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siRNA Suppression of NEDD9 Inhibits Proliferation and Enhances Apoptosis in Renal Cell Carcinoma

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Renal cell carcinoma (RCC) is the most lethal of all genitourinary malignancies. NEDD9/HEF1/Cas-L is a member of the Cas protein family and is known as a biomarker in multiple cancer types. In this study, we demonstrate for the first time that NEDD9 was upregulated in RCC tissue and cell lines. Immunohistochemical analysis and quantitative RT-PCR analysis showed low expression of NEDD9 in normal renal tissues and high expression in RCC tissues. In addition, *in vitro* experiments show that expression of NEDD9 was upregulated in RCC cell lines. Through MTT assay, we observed that NEDD9 knockdown inhibited cell proliferation. Furthermore, flow cytometry analysis showed that NEDD9 downregulation induced apoptosis. Together, our data suggest that abnormal NEDD9 protein expression may be a marker for RCC, and NEDD9 knockdown suppresses cell growth.

Key words: Renal cell carcinoma (RCC); NEDD9; Proliferation; Apoptosis

INTRODUCTION

Renal cell carcinoma (RCC) is the most common type of kidney cancer, comprising 3% of all adult malignancies (1). The incidence and mortality rates of RCC have increased in recent years; 65,150 new cases are diagnosed, and 13,680 people die from these diseases each year (2). However, the incidence and mortality rates of RCC all over the world are still rising with each decade. Therefore, further understanding of the biology of RCC and the development of novel therapeutic approaches for RCC treatment are needed.

Neural precursor cell-expressed, developmentally down-regulated 9 (NEDD9, also known as HEF1 and Cas-L), a member of the Crk-associated substrate family, plays an important role in regulating cell proliferation, apoptosis, adhesion, migration, division, and survival (3–5). NEDD9 has been confirmed to contribute to the development of several cancer types. Recent observations find that NEDD9 is overexpressed in various human malignancies, including primary colorectal cancer, gastric cancer, melanoma, and glioblastoma (6–9). Furthermore, knockdown endogenous NEDD9 suppresses the growth and invasion of breast, gastric cancer cell lines, and human lung cancer cell lines (10–12).

Expression and biological function of NEDD9 in RCC has so far not been reported. Therefore, in the present study,

we determined NEDD9 expression in human kidney cancer tissue and RCC cell lines and investigated biologic functions of NEDD9 in RCC cells such as cell proliferation.

MATERIALS AND METHODS

Cell Lines and Tissue Specimen

Human RCC cell lines 786-O, Caki1, and HK-2 cells were obtained from ATCC (Manassas, VA, USA). HK-2 cells were cultured in KSFM medium (Gibco, USA), and other cells were cultured at 37°C, 5% CO₂ in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Invitrogen), 100 U ml⁻¹ penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 mg ml⁻¹ streptomycin (Sigma-Aldrich).

Human RCC specimens were collected from 11 patients who underwent surgery, during the surgery, according to an approved human protocol at the Second Hospital and Qilu Hospital of Shandong University (China) between January 2013 and December 2014.

Immunohistochemistry

IHC was performed to analyze NEDD9 expression in RCC tissues and adjacent normal renal tissues. In brief, 5-mm paraffin sections were prepared for the experiments. Sections were deparaffinized in xylene, rehydrated, and endogenous peroxidase activity was quenched by 3% hydrogen peroxide

in methanol. The sections were submerged in 10 mM citrate buffer (pH 6.0) and microwaved for antigen retrieval. Block nonspecific binding was performed with normal goat serum to reduce nonspecific antibody binding, and the slides were incubated with NEDD9 rabbit monoclonal primary antibodies. After washing, sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. Color was developed with the DAB Horseradish Peroxidase Color Development Kit.

siRNA Transfection

Knockdown of NEDD9 expression was performed by RNA interference using specific siRNA oligonucleotides. The target sequences genes were: 5'-UCCCAUGCAGGAGACUGCCUCCAGU-3'. Cells were transfected using Lipofectamine RNAiMAX reagent following the manufacturer's instruction (Invitrogen). All experiments were performed 72 h after transfection.

Quantitative RT-PCR

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturers' instruction. RT-PCR of NEDD9 was performed with 1 µg of total RNA and the RevertAid First Strand cDNA Synthesis Kit (Thermo). Primer sequences used were as follows: forward 5'-GGG TAAAAGGTGATAACCCCGT-3', reverse 5'-TGCTGATGAGGGAGGGATGTCGT-3'; primers specific for β-actin were used as a control (forward: 5'-TCC ATC ATG AAG TGT GAC GT-3' and reverse: 5'-GAG CAA TGA TCT TGA TCT TCA T-3'). Quantitative RT-PCR was performed in the Mastercycler ep realplex real-time PCR system (Eppendorf, Hamburg, Germany) using SYBR green (Takara).

Western Blot Analysis

Total cellular protein was extracted from cell lines transfected for 72 h using RIPA lysis buffer (DBI Bioscience, Shanghai, China) following the manufacturer's instructions.

After protein quantitation using BCA Protein Quantitative Kit (DBI Bioscience), equal amounts of proteins were separated by SDS-PAGE and blotted onto to PVDF membranes (0.45 µm; Millipore, Billerica, MA, USA). The membranes were blocked in 5% skim milk and then incubated overnight at 4°C with anti-NEDD9 (1:1,000; Abcam) or anti-tubulin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), followed by incubation with horseradish peroxidase-conjugated IgG. An ECL kit (Millipore) was used for detection.

Cell Viability Assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were performed to test cell proliferation. In a 96-well plate, 1.0×10^4 cells were plated in each well the day before siRNA transfection and incubated for 72 h. Then, 20 µl of 5 mg/ml MTT solution was added to each well and incubated for 4 h at 37°C, the medium was removed gently from each well, and the formazan crystals were solubilized in 150 µl of dimethyl sulfoxide (DMSO). The absorbance values were measured at 570 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). All experiments were performed in triplicate.

Flow Cytometry

Cells were transfected with scramble or NEDD9 siRNA. After 48 h, cells were harvested and fixed with 70% ethanol. DNA was stained with propidium iodide (PI, 50 µg/ml) in the presence of RNase A (50 µg/ml) in the dark for 30 min. Cell cycle distribution was determined using flow cytometry on a FACScan (BD Bioscience, Franklin Lakes, NJ, USA) and analyzed using the ModFit software (BD Bioscience).

The Annexin-V-FITC/PI apoptosis kit (BestBio Biotechnologies, Shanghai, China) was adopted for apoptosis detection according to the manufacturer's protocol. Briefly, 48 h after transfection, 1×10^6 cells were collected and suspended in 500 µl binding buffer, and 5 µl

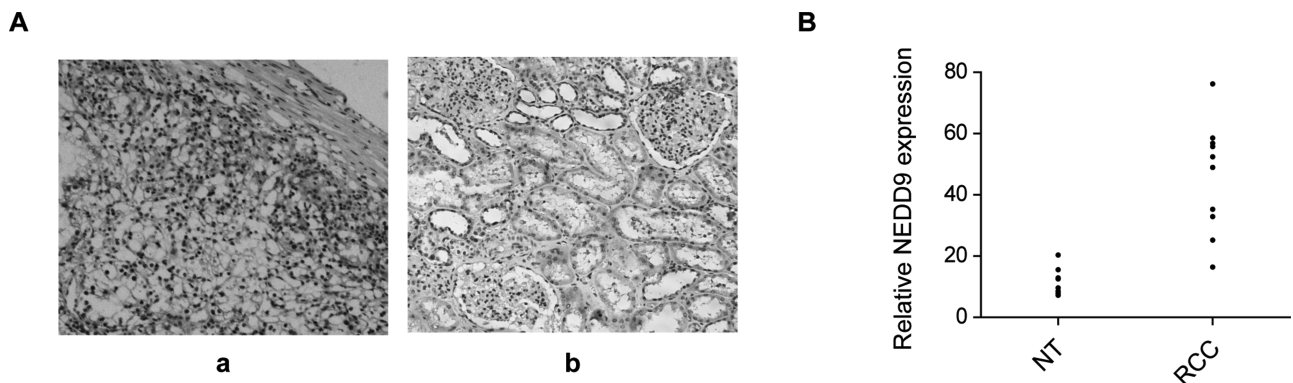


Figure 1. NEDD9 expression analysis in RCC tissues. (A) Immunohistochemical staining of NEDD9 in RCC tissues (a) and adjacent normal renal tissues (b). (B) NEDD9 levels in normal renal tissues and RCC tissues. Normalized NEDD9 mRNA expression was measured by quantitative RT-PCR with β-actin expression as the internal control.

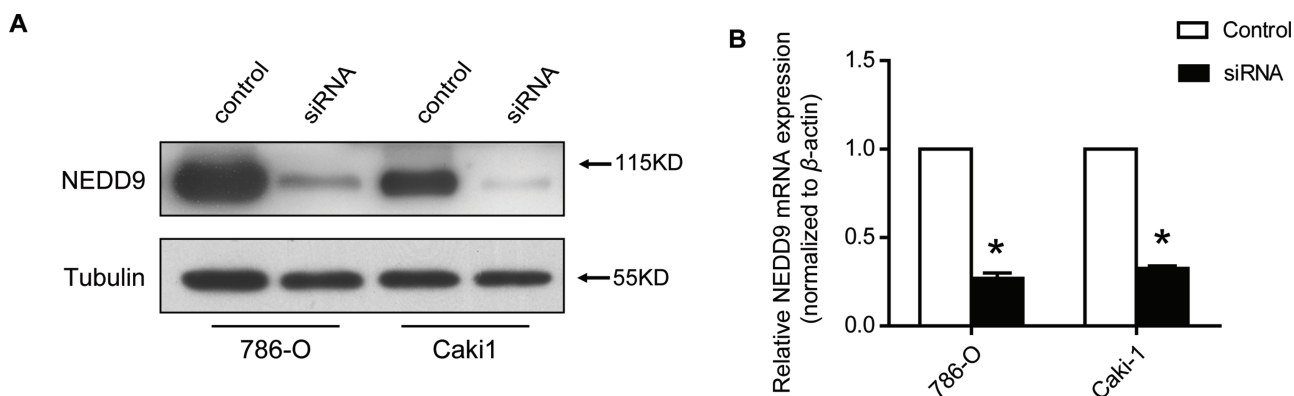


Figure 2. NEDD9 is overexpressed in RCC cell lines. (A) Increased NEDD9 expression was detected in different RCC cell lines compared to the immortalized nonmalignant immortalized renal cell line, HK2. NEDD9 protein levels were normalized to tubulin. Statistical analysis showed increased NEDD9 expression in RCC cell lines. (B) Relative NEDD9 mRNA levels in RCC cell lines were determined by RT-PCR. * $p < 0.001$.

annexin V-FITC and 5 μ l PI were added to each sample and incubated in the dark for 15 min. Data were detected using flow cytometry and analyzed by FlowJo software (Tree Star Inc., Ashland, OR, USA).

Statistical Analysis

All statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used to assess significance for data within two groups.

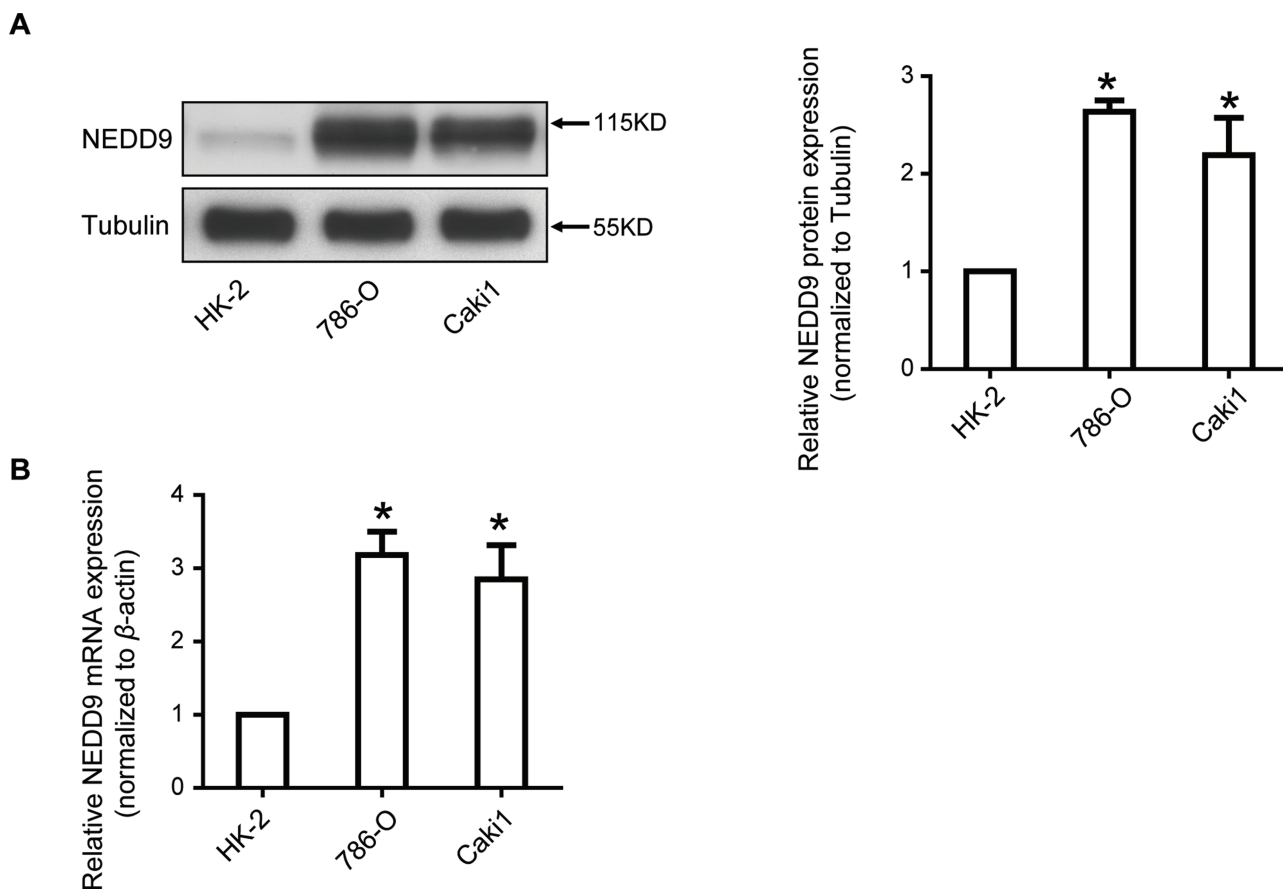


Figure 3. NEDD9 siRNA reduces NEDD9 expression. (A) RT-PCR analysis of NEDD9 mRNA expression in 786-O and Caki1 cells transfected with the specific siRNA targeting NEDD9 for 72 h, respectively. (B) 786-O and Caki1 cells were transfected with scrambled siRNA or NEDD9 siRNA, 72 h later, endogenous NEDD9 and protein levels were assessed by Western blot. * $p < 0.001$.

Multiple statistic comparisons were analyzed using one-way ANOVA followed by post hoc tests.

RESULTS

NEDD9 Is Highly Expressed in RCC Tissues and Cell Lines

We analyzed 11 RCC samples, together with their adjacent normal renal tissues, from RCC patients. Immunohistochemistry showed that the expression of NEDD9 was significantly higher in RCC tissues than that in tumor-adjacent tissues (Fig. 1A). Furthermore, expression of NEDD9 mRNA was assessed using real-time quantitative PCR. NEDD9 was expressed at higher levels in RCC tissues (49.42 ± 6.2) than in matched normal renal tissues (11 ± 1.37 , $p < 0.001$) (Fig. 1B).

In addition, we examined the levels of NEDD9 expression in normal and RCC cell lines. Using quantitative RT-PCR assay, we observed that there was a significant increase in mRNA level of NEDD9 compared to 786-O and Caki1 cells with HK-2 cells (Fig. 2A). To further evaluate the expression of NEDD9 in RCC cells, Western analysis was performed on lysates prepared from two RCC cell lines using a NEDD9 antibody. NEDD9 was more highly expressed in 786-O and Caki1 cells than in HK-2 cells; moreover, the levels of NEDD9 in RCC cell lines were similar to RCC tissues, as determined by Western blot analysis (Fig. 2B).

siRNA-Mediated NEDD9 Knockdown

We detected the effect of knocking down endogenous NEDD9 levels by small interfering RNA (siRNA). Quantitative RT-PCR and Western blot assays confirmed that NEDD9 siRNA transfection could successfully knock down endogenous NEDD9 level (Fig. 3A, B).

NEDD9 Depletion Inhibits RCC Cell Cycle

The effect of the NEDD9 depletion on cell proliferation was evaluated using the MTT assay. We observed the NEDD9 siRNA-treated 786-O and Caki1 cells exhibited a significant decrease in cell viability (786-O control vs. NEDD9 siRNA: 0.72 ± 0.02 vs. 0.44 ± 0.03 , $p < 0.001$; Caki1 control vs. NEDD9 siRNA: 0.7 ± 0.02 vs. 0.51 ± 0.01 , $p < 0.001$) (Fig. 4A). The flow cytometry analysis revealed that NEDD9 siRNA-treated cells underwent a significant increase in the proportion of cells in the G_1 -phase population and a significant reduction in the S-phase population, compared to scramble siRNA group (percentage of cell cycle in G_1 phase: control vs. NEDD9 siRNA, 786-O: 38.39 ± 0.97 vs. 64.43 ± 1.1 , $p < 0.0001$; Caki1: 42 ± 1 vs. 64.6 ± 1.1 , $p < 0.001$; S phase: control vs. NEDD9 siRNA, 786-O: 38.27 ± 5.1 vs. 22.08 ± 1.4 , $p < 0.0001$; Caki1: 43.71 ± 6.20 vs. 22.22 ± 0.85 , $p < 0.001$) (Fig. 4B, C). These results indicated that NEDD9 has an effect on the regulation of the cell cycle of RCC cells.

Effects of NEDD9 Depletion on RCC Cell Apoptosis

As shown by annexin V and PI staining, the NEDD9 siRNA group had a larger number of apoptotic 786-O and Caki1 cells than the scramble siRNA group (percentage of late apoptotic cells: control vs. NEDD9 siRNA, 786-O: 4.91 ± 0.36 vs. 15.5 ± 0.7 , $p < 0.001$; Caki1: 5.36 ± 0.28 vs. 18.51 ± 1.16 , $p < 0.001$, $p < 0.001$) (Fig. 4D, E). Therefore, the NEDD9 knockdown may inhibit the apoptosis of 786-O and Caki1 cells. Taken together, these results suggest that NEDD9 knockdown enhances apoptosis of RCC cells.

DISCUSSION

RCC is the most lethal urologic tumor. The carcinogenesis and development of RCC is a complex process involving multiple factors. RNA interference technology has been identified as an important regulator of cancer progression through various molecular pathways (13). NEDD9 is a member of the Cas family of proteins; as a skeletal protein, NEDD9 is considered a router in the cell signal transduction process. Abnormal NEDD9 expression in tumor cells has been implicated in tumor progression. Moreover, NEDD9 is overexpressed in various human cancers, including breast cancer, glioblastoma, melanoma, and lung cancers (9,14,15). Recently, Lu et al. identified that miR-145 is markedly downregulated in RCC, and it targets NEDD9 (16). However, the pattern of NEDD9 expression in RCC cancer was unknown.

In this study, we examined the endogenous NEDD9 in RCC tissues by immunohistochemical staining. We demonstrated that NEDD9 mRNA levels are significantly higher in RCC tissues than in adjacent normal renal tissues.

We also demonstrated that the expression of NEDD9 protein and mRNA increased in RCC cell lines compared to normal renal cell lines, suggesting that the aberrant expression of NEDD9 is involved in the pathogenesis of RCC. In addition, we showed that siRNA-mediated NEDD9 depletion inhibited the growth of RCC cells.

After binding to FAK and Src, this NEDD9-FAK-Src complex leads to activating the tyrosine phosphorylation of NEDD9 to generate binding sites for effector proteins, including the GTP kinase of Ras and Rho family, which then regulate and activate transcription pathways involved in metastasis and cancer progression (17–20). A previous study also found that NEDD9 suppression results in cancer cell growth inhibition. We therefore hypothesize that NEDD9 may regulate the proliferation of RCC cell lines. We detected cell proliferation and apoptosis using MTT and flow cytometry after knockdown NEDD9 by siRNA. The results showed that NEDD9 knockdown significantly inhibited cell proliferation, by participating in the regulation of the cell cycle and apoptosis. Therefore, it may be that NEDD9 overexpression promotes RCC cell growth.

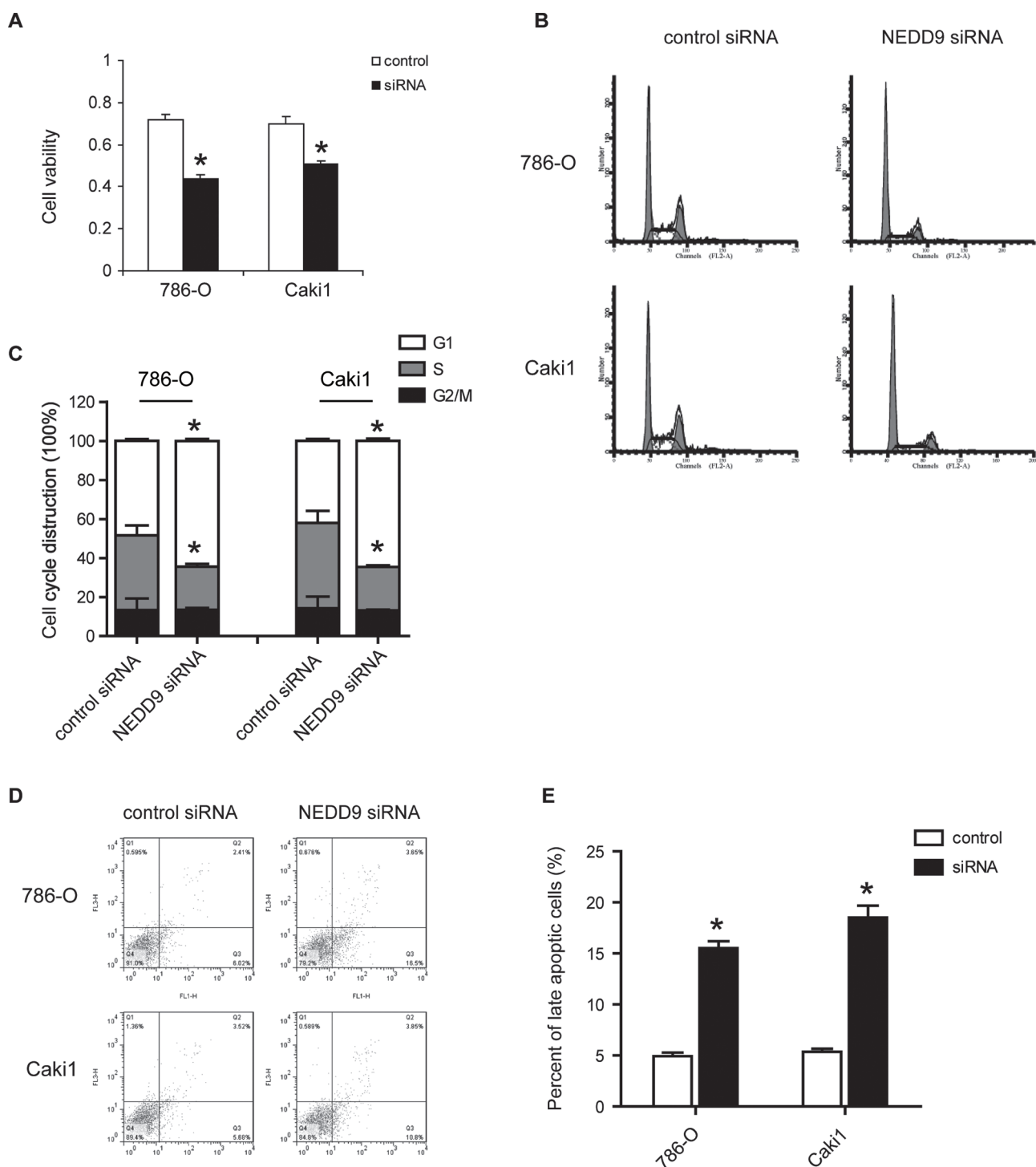


Figure 4. NEDD9 knockdown inhibits RCC cell growth. (A) MTT assays showing that decreased NEDD9 expression inhibited the proliferation of 786-O and Caki1 cells. (B) Flow cytometric analysis shows cell cycle distribution of 786-O and Caki1 cells treated with control and NEDD9 siRNA for 48 h. (C) Bar plots summarizing the percentages of cells in G₁, S, and G₂/M phases in NEDD9-depleted cancer cells. (D) Representative results for 786-O and Caki1 cells showed that knockdown of NEDD9 led to an increase in apoptotic cells compared with untransfected or control cells. (E) Statistical analysis of the flow cytometry data for apoptosis in 786-O and Caki1 cell, respectively. * $p < 0.001$.

In summary, we found that NEDD9 plays an important role in the carcinogenesis of RCC. Furthermore, inhibiting the expression of NEDD9 mRNA and protein by siRNA could inhibit proliferation of RCC cells. Therefore, NEDD9 may be a novel target for prevention and treatment of RCC.

REFERENCES

- Jemal, A.; Siegel, R.; Ward, E.; Hao, Y.; Xu, J.; Thun, M. J. Cancer statistics, 2009. *CA Cancer J. Clin.* 59(4):225–249; 2009.
- Siegel, R.; Naishadham, D.; Jemal, A. Cancer statistics, 2013. *CA Cancer J. Clin.* 63(1):11–30; 2013.
- O’Neill, G. M.; Golemis, E. A. Proteolysis of the docking protein HEF1 and implications for focal adhesion dynamics. *Mol. Cell. Biol.* 21(15):5094–5108; 2001.
- van Seventer, G. A.; Salmen, H. J.; Law, S. F.; O’Neill, G. M.; Mullen, M. M.; Franz, A. M.; Kanner, S. B.; Golemis, E. A.; van Seventer, J. M. Focal adhesion kinase regulates beta1 integrin-dependent T cell migration through an HEF1 effector pathway. *Eur. J. Immunol.* 31(5):1417–1427; 2001.
- O’Neill, G. M.; Seo, S.; Serebriiskii, I. G.; Lessin, S. R.; Golemis, E. A. A new central scaffold for metastasis: Parsing HEF1/Cas-L/NEDD9. *Cancer Res.* 67(19):8975–8979; 2007.
- Kim, S. H.; Xia, D.; Kim, S. W.; Holla, V.; Menter, D. G.; Dubois, R. N. Human enhancer of filamentation 1 is a mediator of hypoxia-inducible factor-1alpha-mediated migration in colorectal carcinoma cells. *Cancer Res.* 70(10):4054–4063; 2010.
- Li, Y.; Bavarva, J. H.; Wang, Z.; Guo, J.; Qian, C.; Thibodeau, S. N.; Golemis, E. A.; Liu, W. HEF1, a novel target of Wnt signaling, promotes colonic cell migration and cancer progression. *Oncogene* 30(23):2633–2643; 2011.
- Liu, Y.; Wang, D.; Zhao, K. L.; Zhu, J. W.; Yin, H. B.; Wei, Y. Z.; Wu, Z. J.; Cheng, G. J.; Wang, F.; Ni, F.; Wang, X. L.; Cao, G. X.; Huang, J.; Cai, J. NEDD9 overexpression correlates with poor prognosis in gastric cancer. *Tumour Biol.* 35(7):6351–6356; 2014.
- Natarajan, M.; Stewart, J. E.; Golemis, E. A.; Pugacheva, E. N.; Alexandropoulos, K.; Cox, B. D.; Wang, W.; Grammer, J. R.; Gladson, C. L. HEF1 is a necessary and specific downstream effector of FAK that promotes the migration of glioblastoma cells. *Oncogene* 25(12):1721–1732; 2006.
- Chen, Z.; Liu, Q.; McGee, M.; Kong, M.; Huang, X.; Deng, Y.; Scheuermann, R. H. A gene selection method for GeneChip array data with small sample sizes. *BMC Genomics* 12(Suppl. 5):S7; 2011.
- Kondo, S.; Iwata, S.; Yamada, T.; Inoue, Y.; Ichihara, H.; Kichikawa, Y.; Katayose, T.; Souta-Kuribara, A.; Yamazaki, H.; Hosono, O.; Kawasaki, H.; Tanaka, H.; Hayashi, Y.; Sakamoto, M.; Kamiya, K.; Dang, N. H.; Morimoto, C. Impact of the integrin signaling adaptor protein NEDD9 on prognosis and metastatic behavior of human lung cancer. *Clin. Cancer Res.* 18(22):6326–6338; 2012.
- Miao, Y.; Li, A. L.; Wang, L.; Fan, C. F.; Zhang, X. P.; Xu, H. T.; Yang, L. H.; Liu, Y.; Wang, E. H. Overexpression of NEDD9 is associated with altered expression of E-Cadherin, beta-Catenin and N-Cadherin and predictive of poor prognosis in non-small cell lung cancer. *Pathol. Oncol. Res.* 19(2):281–286; 2013.
- Iorio, M. V.; Croce, C. M. microRNA involvement in human cancer. *Carcinogenesis* 33(6):1126–1133; 2012.
- Kim, M.; Gans, J. D.; Nogueira, C.; Wang, A.; Paik, J. H.; Feng, B.; Brennan, C.; Hahn, W. C.; Cordon-Cardo, C.; Wagner, S. N. and others. Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene. *Cell* 125(7):1269–1281; 2006.
- Ji, H.; Ramsey, M. R.; Hayes, D. N.; Fan, C.; McNamara, K.; Kozlowski, P.; Torrice, C.; Wu, M. C.; Shimamura, T.; Perera, S. A. and others. LKB1 modulates lung cancer differentiation and metastasis. *Nature* 448(7155):807–810; 2007.
- Lu, R.; Ji, Z.; Li, X.; Zhai, Q.; Zhao, C.; Jiang, Z.; Zhang, S.; Nie, L.; Yu, Z. miR-145 functions as tumor suppressor and targets two oncogenes, ANGPT2 and NEDD9, in renal cell carcinoma. *J. Cancer Res. Clin. Oncol.* 140(3):387–397; 2014.
- Mitra, S. K.; Schlaepfer, D. D. Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr. Opin. Cell Biol.* 18(5):516–523; 2006.
- Kiyokawa, E.; Hashimoto, Y.; Kurata, T.; Sugimura, H.; Matsuda, M. Evidence that DOCK180 up-regulates signals from the CrkII-p130(Cas) complex. *J. Biol. Chem.* 273(38):24479–24484; 1998.
- Klemke, R. L.; Leng, J.; Molander, R.; Brooks, P. C.; Vuori, K.; Cheresh, D. A. CAS/Crk coupling serves as a “molecular switch” for induction of cell migration. *J. Cell Biol.* 140(4):961–972; 1998.
- Sadok, A.; Marshall, C. J. Rho GTPases: Masters of cell migration. *Small GTPases* 5:e29710; 2014.