Silencing DEK downregulates cervical cancer tumorigenesis and metastasis via the DEK/p-Ser9-GSK-3β/p-Tyr216-GSK-3β/β-catenin axis

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Abstract. Cervical cancer is the second most common gynecological malignancy. The mechanisms of the genesis and progression of cervical cancer are complicated and not thoroughly understood. DEK is reported as an oncogene in various cancers, such as acute myeloid leukemia, bladder cancer, breast cancer and hepatocellular cancer. However, its role in cervical cancer has not been well studied. In our study, we confirmed the DEK protein as an oncoprotein in cervical cancer tissues which is correlated to cervical cancer FIGO staging and tumor type. Moreover, *in vitro* loss of DEK inhibited cervical cancer cell proliferation, migration and invasion. We proved that silencing DEK downregulated Wnt/β-catenin and MMP-9, and silencing DEK increased GSK-3β activity via regulating its phosphorylation instead of translation. Silencing DEK reduced p-Ser9-GSK-3β and increased p-Tyr216-GSK-3β, which resulted in β-catenin degradation. Finally, the xenograft model in nude mice proved that silencing DEK impaired cervical cancer cell tumorigenicity. This research unveiled the function of DEK in tumorigenesis and metastasis via the DEK/p-Ser9-GSK-3β/p-Tyr216-GSK-3β/β-catenin axis in cervical cancer and gave insights into DEK-targeting therapy for patients suffering from cervical cancer.

Introduction

Cervical cancer is the second most common type of cancer among women (1), with deaths projected to rise by almost 25% over the next 10 years, according to the World Health Organization (WHO) (2). One reason for the high death rate is that cervical cancer has a high rate of invasion and metastasis (3).

Aberrant activation of oncogenes contribute to cancer progression (4). DEK is one of the increasing number of oncogenes that have been reported (5). DEK is primarily identified as a fusion with the CAN nucleoporin in acute myeloid leukemia (6,7). DEK protein overexpression has been discovered in lung cancer, colorectal carcinoma, hepatocellular carcinoma, breast cancer, bladder cancer and osteosarcoma (8-11). The DEK protein promotes cancer cell proliferation, epithelial-mesenchymal transition (EMT), metastasis, and DNA damage (6,12,13). DEK is an induced target of the human papillomavirus (HPV) E7 oncoprotein in head and neck cancer (10,14). Moreover, DEK upregulate β-catenin in MCF-7 cells (15). On the contrary, DEK expression is regulated by upstream regulators such as ERα, E2F, and NF-Y (16,17). However, the role of DEK in cervical cancer has been reported only rarely.

Glycogen synthase kinase-3 (GSK-3) is a proline-directed serine/threonine kinase that was initially identified as a phosphorylating and an inactivating agent of glycogen synthase (18). Two isoforms, α (GSK3α) and β (GSK3β), show a high degree of amino acid homology (18,19). GSK3β is involved in energy metabolism, neuronal cell development, and body pattern formation (19,20). It can regulate nuclear transcription factor-κB (NF-κB), p53 and β-catenin (21-24). GSK-3β can be inactivated by phosphorylation at the N-terminal Serine 9 (Ser9) residue which is the most frequently examined mechanism that negatively regulates the activity of the kinase (20,25). On the contrary, the phosphorylation of Tyrosine 216 (Tyr216) residue positively regulates GSK-3β activity (20,26). As a component of the protein complex regulating the cellular level of β-catenin, GSK-3β increases β-catenin degradation and maintains cell physiology (27,28). When the Wnt/β-catenin pathway is aberrantly activated, cell behavior changes and cancer cell malignant behavior increases (29). The Wnt/β-catenin pathway increases cancer cell invasion and migration (30,31).

Herein, we report on the function of DEK in tumorigenesis and metastasis of cervical cancer cells. This study

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provides insight into DEK-targeting treatment for cervical cancer patients.

Materials and methods

Tissue specimens. Human cervical cancer samples (n=78) and non-cancer samples (n=15) were collected at the Second Affiliated Hospital of Chongqing Medical University, Chongqing, China. The experiments were approved by the Research Ethics Committee of Chongqing Medical University.

Cell lines. Human cervical cancer cell line SiHa was purchased from the China Center for Type Culture Collection (Wuhan, China). All cells were cultured in RPMI-1640 medium (Gibco, San Diego, CA, USA), containing 10% fetal bovine serum (Gibco) and 1% penicillin and streptomycin solution. All human cervical cancer cell lines were maintained at 37˚C in an atmosphere containing 5.0% carbon dioxide.

Immunohistochemistry (IHC). An IHC evaluation of DEK was performed according to the streptavidin/peroxidase kit instructions (SPlink Detection kits, ZSGB-Bio, Beijing, China). After being deparaffnized and rehydrated, the sections were heated in citrate buffer-induced for 25 min for antigen retrieval. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 10 min. Thereafter, the sections were blocked with goat serum for 10 min and incubated with anti-DEK (1:100) overnight at 4˚C. Then sections were incubated with HRP-conjugated secondary antibodies for 10 min and incubated in horseradish enzyme-labeled chain avidin solution for 10 min at 37˚C. Visualization was performed with a DAB Horseradish Peroxidase Color Development kit and the samples were counterstained with hematoxylin. Staining intensity was graded on a scale of 0-3, as follows: 0 (absence of staining), 1 (weakly stained), 2 (moderately stained), and 3 (strongly stained). The percentage of positive tumor cells was scored as follows: 0 (absence of tumor cells), 1 (<33% tumor cells), 2 (33-66% tumor cells) and 3 (>66% tumor cells). Immunohistochemical score (ranging from 0 to 9) was calculated by multiplying the intensity score and the percentage score. Specimen was determined positive when it was scored 4 or over. Specimen was determined negative when it was scored 3 or under. The same qualified pathologist analyzed all the IHC data to ensure scoring consistency.

Quantitative real-time polymerase chain reaction (PCR). Total RNA was extracted from the cancer cells using an RNA Extraction kit (BioTeke, Beijing, China), according to the manufacturer's protocol. Then RNA was quantified using a Nanodrop spectrophotometer. cDNA was synthesized using a qPCR RT kit (GeneCopoeia Inc., Guangzhou, China). The primers used for DEK and GAPDH amplification were synthesized by GeneCopoeia Inc. The real-time PCR kit was purchased from GeneCopoeia Inc. Each sample was analyzed in triplicate. Quantification of gene transcription was determined using the 2-ΔΔCq method (28). RT-PCR analyses were performed at least three times.

Western blot analysis. Total protein extracted from each sample was separated in 8% polyacrylamide gel, and electrotransferred to polyvinylidene fluoride membranes (Millipore Corp., Billerica, MA, USA). The bands were blocked in 5% powdered milk for 1 h at room temperature. The membranes were incubated with primary antibodies (1:1000-1:2000) against DEK, β-catenin, GSK-3β and p-GSK-3β overnight at 4˚C. After washing with tris-buffered saline containing 0.1% Tween-20 (TBS-T), the membranes were incubated with an anti-rabbit IgG antibody conjugated with horseradish peroxidase (Bioss, China) for 1 h. After washing with TBS-T, the membranes were visualized with an ECL detection system (KeyGen Biotech Inc., Nanjing, China). All of the western blot analyses were repeated at least three times.

Transfections. The shRNA lentiviral vector targeting DEK (LV3-DEK, sense CGAACCAAAUGUCCUGAAA dTdT; antisense UUUCAGGACAUUUGGUUCG dTdT) and Negative control (LV3-NC, 5'-UUCUUCGAACGUGUC AGUTT-3') were provided by Genepharma Co., Ltd. (Shanghai, China). SiHa cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco), and were transduced with the lentivirus (multiplicity of infection = 20), containing 5 μg/ml polybrene. Medium was refreshed 48 h post-transduction and replaced by RPMI-1640 medium, containing 10% fetal bovine serum and 1% penicillin and streptomycin solution. Following transduction for 48 h, stably infection colonies of cells were selected using 10 μg/ml puromycin.

Colony forming assay. Cells were cultured by seeding 1000 cells per well in 6-well plates. All cells were incubated at 37˚C in an atmosphere containing 5.0% carbon dioxide for 14 days, then the number of colonies formed was counted. All experiments were performed in triplicate.

Proliferation assay. Cells were seeded into 96-well plates at a density of 1000 cells per well. Cell proliferation was tested using a CCK-8 Kit (DNDOJAN, Japan) every 24 h after transfection for 6 days (the reactions were incubated for 1 h at 37˚C and 5% CO_2; detection: 450 nm, reference: 630 nm). The experiments were repeated in triplicate.

Wound healing assay. Cell migratory capacity was analyzed using the wound-healing assay *in vitro*. Cells were cultured in 6-well plates and cultivated until 95% confluent. Wounds were incised in the cell monolayer using a sterile pipette tip. At 0 and 72 h after the wounding, cells were observed under the light microscope. The distance between the two wounds were measured and the ability to close the wound channel was expressed as the average percent of wound closure at 72 h, compared to that at 0 h. The experiment was repeated in triplicate.

Transwell membrane based invasion assay. The ability of cells to migrate was analyzed using the Matrigel Transwell assay. For Matrigel migration assays, the upper side of an 8 μm pore, 6.5-mm polycarbonate Transwell filter chamber (Corning Inc., New York, NY, USA) was uniformly coated with Matrigel basement membrane matrix (BD Biosciences, Bedford, MA, USA) for 2 h at 37˚C before the cells were added. The cells were seeded ($2x10^5$ cells/well) in the upper chambers in 200 μl serum-free media, and the lower chambers were filled with

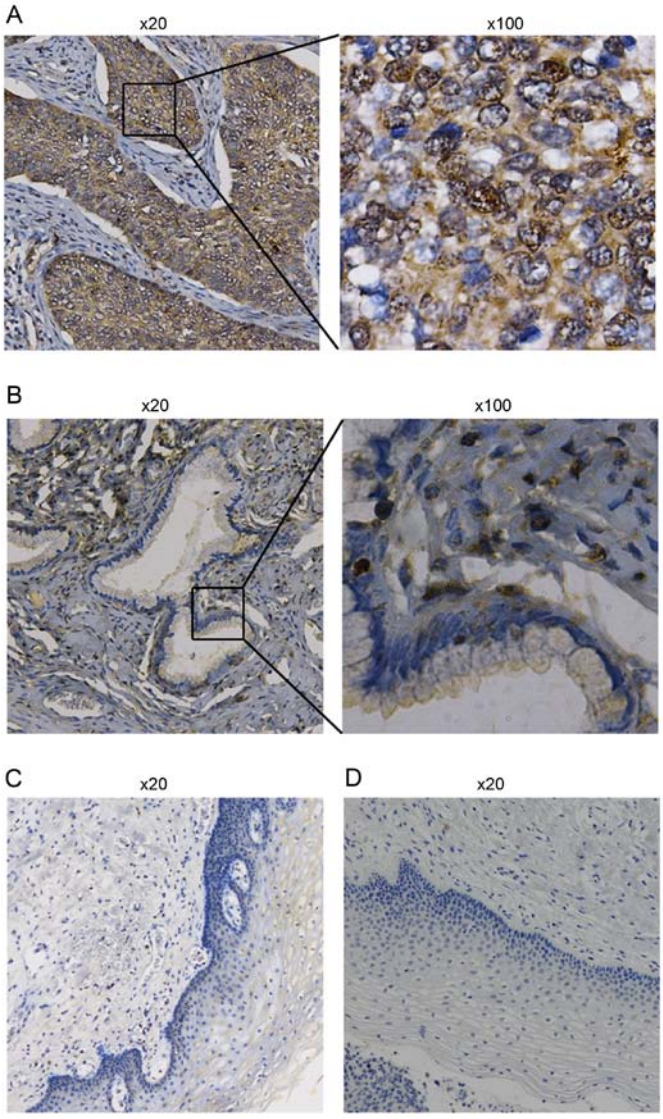

Figure 1. Immunohistochemical visualization of DEK expression in normal/benign and cancerous cervical tissue samples. (A) Squamous carcinoma, (B) adenocarcinoma, (C) normal cervical tissue. (D) Normal tissue adjacent to cervical cancer. DEK was predominantly located in the nucleus of cervical cancer tissues and partially located in the cytoplasm. DEK expression was significantly higher in cervical cancer tissues.

750 μl complete media containing fetal bovine serum, which can induce cell migration. After 24 h, cells that invaded to the lower surface of the filter were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet, and counted using a microscope. Each experiment was performed in triplicate and repeated thrice.

Dual-luciferase reporter gene assay. Luciferase reporter gene assay was performed using Dual-Luciferase Reporter assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol. TOPflash reporter plasmid was provided by Shanghai Qcbio Science & Technologies Co., Ltd. (Shanghai, China). Reporter assay was performed 48 h post-transfection using the Dual Luciferase Assay System (Promega). Firefly luciferase activity was normalized for transfection efficiency using the corresponding *Renilla* luciferase activity. The experiment was repeated in triplicate.

Table I. Association of DEK expression with clinicopathological characteristics in 78 patients with EOC.

Characteristics	No. of pts. (n=78)	DEK expression Low no. (%)	DEK expression High no. (%)	P-value
Age (years)				0.452
<51	48	23 (47.92)	25 (52.08)	
≥51	30	17 (56.67)	13 (43.33)	
FIGO stage				0.043
I-II	52	22 (42.31)	30 (57.69)	
III-IV	26	5 (19.23)	21 (80.77)	
Grade				0.157
1-2	51	18 (35.29)	33 (65.71)	
3	27	14 (51.85)	13 (48.15)	
Tumor type				0.038
Squamous carcinoma	63	10 (15.87)	53 (84.13)	
Adenocarcinoma	15	6 (40.00)	9 (60.00)	

Pts., patients; FIGO, International Federation of Gynecology and Obstetrics.

In vivo tumor xenograft study. Six-week-old female BALB/c nude mice were purchased from the Experimental Animal Center of Chongqing Medical University. The research protocol used in this study was approved by, and the mice were maintained, in accordance with the institutional guidelines set forth by the Committee on the Use and Care on Animals (Chongqing Medical University). Cells were infected with the indicated lentiviral vectors and injected ($5x10^6$ cells per mouse in 200 μl) subcutaneously into the left armpit of 6-week-old BALB/c nude mice. Twenty-one days later, the animals were sacrificed to confirm the the weight of the established tumors.

Statistical analysis. All statistical analyses were performed using SPSS software v17.0 (Chicago, IL, USA). Comparisons between groups were analyzed using a Student's t-test or a Mann-Whitney U test. The Chi-squared test was used to compare the associations between DEK overexpression and clinicopathological variables of cervical cancer and non-tumor samples. All experiments were performed in triplicate. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

DEK is overexpressed in cervical cancer tissues. To evaluate the expression of DEK, IHC analyses were performed in 78 cervical cancer and 15 non-cancer cervical tissue samples (the clinicopathological parameters of the tumor specimens examined in the study are summarized in Table I). DEK was shown to be expressed at significantly higher levels in cervical cancer tissues than that in normal cervical tissues (P<0.05, Fig. 1, Table I). According to the FIGO staging system, the

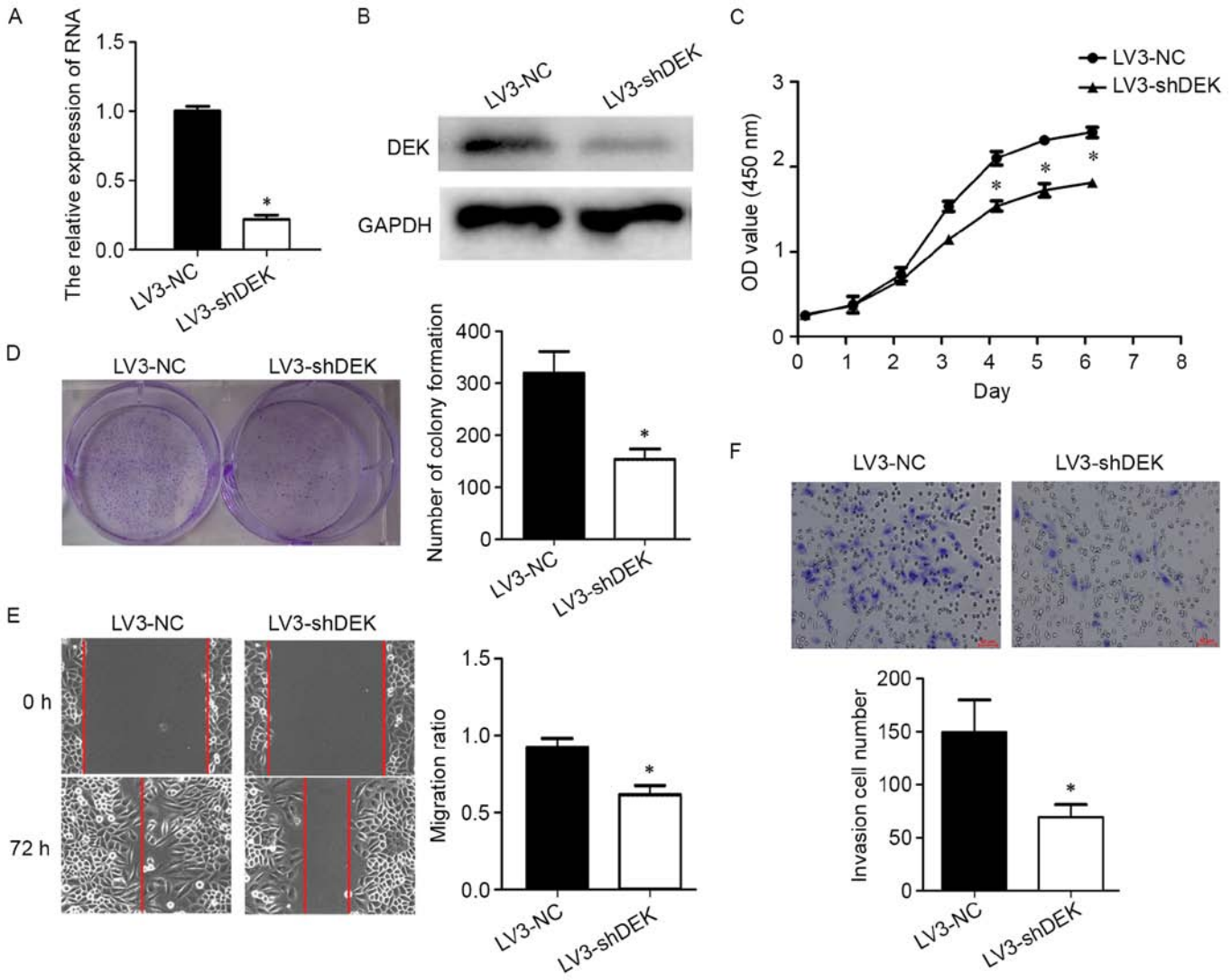

Figure 2. The effect of DEK silencing on *in vitro* cell proliferation, migration, and invasion. The effect of lentivirus-shRNA on silencing DEK in SiHa cells. DEK was significantly silenced at (A) the RNA level and (B) the protein level. Cell proliferation was examined by CCK-8 assay. (C) Silencing DEK resulted in significant growth-inhibition after day 4. (D) Colony forming assay was performed. Colony forming ability was impaired in DEK-silenced cells. Proliferation of DEK-silenced cells was clearly inhibited. Downregulation of DEK resulted in a decrease in (E) cell migration and (F) invasive cells. *P<0.05 vs. the NC.

IHC results indicated that high DEK expression was positively correlated with FIGO stage classification (P<0.05). Patients having higher FIGO stages tended to overexpress DEK. Additionally, the positive levels of DEK expression was higher in squamous cell carcinoma than in adenocarcinoma (P<0.05). However, there was no obvious correlation between DEK expression and patient age.

Silencing DEK inhibits SiHa cell proliferation. In this study, DEK was silenced using the shRNA lentiviral vector (LV3-DEK). The efficiency was tested by qPCR and western blotting. In the silenced group, DEK expression at mRNA level was 78% lower compared to the negative control group (LV3-NC) (Fig. 2A and B).

To understand better the role of DEK in cervical cancer cells, cell proliferation was analyzed by CCK-8 and colony-forming assays. The results showed that cell proliferation was inhibited in the LV3-DEK group (Fig. 2C). The OD value of LV3-shDEK group was significantly lower than that of LV-NC group from day 4. Consistent with the CCK-8 assay, the colony-forming ability was reduced in the LV3-DEK group (Fig. 2D).

Silencing DEK impaired SiHa cell migration and invasion. Cell migration capability was determined with a wound healing assay. After 72 h, the wound was filled in the LV3-NC group, while there was still a gap in the LV3-DEK group (Fig. 2E). Moreover, cell invasion capability was observed using a Matrigel-Transwell assay (Fig. 2F). It was found that silencing DEK impaired the migration and invasion capacity of cervical cancer cells.

Silencing DEK downregulates the Wnt pathway. The Wnt pathway is a classic signaling pathway which regulates cell proliferation, migration and invasion. Previous studies have reported that DEK regulates the Wnt signaling pathway in acute leukemia cells (5). However, whether DEK has an effect on cervical cancer cells is not clear. Thus, we investigated Wnt pathway activity by luciferase reporter assay. The Wnt pathway was found to be inhibited when DEK was silenced (Fig. 3A).

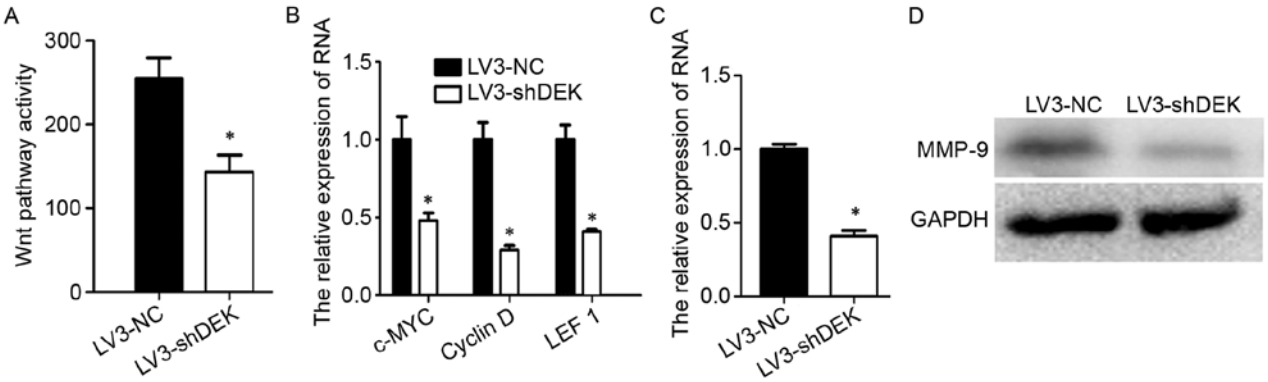

Figure 3. Silencing DEK inhibits the Wnt pathway. (A) Wnt luciferase reporter assay results indicated that silencing DEK downregulated the activity of the Wnt pathway. (B) The Wnt pathway-targeted genes decreased at the mRNA level in DEK-silenced cells. Silencing DEK inhibited MMP-9 expression at both (C) the RNA level and (D) the protein level; *P<0.05 vs. the NC or mock group.

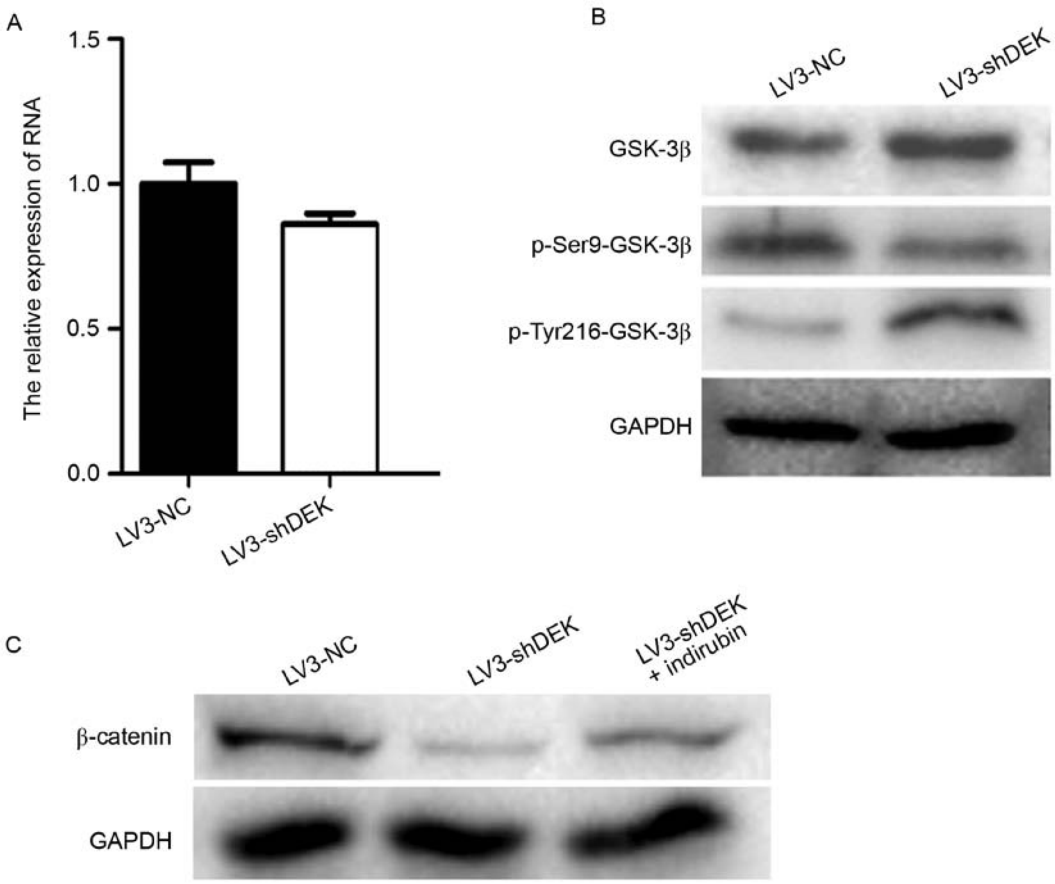

Figure 4. Silencing DEK downregulated the Wnt/β-catenin pathway by mediating p-GSK-3β. There were no statistical difference between the LV3-NC and LV-3shDEK groups at (A) the RNA level and (B) the protein level of DEK. Silencing DEK reduced p-Ser9-GSK-3β protein and increased p-Tyr216-GSK-3β. (C) The inhibition of silencing DEK on β-catenin was partially reversed when indirubin, a GSK-3β inhibitor, was added.

At the same time, silencing DEK reduced the expression of c-MYC, cyclin D and LEF1 (Fig. 3B), the downstream targets of Wnt signaling. MMP-9 has been reported as a Wnt targeting gene, and is closely related to tumor metastasis (32). We examined MMP-9 in cervical cancer cells. Silencing DEK resulted in reduced expression of MMP-9 at the mRNA level (Fig. 3C) and the protein level (Fig. 3D). These data indicated that DEK promoted cervical cancer cell metastasis via upregulating the Wnt pathway and MMP-9 expression.

Silencing DEK downregulates the Wnt/β-catenin pathway by mediating p-GSK-3β. In order to identify which proteins interacted with DEK, we utilized String10.0 (http://string.embl.de/). We found that GSK-3β was predicted to interact with DEK, although this had not yet been proven. Therefore, we investigated the relationship between DEK and GSK-3β. First we found that the expression of GSK-3β at the mRNA and protein levels displayed no significant difference between the LV3-DEK cells and LV3-NC cells (Fig. 4A and B). It has

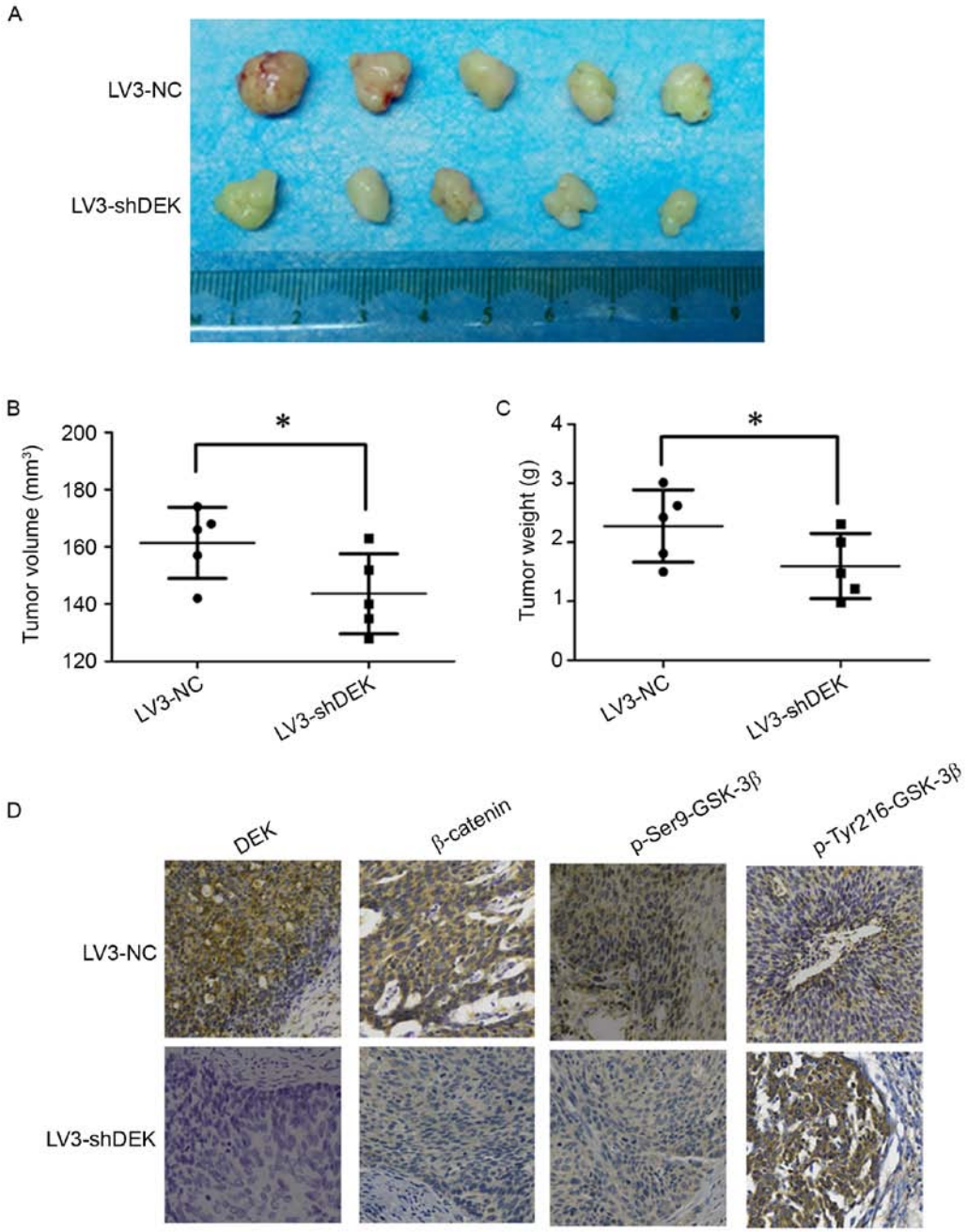

Figure 5. *In vivo* tumor xenograft study. (A-C) Average tumor volume and weight 21 days after LV3-NC and LV3-shDEK treated tumor cells were transplanted. (D) Representative microphotographs of immunohistochemical analysis of the expression of DEK, β-catenin, p-Ser-GSK-3β and p-Tyr216-GSK-3β in tumor xenografts. *P<0.05 vs. the NC or mock group.

been reported that, in the normal state, GSK-3β, Axin, CK-I and β-catenin form a compound and promote β-catenin degradation. When Wnt signaling terminates, the compound disintegrates and β-catenin accumulates and becomes active. Thus, we hypothesized that DEK regulated the Wnt/GSK-3β/β-catenin pathway via regulation of GSK-3β phosphorylation. Levels of p-Ser9-GSK-3β and p-Tyr216-GSK-3β were measured in the LV3-DEK and control groups to confirm this hypothesis. The results showed that p-Ser9-GSK-3β expression was lower in the LV3-DEK group than that in LV3-NC group (Fig. 4B) and that p-Tyr216-GSK-3β expression was higher in DEK silenced cells (Fig. 4B). This indicated that DEK regulated the Wnt/β-catenin by mediating GSK-3β phosphorylation rather than GSK-3β translation. To further prove this idea, indirubin, a powerful inhibitor of GSK-3β (33), was added. The result showed that DEK-induced downregulation of β-catenin could be partially reversed (Fig. 4C).

DEK impaired in vivo tumorigenesis. Xenograft tumorigenesis in nude mice was used to explore the effect of DEK on tumor formation in cervical cancer. The LV3-DEK cells and LV3-NC cells were implanted subcutaneously into the left armpit of nude female mice. Twenty-one days after transplantation, the tumors were harvested from mice (Fig. 5A). The average volume and weight of tumors in the LV3-DEK group were significantly smaller and lighter than those of

the LV3-NC group (Fig. 5B and C). The expression of DEK, p-Ser9-GSK-3β and β-catenin was reduced in the LV3-DEK, compared to the LV3-NC group (Fig. 5D). Conversely, p-Tyr216-GSK-3β expression was increased in the LV3-DEK group (Fig. 5D). These data showed that silencing DEK blocked *in vivo* tumor formation and inhibited the *in vivo* expression of p-GSK-3β.

Discussion

Cervical cancer is the most common gynecological malignancy and is intimately linked with HPV infection (34). DEK has been reported as an oncogene in acute leukemia, lung cancer, hepatocellular carcinoma, breast cancer, and other forms of cancer (35,36). The research has focused on its role in apoptosis, metastasis, and DNA damage. However, the role of DEK in cervical cancer has not been well established. In the present study, it was found that DEK is significantly overexpressed in cervical cancer tissues, compared to non-cancerous cervical tissue. Furthermore, high DEK expression in samples had a positive correlation with FIGO staging and tumor type. A higher proportion of DEK positive staining was found in squamous carcinoma, compared to adenocarcinoma. These results demonstrate that DEK is an oncogene in cervical cancer and is related to squamous carcinoma, which is highly linked to HPV infection. Determine whether or not DEK expression is correlated to HPV infection, however, requires further research.

DEK promotes proliferation, epithelial-mesenchymal transition (EMT), and metastasis in various cancer cells. In order to investigate the function of DEK in cervical cancer, the present study employed a lentiviral vector to inhibit DEK expression in functional assays. The results showed that silencing DEK inhibited cervical cancer cell proliferation, migration and invasion. The results indicate that DEK acts as a promoter in cervical cancer cell metastasis, a result in line with a previous study of DEK in acute leukemia cells, hepatoma cells, and colorectal cancer cells. These results partially explain the relationship between overexpression of DEK and FIGO staging in cervical cancer tissues.

The Wnt pathway is a classic signaling pathway which regulates cell proliferation, migration and invasion. Thus we investigated the effect of DEK on Wnt/β-catenin pathway. Our results showed that silencing DEK inhibited not only Wnt pathway activity but also MMP-9 expression. MMP-9 is a downstream target for Wnt signaling, which is also closely related to tumor metastasis (32,37). Taken together, these data could be the explanation to the decrease of invasion and migration after DEK silencing in SiHa cells.

Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine protein kinase (25) that plays an important role in early embryo development, neurodegenerative disease, diabetes, inflammatory conditions and oncogenesis (38). It has also been reported to regulate transcription factors, such as nuclear transcription factor-κB (NF-κB), p53 and β-catenin (19,22,23). While GSK-3β can be inactivated by phosphorylation at the N-terminal Serine 9 residue, phosphorylation at the Tyrosine 216 residue activates GSK-3β (20). In the present study, we found that DEK regulated GSK-3β at post-translational protein modification level rather than at the translation level. DEK inhibited GSK-3β activity such that the downstream target was activated. Under normal conditions, GSK-3β, Axin, CK-I and β-catenin form a compound and promote β-catenin degradation (27). In the absence of Wnt signaling, the compound disintegrates and β-catenin accumulates and becomes active. Therefore, abnormally high DEK expression can phosphorylate GSK-3β at Ser9 and inhibit Tyr216 phosphorylation, thereby inactivating GSK-3β. Consequently, β-catenin accumulated and aberrantly increased downstream target genes, which facilitated tumor metastasis, proliferation and other malignant behavior. These data explained the results of high positive rate in IHC staining in cervical cancer samples and the phenotype of decreased proliferation, migration and invasion in DEK silenced cervical cancer cells.

In summary, the results reported herein highlight the determination that DEK is an oncogene in cervical cancer and may serve as a novel target for cervical cancer research and treatment.

Acknowledgements

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