. The results indicated that 786-O and Caki-1 cell viability was steadily reduced following dsDIRAS1-755 treatment compared with that of the negative control (NC) group. Overexpression of DIRAS1 suppressed the proliferation of cultured RCC cells at varied concentrations and time points (Figures 4A and 4B). Clonogenicity assays revealed that



**Figure 2. Promoter Hypermethylation of DIRAS1 Was Validated in RCC Cells**

(A) Regions analyzed by BSP are indicated. (B) Quantitative real-time PCR indicated that 5-Aza-dc treatment for 96 hr increased the expression of DIRAS1 in RCC cells. (C) CpG islands of the DIRAS1 promoter region were aberrantly hypermethylated in ACHN, 786-O, and Caki-1 cells. White circles represent nonmethylated and black circles represent methylated CpG sites. Rows represent individual clones, columns represent individual CpG sites within the DIRAS1 promoter region. Error bars represent the SD from three independent experiments. \* $p < 0.05$ .

the quantity of developed cell colonies was significantly reduced after dsDIRAS1-755 transfection in 786-O and Caki-1 cells (Figures 4C and 4D).

To confirm the preceding findings, nude mice ( $n = 8$ ) were implanted with Caki-1 cells ( $1 \times 10^6$ /mouse) by subcutaneous injection. Treatment (dsDIRAS1-755 or NC) was started 7 days after injection when palpable tumors were detected. The tumor volume and body weight were examined to evaluate the effects of the treatment. The results showed that the tumor volume was significantly lower in xenografts treated with dsDIRAS1-755 than in those treated with NC (Figure 4E). Four weeks later, the tumor xenografts were dissected and weighed (Figure 4F), and the statistical analysis indicated that a significant reduction in tumor weight was induced by the dsDIRAS1-755 treatment (Figure 4G). Altogether, these results indicated that DIRAS1 negatively modulates the growth of RCC cells.

#### DIRAS1 Promotes Apoptosis in RCC Cells

The potential role of DIRAS1 in apoptosis was studied after the transfection of dsDIRAS1-755 or NC in 786-O and Caki-1 cells. The results indicated that both early apoptotic (annexin V positive and phosphatidylinositol [PI] negative) and late apoptotic (annexin V positive and PI positive) indexes were significantly higher in the dsDIRAS1-755 group than in the NC group (Figures 5A and 5B). Considering that apoptosis is often mediated by the activation of caspase-3 and subsequent poly ADP-ribose polymerase (PARP) proteolysis, western blot analysis was then performed to detect the degree of caspase activation. The result suggested that the cleavage of caspase-3 and PARP was greater in dsDIRAS1-755-transfected cells compared with that in control cells (Figure 5C). It has been well documented that the MAPK-ERK1/2 pathway participates in the regulation of apoptosis; thus, we hypothesized that the upregulation of DIRAS1 might inhibit

ERK1/2 pathway signaling. As shown in Figure 4C, the phosphorylation level of ERK1/2 was substantially lower in the dsDIRAS1-755 group compared with that in the NC group.

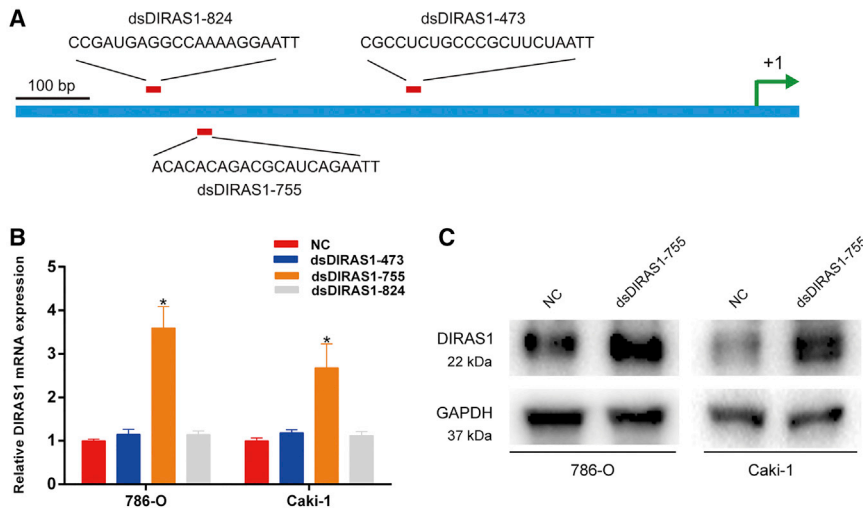
#### DIRAS1 Inhibits Cell Migration and Invasion of RCC Cells

To investigate whether endogenous DIRAS1 inhibits cancer cell migration and invasion, 786-O and Caki-1 cells were transfected with dsDIRAS1-755 or NC. The results of transwell assays indicated that overexpression of DIRAS1 by dsDIRAS1-755 inhibited cell migration and invasion abilities; a more than 50% reduction in the number of migratory and invading cells was detected in the DIRAS1-overexpressing cell population compared with the NC group (Figures 6A and 6B).

We used the RNAi technique to knock down DIRAS1 expression. Caki-1 cells were treated with small interfering RNAs (siRNAs) targeting DIRAS1 (siDIRAS1-1 and siDIRAS1-2) or NC. The results showed that compared with NC-transfected cells, transfection with siDIRAS1-1 or siDIRAS1-2 decreased DIRAS1 expression at both the mRNA and the protein expression levels in Caki-1 cells (Figures 6C and 6D). Transwell assays showed that the knockdown of DIRAS1 expression significantly restored cell migration and invasion abilities (Figure 6E).

#### Vector-Based Overexpression of DIRAS1 Recapitulates RNAi Results

To confirm the results revealed by RNAa-mediated activation of DIRAS1, we introduced a DIRAS1 expression vector (pDIRAS1) to upregulate DIRAS1 expression in 786-O and Caki-1 cells. The results of western blotting suggested that compared with that of the control (pNull), pDIRAS1 dramatically increased DIRAS1 protein expression (Figure 7A). Functional studies showed that DIRAS1 vector transduction suppressed cell viability (Figure 7B), promoted apoptosis (Figure 7C), and inhibited cell migration and invasion (Figure 7D) compared with these parameters in pNull cells, which recapitulated the results obtained by RNAa-mediated DIRAS1 induction. These data support that DIRAS1 functions as a putative tumor suppressor in RCC cells.



**Figure 3. DIRAS1 Expression Was Upregulated by RNAi in RCC Cell Lines**

(A) Schematic representation of the DIRAS1 promoter. The location of the dsRNA target site relative to the transcription start site is indicated. (B) Results of quantitative real-time PCR revealed that compared with DIRAS1 expression in NC cells, dsDIRAS1-755, but not dsKLF4-473 or dsKLF4-824, significantly upregulated DIRAS1 expression. (C) Western blot analysis revealed that the DIRAS1 protein level was upregulated by dsDIRAS1-755 in 786-O and Caki-1 cells. Error bars represent the SD from three independent experiments. \* $p < 0.05$ .

## DISCUSSION

To our knowledge, the expression pattern and biological role of DIRAS1 in RCC have not been established. In this study, we have revealed that both mRNA and protein expression levels of DIRAS1 are downregulated in RCC tumor tissues and cell lines. The magnitude of downregulation in RCC cell lines is greater than that seen in primary RCCs; this is probably because HK-2 does not always accurately represent normal human renal proximal tubular cells' physiology.<sup>17</sup> The restoration of DIRAS1 expression by RNAi or vector-mediated techniques suppresses RCC cell viability, colony formation, migration, and invasion, which indicates that DIRAS1 functions as a tumor suppressor in RCC cells. Promoter hypermethylation might contribute to the inactivation of DIRAS1 during the tumorigenesis of RCC.

The DIRAS family consists of DIRAS1, DIRAS2, and DIRAS3 (ARHI), sharing a highly conserved G domain and a carboxyl-terminal CAAX motif.<sup>5</sup> DIRAS1 has been suggested as a tumor suppressor in several human cancers, including colorectal cancer,<sup>9</sup> esophageal squamous cell carcinoma,<sup>5</sup> and glioma.<sup>6</sup> The mechanism underlying these tumor-suppressive activities might contribute to its ability to bind to SmgGDS, a protein that induces the activation of several oncogenic GTPases.<sup>18</sup> The ERK1/2, p38 mitogen-activated protein kinase (MAPK), and nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathways can be inhibited by the restoration of DIRAS1 expression.<sup>5,18</sup> Studies have indicated that downregulation of DIRAS1 is attributed to DNA copy number loss, LOH, and hypermethylation.<sup>5,9</sup> The expression of DIRAS1 also has important prognostic value. DIRAS1 downregulation has been associated with metastasis and poor prognosis in patients with colorectal cancer and esophageal squamous cell carcinoma.<sup>5,9</sup> In the present study, we have shown that DIRAS1 is downregulated in RCC cells and upregulation of DIRAS1 inhibits the tumor growth and metastasis, supporting the tumor suppressive role of DIRAS1 in human cancers.

The use of constructed plasmid vectors is a conventional approach to upregulate the expression of a tumor suppressor gene and has been widely used to explore the molecular function of a targeted gene in human cancers. However, an exogenous vector is not an exact mimic of the natural gene.<sup>19,20</sup> A plasmid vector rarely contains introns or UTR elements, which may have vital effects on the biological function of the endogenous gene.<sup>19</sup> RNAi is triggered by small RNAs that target gene promoters and induce the transcription of mRNA, exerting the opposite effect of RNAi. Previous studies have validated that RNAi is able to induce robust and prolonged expression of tumor suppressor genes and thus leads to antigrowth effects *in vitro* and *in vivo* in various cancer cells.<sup>11,12,15</sup> Intravesical or rectal delivery of small RNAs preferentially accumulated at the tumor site and demonstrated excellent antitumor activity,<sup>21,22</sup> which provided a preclinical proof of concept for novel cancer therapy. Consistent with this result, we observed that small RNAs targeting the DIRAS1 promoter upregulated the expression of DIRAS1 and inhibited the growth and metastasis of RCC cells. The findings from the experiments with DIRAS1 RNAi are consistent with those induced by vector-mediated DIRAS1 overexpression, indicating that RNAi promoted the expression of a functional DIRAS1 protein.

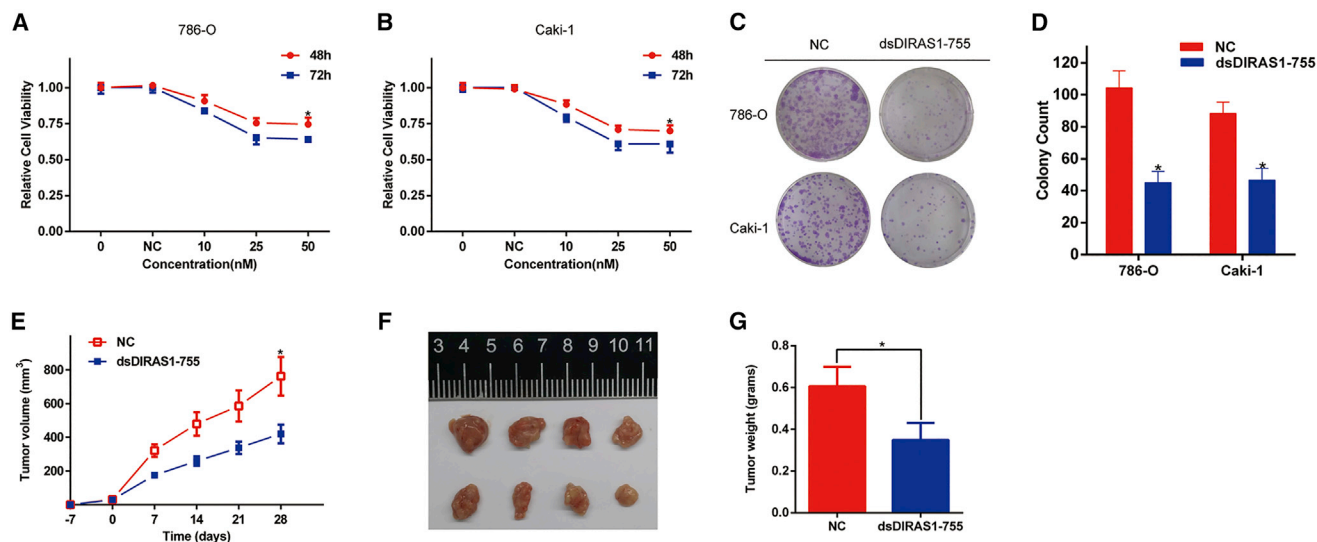
In conclusion, DIRAS1 is significantly downregulated at both the mRNA and the protein expression levels in RCC cell lines and tissues. RNAi and vector-mediated overexpression techniques both identified DIRAS1 as an inhibitor of tumor cell growth and migration in RCC. The downregulation of DIRAS1 in RCC cells was associated with promoter hypermethylation. This study reveals that DIRAS1, functioning as a putative tumor suppressor in RCC, could potentially be a therapeutic target and RNAi could be a therapeutic strategy for RCC.

## MATERIALS AND METHODS

### Reagents and Transfection

One kilobase of the human DIRAS1 promoter was scanned for dsRNA target sites according to the previously reported rational design rules.<sup>11,13</sup> The siRNAs targeting human DIRAS1 mRNA (named siDIRAS1-1 and siDIRAS1-2) were also designed. All dsRNA, siRNA, and NC sequences are listed in Table S1. These





**Figure 4. DIRAS1 Overexpression Mediated by RNAi Inhibited RCC Cell Viability and Tumorigenicity *In Vitro* and *In Vivo***

(A) CCK-8 assay revealed that the relative cell viability in dsDIRAS1-755-treated 786-O cells was lower than that in NC-treated 786-O cells. (B) The relative cell viability in dsDIRAS1-755-treated Caki-1 cells was lower than that in NC-treated Caki-1 cells. (C) Representative wells from the colony formation assay are presented. (D) Colony formation rate was reduced in dsDIRAS1-755-transfected groups compared with that in NC-transfected groups. Error bars represent the SD from three independent experiments. (E) Tumor xenograft model. The growth curve and tumor weight indicated that tumors in the dsDIRAS1-755-transfected group had a significant slower growth pattern than those in the NC-transfected group. (F) Tumor xenografts were dissected. (G) Statistical analysis indicated a significant reduction in tumor weight. Error bars represent the SD from four nude mice. \* $p < 0.05$ .

RNA duplexes were chemically synthesized by GenePharma, Shanghai, China. The DIRAS1 plasmid was constructed by inserting the human DIRAS1 complementary DNA lacking the 3' UTR into pIRES2-EGFP (Clontech, USA) (the overexpression clone of DIRAS1 would be termed pDIRAS1). Oligonucleotide and vector transfection was performed with Lipofectamine 2000 reagent (Invitrogen, USA) following the manufacturer's protocol.

#### Cell Lines and Cell Culture

The human RCC cell lines ACHN, 769-P, 786-O, and Caki-1 were obtained from the Shanghai Institute of Cell Biology, Shanghai, China. Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) under a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

#### Human Clinical Samples

Twenty-five paired RCC tissues and matched nontumor tissues were obtained from patients with RCC who underwent radical nephrectomy at the First Affiliated Hospital of Medical College, Zhejiang University. The studied samples were collected between January 2013 and October 2013 after obtaining patient informed consent and ethical approval from the Ethics Committee of Zhejiang University. Clinicopathological characteristics of the patients are presented in Table S2.

#### RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated using TRIzol reagent (TaKaRa, China) and then transcribed into cDNA with the PrimeScript RT Reagent Kit (TaKaRa, China). An ABI 7500 FAST Real-Time PCR system

(Applied Biosystems, USA) was used for quantitative real-time PCR with a SYBR Green PCR Kit (Takara, China). Relative quantification of mRNA expression was based on the  $2^{-\Delta\Delta C_t}$  method after normalization to the endogenous reference GAPDH. The primers are shown in Table S1.

#### Cell Growth and Cell Viability Assay

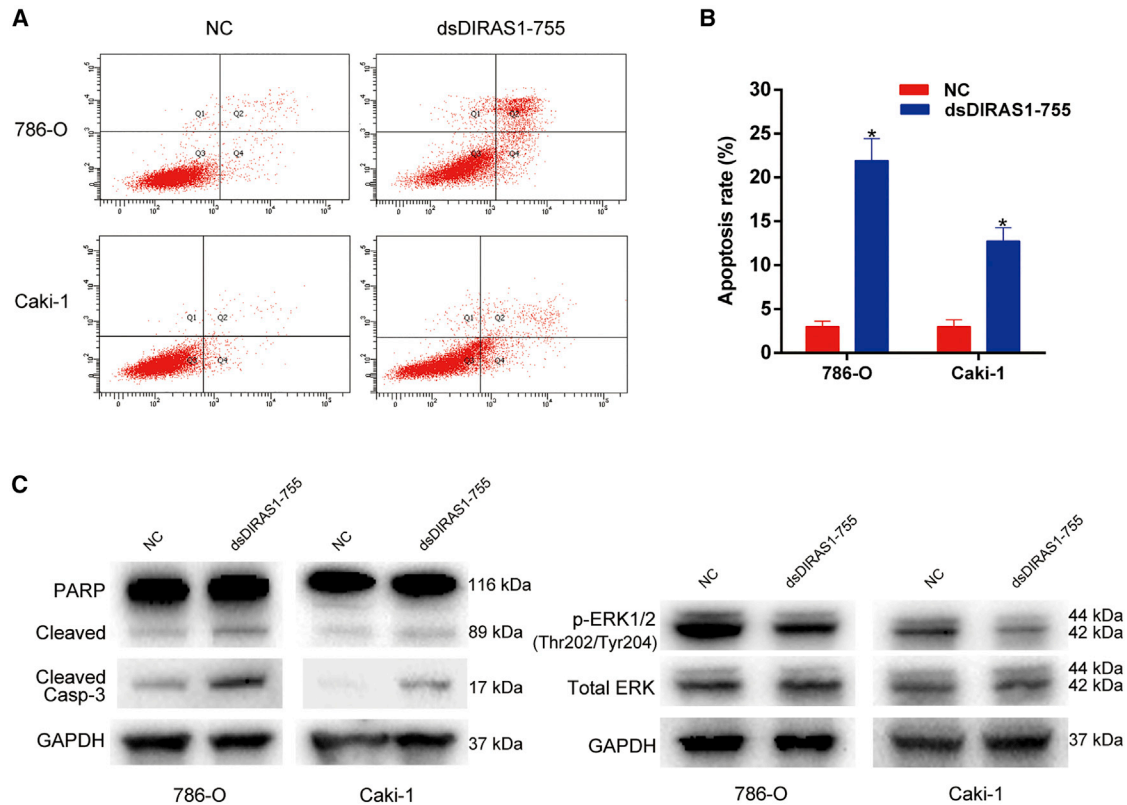
Caki-1 or 786-O cells were plated in 96-well plates at 3,000 cells per well and incubated overnight. The cells were treated with RNA duplex at concentrations ranging from 10 to 50 nM for 2–3 days. After CCK-8 (Dojindo Laboratories, Japan) reagent was added to each well, the 96-well plate was incubated at 37°C for 30 min and the absorbance at 450 nm was determined spectrophotometrically using a MRX II absorbance reader (Dynerx Technologies, USA).

#### *In Vitro* Colony Formation Assay

After transfection with RNA duplex (50 nM) for 24 hr, 786-O or Caki-1 cells were trypsinized to a single-cell suspension and seeded into 6-well plates at 500 cells per well. The 6-well plates were maintained under standard culture conditions for 2 weeks. The colonies were fixed using ice-cold methanol and then stained with 0.1% crystal violet solution to identify the colony number.

#### *In Vivo* Tumorigenicity Assays

Male BALB/c-nude mice (4 weeks old) were purchased from the Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai, China. Caki-1 cells ( $1 \times 10^6$  cells in 100  $\mu$ L of PBS) were injected subcutaneously into mice. When palpable tumors



**Figure 5. DIRAS1 Overexpression by RNAa Promoted Apoptosis in RCC Cells**

(A) Representative images of apoptosis assay are presented. (B) Statistical analysis showed that the apoptotic index was significantly increased in the dsDIRAS1-755 group compared with that in the NC group. (C) Western blot analysis revealed that cleavage of caspase-3 and PARP was increased while the phosphorylation level of ERK1/2 was reduced in dsDIRAS1-755-transfected cells compared with those in control cells. \* $p < 0.05$ .

were detected, mice were randomly divided into two groups and injected intratumorally with 30  $\mu\text{g}$  of dsRNA or NC encapsulated by Lipofectamine 2000 (30  $\mu\text{L}$ ) every 3 days for 4 weeks. Tumor size was monitored by caliper measurements of the two largest perpendicular diameters and then calculated with the formula  $V = (\text{width}^2 \times \text{length} \times 0.52)$ . Animal studies were performed according to institutional guidelines and were approved by the Animal Studies Committee at the Medical College of Zhejiang University.

#### Cell Apoptosis Analysis via Flow Cytometry

Caki-1 or 786-O cells were harvested and washed with ice-cold PBS after treatment with RNA duplex (50 nM) for 72 hr. Cells were then resuspended in mixed binding buffer (containing annexin V-fluorescein isothiocyanate (FITC) and PI) for 15 min in accordance with the manufacturer's instructions. Analysis was performed with the BD LSRII flow cytometer system with FACSDiva Software (BD Biosciences, USA) to quantify the percentage of apoptotic cells.

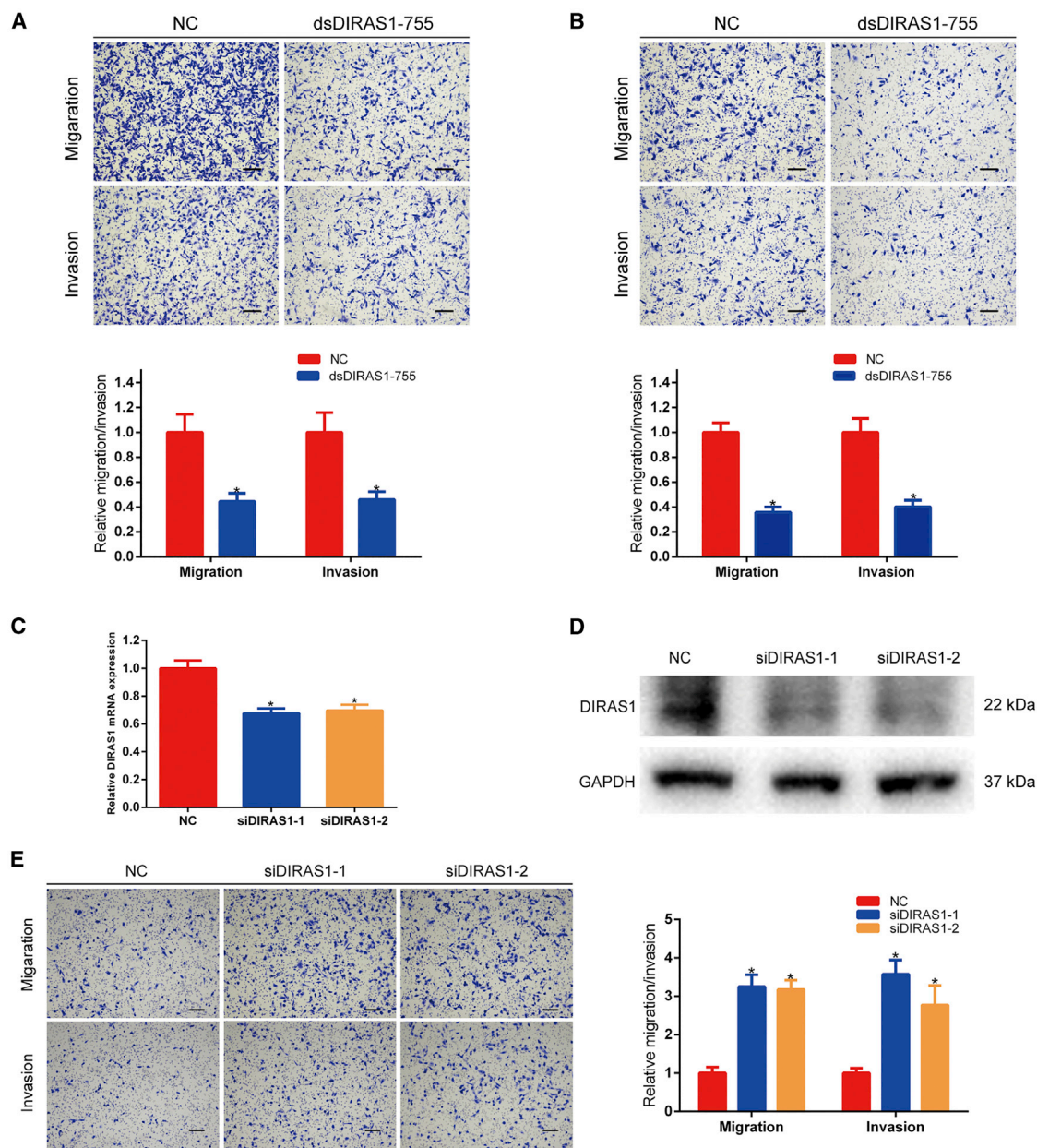
#### Cell Migration and Invasion Assay

The cell migration and invasion assay was carried out in transwell chambers as previously reported.<sup>23</sup> The invasion assay used

inserts coated with Matrigel (BD Biosciences, USA), while the migration assay used non-coated inserts. After transfection with RNA duplex (50 nM) for 24 hr,  $4 \times 10^4$  cells (except for the RNA interference study, in which  $1 \times 10^4$  cells were used) suspended in 0.2 mL of serum-free medium were added to the inserts. The lower compartment was filled with 0.6 mL of RPMI 1640 medium containing 10% FBS as a chemoattractant. After incubation for 24 hr, the cells on the lower surface of the membrane were fixed with ice-cold methanol and then stained with 0.1% crystal violet. Five visual fields at 200 $\times$  magnification were selected randomly for each insert and counted under a light microscope (Olympus, Japan).

#### DNA Methylation Analysis and 5-Aza-CdR Treatment

BSP was carried out as previously described.<sup>24</sup> After bisulfite conversion, the CpG islands of DIRAS1 were amplified by PCR with the primers 5'-GTGGGTTTTAGGTGTATTTATTG-3' (forward) and 5'-ACTTAAAAAACAATCCAACC-3' (reverse). Then, the PCR products were cloned into the pUC18 T-vector and underwent bacterial amplification. Eight randomly selected clones were subjected to DNA sequencing (Sangon Biotech, China). Caki-1, 786-O, and ACHN cells were treated with 10  $\mu\text{M}$  5-Aza-CdR (Sigma,



**Figure 6. DIRAS1 Overexpression by RNAa Inhibited Cell Migration and Invasion in RCC Cells**

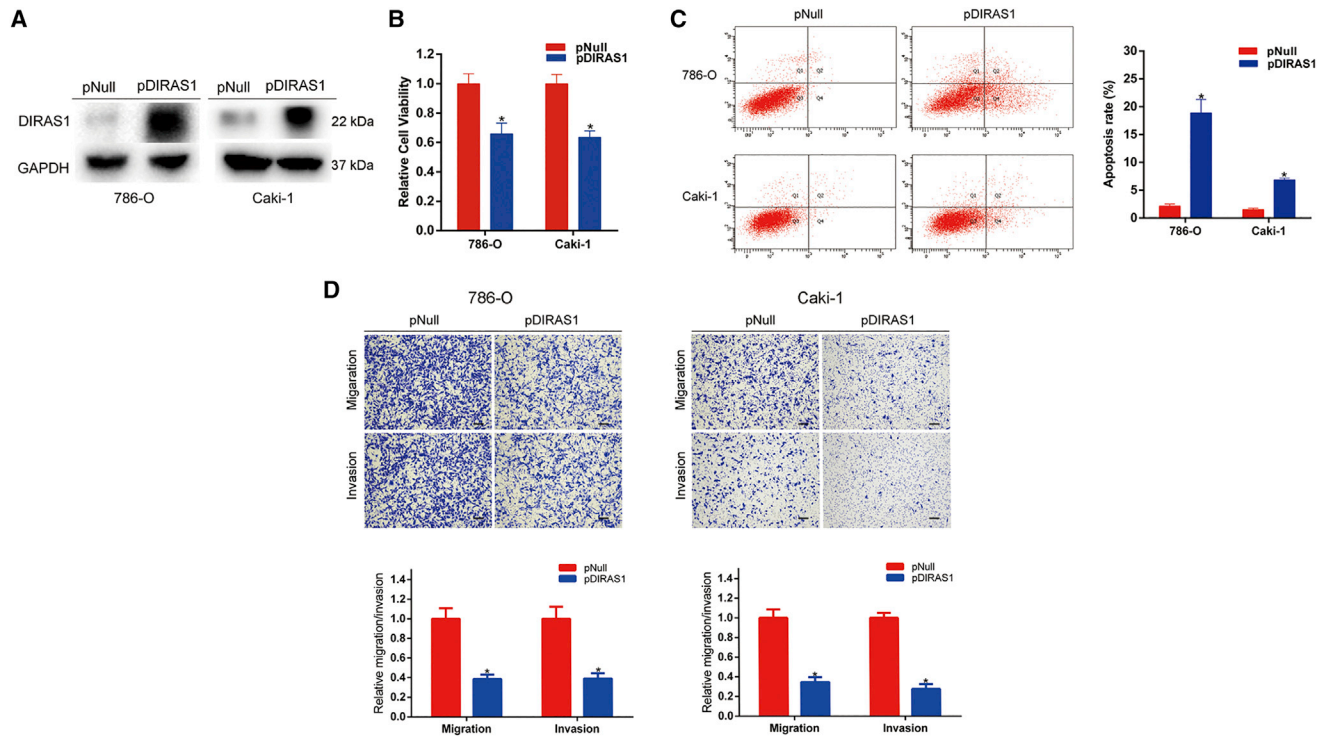
(A and B) Representative micrographs of transwell assays are presented. DIRAS1 overexpression by RNAa impaired the motility of 786-O (A) and Caki-1 (B) cells. (C and D) Compared with NC, transfection of siDIRAS1-1 or siDIRAS1-2 in Caki-1 cells dramatically decreased DIRAS1 expression at both mRNA (C) and protein (D) expression levels. (E) Knockdown of DIRAS1 expression promoted the motility of Caki-1 cells. Error bars represent the SD from three independent experiments. \* $p < 0.05$ .

USA) for 96 hr. Total RNA was extracted to analyze the expression of DIRAS1.

**IHC Staining**

A TMA containing 90 pairs of RCC and nontumor adjacent tissues was obtained from Xinchao Biotech, Shanghai, China. After sections were dewaxed and rehydrated, antigen retrieval was per-

formed by heating the slides in 10 mM citrate buffer. The slides were blocked with bovine serum albumin (Sango Biotech, China) and then incubated with anti-DIRAS1 antibody (Proteintech, USA) overnight at 4°C. After incubation with secondary antibody for 1 hr at room temperature, a 3, 3-diaminobenzidine (DAB) solution was used for brown color development. The strength of positivity was semi-quantified by assessing both the intensity and the



**Figure 7. Vector-Based Overexpression of DIRAS1 Recapitulates RNAa Results**

(A) Western blot analysis revealed that DIRAS1 protein expression level was upregulated by pDIRAS1 vector in 786-O and Caki-1 cells. (B) CCK-8 assay revealed that the relative cell viability in the pDIRAS1-treated group was lower than that in the pNull-treated group. (C) DIRAS1 overexpression by the vector promoted apoptosis in RCC cells. Representative images of apoptosis assays are presented. (D) DIRAS1 overexpression by the vector inhibited cell migration and invasion in RCC cells. Representative micrographs of transwell assays are presented. Error bars represent the SD from three independent experiments. \* $p < 0.05$ .

proportion of positive cells. Staining intensity was graded as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong); The proportion of positive staining cells is a visual estimation, depicted as a percentage. The score was calculated by multiplying the staining intensity by the percentage of positive tumor cells, with a maximum score value of 3 ( $3 \times 100\%$ ).

#### Western Blot Analysis

Western blot analysis was performed as previously reported,<sup>25</sup> with the following primary immunoblotting antibodies: anti-GAPDH (Sango Biotech, China), anti-DIRAS1 (Proteintech, USA), anti-ERK, anti-p-ERK1/2, anti-caspase-3, and anti-PARP (Cell Signaling Technology, USA).

#### Statistical Analysis

The data are presented as the means  $\pm$  SD. Differences between groups were evaluated with Student's *t* test. All analyses were performed with SPSS16.0 software (IBM, USA), and a two-tailed value of  $p < 0.05$  was considered statistically significant.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and can be found with this article online at <https://doi.org/10.1016/j.omtn.2018.07.019>.

#### AUTHOR CONTRIBUTIONS

X.X., J.L., X.Z., and S.W. performed experiments; X.X. and L.X. designed research, analyzed data, and edited the manuscript for intellectual content. All authors read and approved the final manuscript.

#### CONFLICTS OF INTEREST

The authors declare no competing interests.

#### ACKNOWLEDGMENTS

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