Stable silencing of *dll4* gene suppresses the growth and metastasis of esophagus cancer cells by attenuating Akt phosphorylation

XIANZHI GUO[1*], YINGCHUN DUAN[1*], XIAOLEI YE[2*], LINA HU[1], TONG XU[1], LI TONG[2] and MINGHUA YU[1]

[1]Department of Medical Oncology, Fudan University Pudong Medical Center, Shanghai 201399;
[2]Ningbo Institute of Medical Sciences, Ningbo University, Ningbo, Zhejiang 315020, P.R. China

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Abstract. δ-Like ligand 4 (DLL4) has recently been reported to be involved in the process of cancer angiogenesis and considered to play a vital role in vascular endothelial growth factor (VEGF) signaling, while the role of DLL4 in cancer metastasis and growth has not been systematically studied. In the present study, the esophagus cancer cell line Eca109 was infected *in vitro* with a lentiviral vector loaded with *dll4*-shRNA to obtain a stable cell line of DLL4 expression which was downregulated through puromycin screening. The migration and invasion ability of the Eca109 $^{dll4\text{-shRNA}}$ cells were evaluated by scratch and Transwell assays, respectively. The underlying signaling pathway was further explored by western blotting. Subsequently, to explore the role of *dll4* in the development of esophagus cancer cells *in vivo*, a xenograft model was established by intraperitoneal injection with Eca109 $^{dll4\text{-shRNA}}$ cells containing luciferase activity in nude mice. Then, small animal imaging system was used to evaluate the volume and metastatic potential of the tumors. Additionally, the overall survival rate of the nude mice was also recorded. Following infection with lentivirus, the expression of DLL4 in the Eca109 cells could be stably silenced through screening with puromycin, which was confirmed by western blotting. The scratch and Transwell assays demonstrated that downregulated DLL4 significantly diminished the aggressive invasion and migration properties of the Eca109 cells. The underlying mechanisms may be attributed to the inactivation of the PI3K/Akt/E-cadherin pathway by western blotting. Finally, the results from the *in vivo* study indicated that the tumor growth rate in the Eca109 $^{dll4\text{-shRNA}}$ group, as displayed by the tumor volume and the weak staining of the proliferating cell nuclear antigen (PCNA), was significantly slower than the control group, and the metastasis ability of the Eca109 $^{dll4\text{-shRNA}}$ cells was also dramatically abolished *in vivo*. It was also observed that downregulated DLL4 led to the formation of less pulmonary nodules in mice lungs and to a prolonged survival rate of nude mice. In summary, this study revealed that DLL4 has pathophysiological roles on the progression of esophagus cancer cells, including migration, invasion and apoptosis, which indicated that DLL4 may be considered as a potent therapeutic target for the treatment of malignant esophageal cancer.

Introduction

Esophageal cancer, is the fourth leading cause of cancer-related deaths, causing ~220.000 deaths worldwide per year, with an annual mortality rate almost matching its incidence (1). Early diagnosis of esophageal cancer has been proved to be difficult, along with the unavailable therapeutic responses (2). At the early stages of esophageal cancer, certain changes in cell-cell and cell-matrix interactions lead to abnormal cell behavior and result in the invasive and malignant cell transformation. In the late stages of tumor development, metastasis is the predominant complication rendering esophageal cancer difficult to control, which has been identified as the main cause of high mortality (3). Therefore, it is urgently required to elucidate the underlying mechanism of disease progression and metastasis.

Normal cells constantly monitor the cellular microenvironment. They are equipped with an inherent molecular defense to detect changes and then block several oncogenic signaling cascades in the internal and external cell environment (4). During embryonic development, epithelial cells escape the structural constraints imposed by tissue architecture and adopt a phenotype more amenable to cell movement, which has been known as epithelial-mesenchymal transition (EMT). In addition, the progression of carcinomas to invasive and metastatic disease may involve localized occurrences of EMT (5). Research has demonstrated that the Notch signaling pathway participated in the invasion and metastasis of esophageal carcinoma through EMT.

The Notch pathway plays an important role in normal embryonic development, in maintaining the characteristics of cancer stem cells and in cell metastasis (6). DLL4 belongs to the Notch protein family, along with DLL1, DLL3, Jagged 1

Correspondence to: Dr Minghua Yu, Department of Oncology, Shanghai Pudong Hospital, Fudan University, 2800 Gongwei Road, Pudong, Shanghai 201399, P.R. China
E-mail: ymh3011@hotmail.com

*Contributed equally

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(JAG1) and JAG2. In recent years, Notch receptor ligands have been frequently reported to be involved in tumor proliferation and metastasis. Specifically, DLL1 promoted the metastasis of melanoma by regulating the adhesion molecules (7) and Jag1 played an important role in bone metastasis of breast cancer (8). Breast cancer cells with high Jag1 expression directly affected osteoblasts and osteoclasts, increasing the secretion of IL6 and TGF-β and finally promoting the proliferation and metastasis of cancer cells (8,9). Similarly, Jag2 promoted cancer metastasis by regulating the Notch signaling pathway in several types of cancer, such as pancreatic, bladder, breast and lung cancer (10-13). Conversely, the role of DLL4 in normal vascular development was first established by Reinacher-Schick *et al* (18).

Previous studies, have mainly focused on the effectiveness of DLL4 in regulating angiogenesis. However, the role that DLL4 plays in tumor metastasis has not been fully explored. In 2014, Huang *et al* (14) reported that the overexpression of DLL4 promoted the migration and invasion of renal cell carcinoma by activating Notch signaling cascades, which promoted further studies of DLL4 in regulating tumor metastasis. Similarly, evidence indicated that DLL4 was highly expressed in breast cancer patients and furthermore, DLL4 expression was also positively related to lymphatic metastasis (15). Additionally, by detecting DLL4 plasma levels, total *dll4*$^{+}$ breast cancer cell and vasculature activity can be reliably estimated, which indicated that DLL4 could be regarded as a novel target in tumor therapeutic management (16).

In the present study, in order to elucidate the mechanism which DLL4 is involved in cell invasion and metastasis in esophagus cancer, initially, the *dll4* gene was stably silenced in the Eca109 cells, then the expression of DLL4 in esophagus cancer and adjacent non-cancerous tissues was detected and further analysis of its correlation *in vitro*. Notably, we observed that downregulated DLL4 suppressed the expression of p-Akt, and subsequently, led to the induction of migration and invasion, as well as apoptosis of esophagus cancer cells. Stable silencing of *dll4* also reduced esophageal tumor growth and extended survival in xenograft animal models by affecting the phosphorylation of Akt. These findings associated DLL4 with metastasis in esophagus cancer and also provided a promising target for treating late stage patients with esophagus cancer.

Materials and methods

Cell culture. 293T cells and Eca109 cells were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). 293T and Eca109 cells were separately maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin solution at 37˚C in a humidified atmosphere of 5% CO_2.

dll4 shRNA construction and lentiviral production. Three target sequences for *dll4* mRNA were chosen according to the RNAi Consortium (TRC) shRNA Library. The *dll4* shRNA single-strand oligonucleotides are listed as follows: shRNA1 forward, 5'-CCGGCTCTCCAACTGCCCTTCAATTCTCGAGAATTGAAGGGCAGTTGGAGAGTTTTTG-3' and reverse, 5'-AATTCAAAAACTCTCCAACTGCCCTTCAATTCTCGAGAATTGAAGGGCAGTTGGAGAG-3'; shRNA2 forward, 5'-CCGGGTGTCGGATATCAGCGATATGCTCGAGCATATCGCTGATATCCGACACTTTTTG-3' and reverse, 5'-AATTCAAAAAGTGTCGGATATCAGCGATATGCTCGAGCATATCGCTGATATCCGACAC-3'; shRNA3 forward, 5'-CCGGACCACACATTGGACTATAATCCTCGAGGATTATAGTCCAATGTGTGGTTTTTTG-3' and reverse, 5'-AATTCAAAAAACCACACATTGGACTATAATCCTCGAGGATTATAGTCCAATGTGTGGT-3'. Additionally, shRNA without homology to *dll4* gene and other genes was selected as the negative control (shScramble), and its sequence was as follows: shScramble forward, 5'-CCGGACTCATCTATTCGTCCACTCCCTCGAGGGAGTGGACGAATAGATGAGTTTTTTG-3' and reverse, 5'-AATTCAAAAAACTCATCTATTCGTCCACTCCCTCGAGGGAGTGGACGAATAGATGAGT-3'.

Two single-stranded, complementary oligonucleotides containing the target sequences were chemically synthesized by Sangon Biotech (Shanghai, China) and annealed. The double-stranded oligonucleotides were then inserted into pLKO lentiviral vector between the *Age*I and *Eco*RI restriction sites [New England Biolabs (NEB), Ipswich, MA, USA]. pLKO lentivirus vector contains CMV promoter-driven enhanced green fluorescent protein (eGFP) reporter gene and puromycin selectable marker. After ligation, plasmid was transformed into *E. coli* DH5α competent cells (maintained by our own laboratory) for plasmid amplification. The plasmids from positive colonies were identified by *Xho*I (NEB) digestion and confirmed by DNA sequencing (Boshang Biotech, Hangzhou, China).

Recombinant lentiviruses were produced by co-transfecting with three combinant lentiviral vectors, Δ8.91 and pVSVG (10:10:1, mass ratio) in 293T cells and the transfection reagent X-tremeGENE™ HP DNA (Roche Diagnostics, Indianapolis, IN, USA) was used to facilitate the transfection. The culture supernatants containing the lentiviral particles were harvested at 24, 48 and 72 h after transfection, mixed and centrifuged at 10,000 x g at 4˚C for 10-15 min to discard cell debris. Eca109 cells were subcultured at a density of $1x10^6$ cells/6 cm dish. The cells were divided into 5 groups: the control group (infected with vehicle plasmids), the vector group (infected with shScramble lentiviral vector) and three *dll4*-shRNA groups (infected with 3 different shRNA sequences designed for *dll4*). Subsequently, the Eca109 cells were infected with the same titer virus for 3 days continuously, followed by screening with 2 μg/ml puromycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The cells were maintained and allowed to grow for 7-9 days, changed to fresh medium without puromycin, and then passaged for the following assays.

Western blotting. After being washed with cold PBS for 3 times, the cells were lysed in RIPA buffer which composed of 0.6 M Tris-HCl (pH 6.8), 10% SDS and protease inhibitor cocktail. Cell lysates were collected and centrifuged at 10,000 x g for 15 min at 4˚C. The supernatants were transferred, mixed with 2X sampling buffer and boiled for 10 min. Following the BCA assay, protein was separated by SDS-polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was

blocked with 5% non-fat milk (Nestlé S.A, Vevey, Vaud, Switzerland) in TBS-T (10 mM Tris-Cl, pH 7.5, 100 mM NaCl and 1% Tween-20) at room temperature for 1 h. Subsequently, the membranes were incubated with primary antibodies such as DLL4 (1:1,000; cat. no. ab7280; Abcam, Cambridge, MA, USA), AKT (1:1,000; cat. no. 9272S; Cell Signaling Technology, Inc. Danvers, MA, USA), p-AKT (1:1,000; cat. no. 9271S; Cell Signaling Technology), E-cadherin (1:500; cat. no. 743712; BD Biosciences, Franklin Lakes, NJ, USA) and β-actin (1:1,000; cat. no. 4767; Cell Signaling Technology) at 4˚C overnight. The membrane was washed by TBS-T three times (3x10 min), and then incubated with HRP-labeled secondary antibody (1:2,000; cat. no. 7074s; Cell Signaling Technology) for 1 h at room temperature. After being washed again for three times (3x10 min), the blots were subjected to chemiluminescence using an ECL kit (cat. no. 6883s; Cell Signaling Technology) and signals were recorded under the C-DiGit Western Blot Scanner (LI-COR Biosciences, Lincoln, NE, USA).

DAPI staining. The cells were plated in a 6-well-dish with coverslip to perform the DAPI staining. Twenty-four hours later, the cells were washed by 1X PBS for 3 times and stained with DAPI (1 μg/ml, dissolved with 1X PBS) for 10 sec, then fixed with 4% paraformaldehyde. The coverslips were washed by 1X PBS for another 3 times, and then were observed under a fluorescence microscope.

Annexin V-FITC/PI double-staining. The Annexin V-FITC/PI double-staining was used to detect cell apoptosis. The Eca109 cells treated with shScramble or *dll4*-shRNA1 for 48 h were collected, washed twice with PBS, and then suspended with 1X binding buffer to adjust the cell concentration to $1x10^8$/ml. Samples were treated according to the Annexin V/PI apoptosis detection kit and analyzed by flow cytometry. Cells (10,000) were analyzed each time. The experiment was repeated three times, and representative data from one experiment were presented.

Scratch wound healing assay. The migration ability of the Eca109 cells was assessed by scratch assay as follows: when cells grew to 100% confluency on the 6-well plate, straight scratches were made by a sterile 10 μl pipette tip. The medium was discarded and cells were washed once with PBS to remove the floating cells. For minimizing the interference of cell proliferation, 10% FBS complete medium was replaced by RPMI-1640 without FBS. After 24-h incubation, the plate was removed from the incubator and images were captured under the microscope (Olympus IX71 inverted system microscope; Olympus Corp., Tokyo, Japan). The average wound closure rate was calculated using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). All the experiments were repeated three times.

Matrigel invasion assay. A 24-well plate with inner chamber (Corning Inc., Corning, NY, USA) was used for the invasion assay. The 8-μm pore membranes were pre-coated with Matrigel (BD Biosciences), which was mixed with RPMI-1640 without FBS at a dilution of 1:8. After incubation and hydration with RPMI-1640 for 0.5 h, the Eca109 cells of the control group and the *dll4* shRNA group at the density of $2x10^4$/chamber were inoculated in the inner chamber with RPMI-1640 with 1% FBS and 0.1% bovine serum albumin (BSA). Subsequently, 500 μl RPMI-1640 containing 10% FBS was placed in the lower chamber. After incubation at 37˚C for 36 h, cells on the upper surface of the filters were removed using a cotton swab. The chamber was kept at room temperature for 30 min and then immersed with 0.5% crystal violet containing 1% methanol for another 30 min. The crystal violet was washed with PBS for three times. Cells on the lower chamber were counted under a microscope in four randomly selected fields. All of the experiments were repeated three times.

Xenograft model. Mice were treated according to the NIH Guide for the Care and Use of Laboratory Animals (17) and the animal study was approved by the Ethics Committee of Ningbo University. The mice were housed in a temperature-controlled room with proper dark-light cycles, fed with a regular diet and maintained under the care of the Laboratory Animal Unit of Ningbo University, China. The mice were acclimated for 1 week before the beginning of the experiment. The Eca109 control, Eac109 vector as well as Eca109 with *dll4*-silenced cells grown in logarithmic phase were trypsinized, resuspended and harvested. After being stained with trypan blue, the cells were counted under a hemocytometer to assess their viability. Viable cell concentration was adjusted with culture medium to $1x10^7$ cells/ml. The nude mice were randomly divided into 3 groups (n=10 of each), corresponding to the 3 different cell lines. Each nude mouse was inoculated with 0.5 ml of cell suspension. The needle was stopped internally for 5 sec, rotated and pulled out to avoid leakage of the cell suspension. The activity, diet and mental state of nude mice were observed daily. The tumor volume was evaluated by a caliper every 5 days. At the 30th day of the experiment, the mice were intraperitoneally injected with 200 μl of D-luciferin (15 mg/ml), anaesthetized with isoflurane by a gas manifold at air flow rate of 2%, and then placed under the animal imaging system to record bioluminescent signals after 10-15 min of anesthesia. At the end of the experiment, the mice were sacrificed by cervical vertebra dislocation. The tumors and pulmonary nodules were stripped and counted, and then tumors were frozen under liquid nitrogen. Immunohistochemistry (IHC) and western blot analysis were performed to detect the expression of proliferating cell nuclear antigen (PCNA) and DLL4, respectively.

IHC assay. IHC assay was performed to detect the protein expression in xenografts. Anti-rabbit polyclonal immunoglobulin G antibody against PCNA (1:200; cat. no. ab18197; Abcam) was used as the primary antibody. Immunohistochemical kit (SP-9001) was obtained from Beijing Zhongshan Golden Bridge Biotechnology, Co., Ltd., (Beijing, China). For each sample, one score was given according to the percent of positive cells as follows: no positive cells, 0; <5% of the cells, 1 point; 5-35% of the cells, 2 points; 36-70% of the cells, 3 points; >70% of the cells, 4 points. To achieve objectivity, the intensity of positive staining was also used in a 4-scoring system as follows: 0 (negative staining), 1 (weak staining exhibited as light yellow), 2 (moderate staining exhibited as yellow brown), and 3 (strong staining exhibited as brown). A final score was

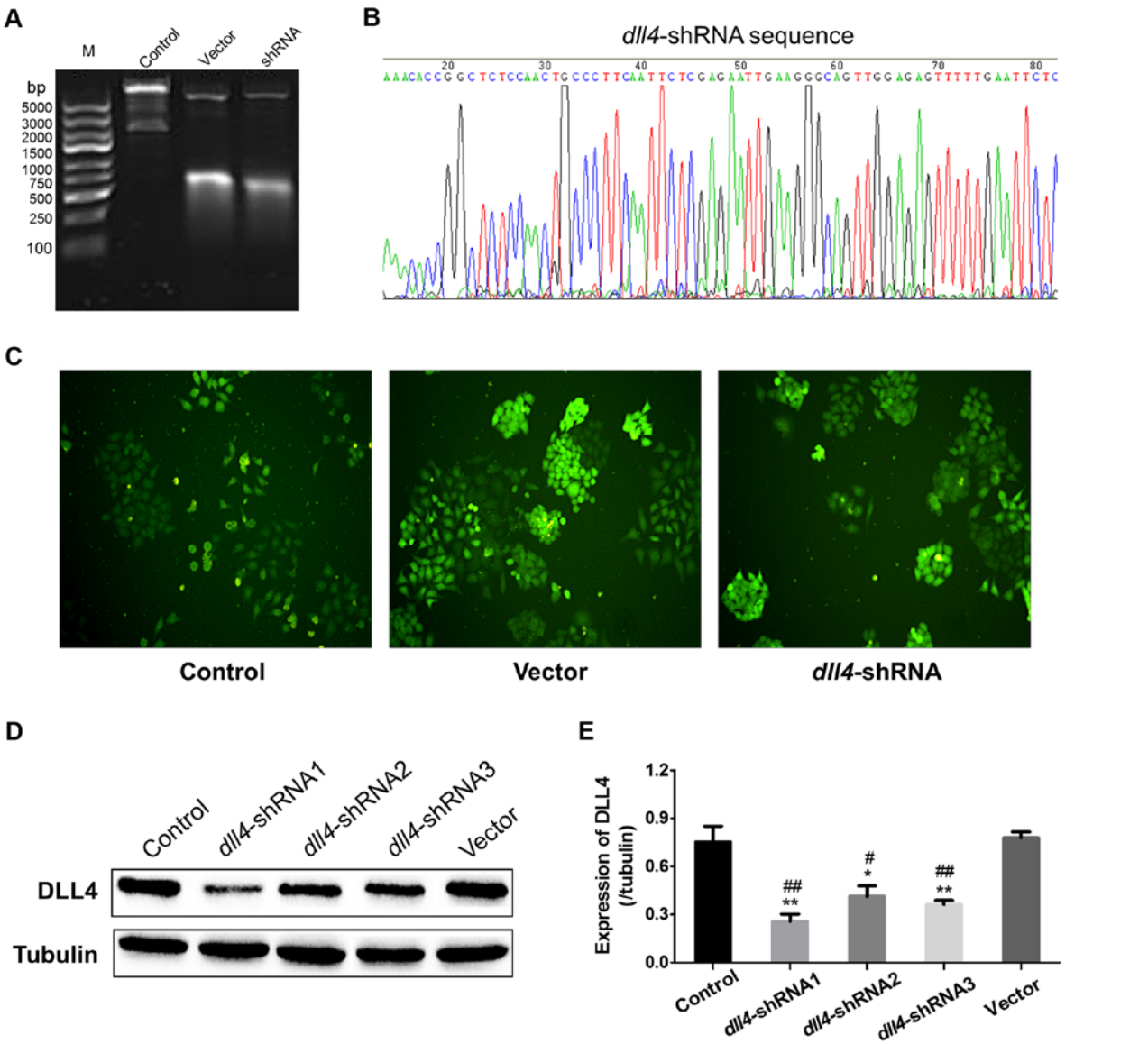

Figure 1. Stable silence of δ-like ligand 4 (DLL4) in Eca109 cells. (A) The plasmids were verified by enzyme digestion. (B) Sequencing data for *dll4*-shRNA plasmids. (C) Expression of green fluorescence protein in the Eca109 cells following infection with *dll4*-shRNA lentivirus particles; scale bar, 40 μm. (D) Western blotting detected the expression of DLL4 in the Eca109 cells after screening with puromycin. (E) Histogram of the DLL4 protein quantification of 1D, using tubulin as an internal control.

then calculated by multiplying the above two scores. If the final score was equal or >4, the tumor was considered as high expression, otherwise, it was considered as low expression.

Statistical analysis. All statistical analyses were performed using the SPSS 13.0 statistical software package (SPSS, Inc., Chicago, IL, USA). The data evaluated were normal distribution and variance homogeneity, and were expressed as the means ± SD. Survival curves were plotted by the Kaplan-Meier method and compared by the log-rank test. The significance of various variables for survival was analyzed using the Cox proportional hazard model in the multivariate analysis. *In vitro* data statistical analysis was performed using two-tailed Student's t-test or one-way ANOVA followed by the Tukey's test by GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ in all cases was considered to indicate a statistically significant difference.

Results

Construction and identification of stable knockout DLL4 cells. The primary plasmids of pLKO-EGFR-TRC as well as the recombinant plasmids were digested by *Xho*I at 37˚C for 2 h, ensuing with an examination by 0.8% agarose gel electrophoresis. The primary plasmids of DNA fragment were identified to be 7,872, 2,155 and 190 bp, while fragment of the recombinant plasmids after digestion should be changed to 7,872, 190, 303 and 42 bp, since the agarose concentration was at low concentration of 0.8% for distinguishing the large fragments, the small fragments basically run out of the agarose, therefore, it was difficult to detect both the large and small fragments in one gel, however, we could still judge that the recombinant plasmid was successfully constructed according to the typical bands at large size (Fig. 1A). After plasmids sequencing to identify the insertion sequence (Fig. 1B), the lentivirus was

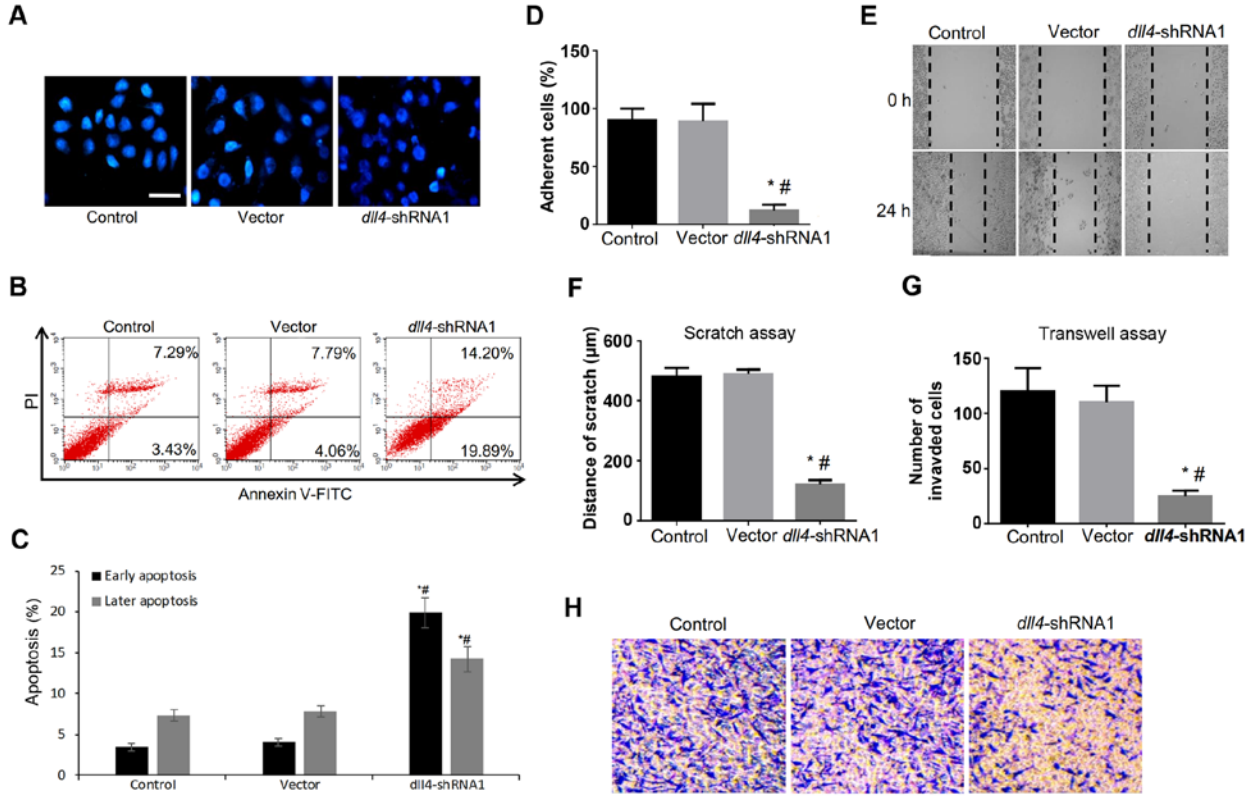

Figure 2. The role of δ-like ligand 4 (DLL4) in regulating cell adhesion, migration and invasion. (A) DAPI staining indicated that *dll4*-shRNA1 significantly induced cell apoptosis compared with the control group; scale bar, 20 μm. (B) Annexin V/PI double staining indicated the increased apoptosis rate in the *dll4*-shRNA1 group. (C) Histogram indicating the percentage of later and early apoptosis cells in 3 groups; *P<0.05 vs. vector group, $^{\#}$P<0.05 vs. control group. (D) Proportion of adherent cells after downregulation of the expression of DLL4 in the Eca109 cells; *P<0.05 vs. vector group, $^{\#}$P<0.05, vs. control group. (E) Twenty-four hours scratch assay for investigating role of DLL4 in regulating cell migration. (F) Quantitative results for the scratch assay; *P<0.05 vs. vector group, $^{\#}$P<0.05 vs. control group. (G and H) Transwell assay for investigating the role of DLL4 in regulating cell invasion (24 h) and quantitative results for the Transwell assay; *P<0.05 vs. vector group, $^{\#}$P<0.05 vs. control group.

packaged by the recombinant plasmids coupled with VSV-G and Δ8.91. Using lentivirus infection and screening by puromycin, the cell lines of control, vector and *dll4*-shRNA were successfully constructed and observed under a fluorescence microscope (Fig. 1C). Finally, the expression of DLL4 was detected by western blotting and the results indicated that *dll4*-shRNA1 could lead to an ~85% downregulation of DLL4 in the Eca109 cells (Fig. 1D and E). Therefore, *dll4*-shRNA1 was chosen for the stable silencing of *dll4* in Eca109 cells.

The role of DLL4 in regulating cell apoptosis, migration and invasion. In cancer, cell invasion allows cancer cells to acquire the ability to enter lymphatic and/or blood vessels for dissemination into the circulation, followed by invasion to distant organs for metastatic growth. To examine the role of DLL4 in esophagus carcinogenesis, we examined the effects of *dll4* knockdown on the proliferation, migration and invasion of the Eca109 cell line.

After DAPI and Annexin V-FITC/PI staining, a trend of apoptosis induction was observed in the *dll4*-shRNA group (Fig. 2A and B). There was a significant difference in cell apoptosis rate between the *dll4*-shRNA group and the control or vector group (34.09 vs. 10.72 or 11.85%) (Fig. 2C). Cell adherent assay also indicated the abolished ability of cell adhesion to fibronectin after the downregulation of DLL4 (Fig. 2D). Furthermore, the scratch assay (Fig. 2E) and the Transwell assay demonstrated that migration (Fig. 2F) and invasion (Fig. 2G and H) of the Eca109 cells significantly decreased after the *dll4* silencing. These results indicated that DLL4 may play a vital role in regulating the apoptosis and invasion ability of esophagus cancer cells.

Knockdown of dll4 attenuates the phosphorylation of Akt and downregulates the expression of E-cadherin. Given that DLL4 positively correlated with enhanced esophagus cancer cell migration and invasion, we examined whether p-Akt or E-cadherin is an underlying mechanism. Dysregulation of E-cadherin has been considered to be the major critical molecule in epithelial-mesenchymal transition (EMT)-mediated cancer cell metastasis. In the present study, we explored the role of DLL4 in the regulation of p-Akt and E-cadherin expression in the Eca109 cell. The expression of Akt and p-Akt was detected by western blotting. The results indicated that p-Akt was significantly decreased after the *dll4*-gene silencing (Fig. 3). In contrast, as a key molecule that regulates cell migration, the expression of E-cadherin significantly increased in the *dll4*-shRNA1 group, which indicated that E-cadherin may be responsible for regulating cell adhesion in esophagus cancer (Fig. 3).

Downregulation of DLL4 abolishes the growth and metastasis of Eca109 cells. To examine the effect of DLL4 on tumor

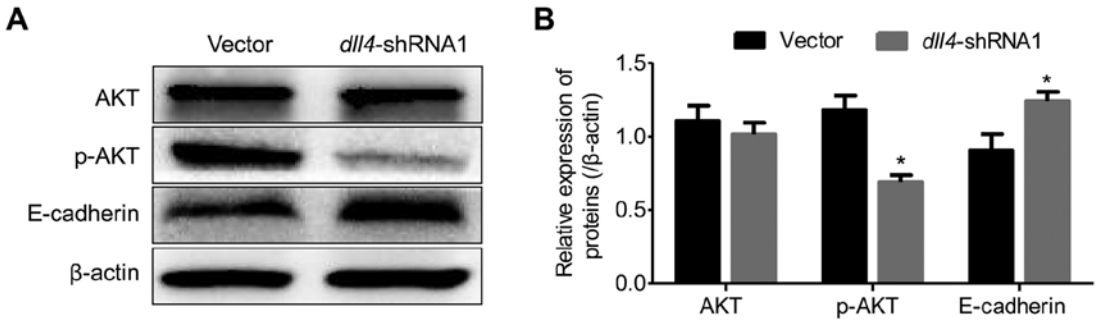

Figure 3. Downregulation of DLL4 attenuates the phosphorylation of Akt and upregulates the expression of E-cadherin. (A) The downregulation of DLL4 decreased the expression of p-Akt but had no effect on total Akt expression. Furthermore, *dll4*-shRNA treatment also upregulated the expression of E-cadherin. (B) The quantitative results of the western blot analysis (*P<0.05) vs. vector group.

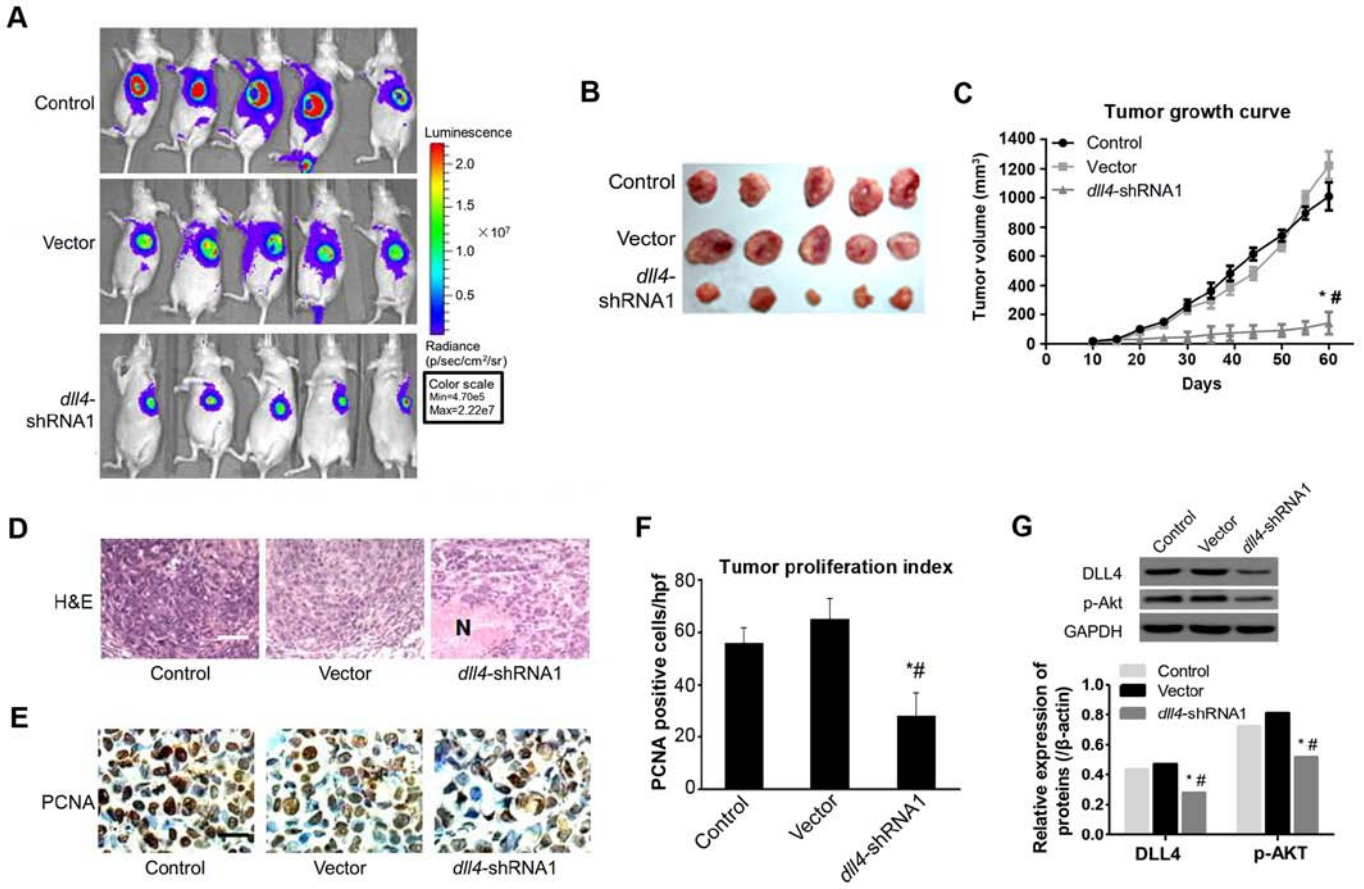

Figure 4. Tumor growth properties and mice survival rate after inoculation with Eca109 $^{dll4\text{-shRNA}}$ cells. (A) Bioluminescence imaging of implanted, luciferase-expressing Eca109 vector, shScramble or *dll4*-shRNA1 cells. (B) Macroscopic appearance of stripped tumors, whose volumes were recorded every 5 days during experiment, *dll4*-shRNA1 group shown more smaller volume than control or vector group (P<0.01). (C) Tumor growth curve after inoculation with Eca109 $^{dll4\text{-shRNA}}$ cells. (D) Hematoxylin and eosin (H&E) staining of tumors derived from xenograft, N, indicated extensive necrosis. Upper panel, the original images of mice lungs section was acquired under the light microscope after H&E staining, scale bar indicated 100 μm. Down panel, the percentage of pulmonary nodules and the number of large foci of lung tissue. (E) Immunohistochemistry (IHC) was performed to measure the expression of proliferating cell nuclear antigen (PCNA) in 3 groups, images were captured under the light microscope. Scale bar, indicated 20 μm. Proliferative index of tumor 60 days after inoculation. Proliferative index is estimated by the number of PCNA-positive cells per high-power field. Positive staining for PCNA is mainly located within the nuclei (magnification, x400). (F) The proliferative index of each tumor was calculated by averaging the number of PCNA-positive cells in 5 random bright fields of microscope (magnification, x400), counted in a blinded fashion by a single observer under the supervision of a pathologist; *P<0.05 vs. vector group, #P<0.05 vs. control group. (G) Western blotting detected the expression of DLL4 and p-Akt in xenografts. The tumor tissue lysates were probed with polyclonal anti-DLL4, monoclonal anti-p-Akt (ser473), and anti-β-actin as internal control. A representative of 3additional experiments is shown. Data depicted were derived from 3 independent experiments using at least 5 animals for each cell line injected; *P<0.05 vs. vector group, #P<0.05 vs. control group.

growth, we subcutaneously injected Eca109 cells transfected with vector or *dll4* shRNA into nude mice, and then monitored tumor growth for 60 days. As displayed in Fig. 4A, the tumor formation rate was 100%. Palpable tumors size was assessed during the experiment in the scrambled control sides. Downregulation of DLL4 had significant effect on tumor

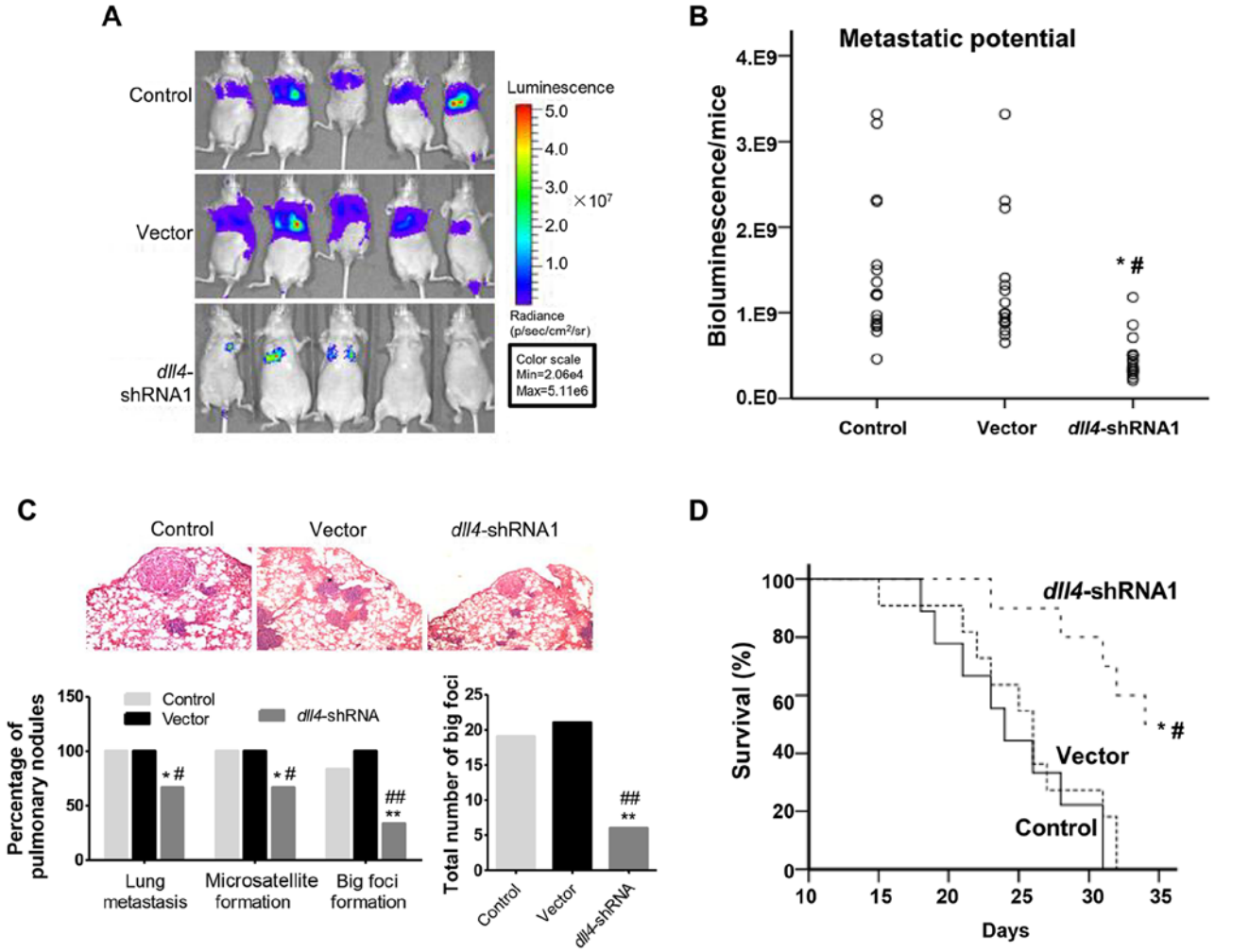

Figure 5. Tumor metastatic potential of dll4-knockdown cells. (A) Bioluminescence imaging of tail vein injected, luciferase-expressing Eca109 vector, control or Eca109 $^{dll4\text{-shRNA}}$ cells. Results were obtained 5 weeks after injection. Data depicted were derived from 3 independent experiments using at least 5 animals for each cell line injected; (B) Metastatic potential of Eca109-luc vector/control/*dll4*-shRNA1 cells as indicated by the quantification of the bioluminescence signal (photons/second/square centimeter) from each animal; (C) hematoxylin and eosin (H&E) staining of lungs and summary of aggressive features of experimental metastatic tumors derived from *dll4*-shRNA1 and control cells. Metastasis is lower in animals that received *dll4*-shRNA1 expressing cells through tail vein injection; *P<0.05, **P<0.01 vs. vector group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. control group. (D) Survival of mice with tail vein implantation of tumors derived from Eca109-luc cells stably expressing control or *dll4*-shRNA1 or empty vector. Asterisks indicate that the survival rate in the *dll4*-shRNA1 group was significantly different from the vector or control group; *P<0.05 vs. vector group, $^{\#}$P<0.05 vs. control group.

size (Fig. 4B) and growth curve (Fig. 4C). Compared with the vector (1,222.73±95 mm^3) and control group (1,009.5±95 mm^3), the *dll4*-shRNA group had significantly smaller tumor volumes at day 60 (P<0.05). Stripped xenografts were then subjected to pathological sections and stained with hematoxylin and eosin (H&E) (Fig. 4D) and PCNA was stained to evaluate the proliferation index of cancer cells. As displayed in Fig. 4F, the PCNA index was significantly lower in the *dll4*-shRNA group than in the vector and control group (P<0.05), which was also in consistense with the observation results from the tumor growth curve. Furthermore, to determine whether *dll4* knockdown would alter the expression of DLL4 and p-Akt *in vivo*, Akt and DLL4 expression were detected by western blotting. Densitometric analysis of western blotting revealed that the expression of both DLL4 and p-Akt was significantly lower in the *dll4* knocked-down cells (P<0.05; Fig. 4G).

The lung colonization ability of the Eca109 $^{dll4\text{-shRNA}}$ cells was then investigated after mice tail vein injection. Survival rates were examined and observed by small animal imaging systems (Fig. 5A). The control and vector groups, presented a significant amount of bioluminescence signal in the lung area, whereas in the *dll4*-shRNA group, little signals were detected in some mice. Subsequently, we quantified the bioluminescence signal in mice lung regions (Fig. 5B). In the *dll4*-shRNA group, the signal intensity was significantly lower, than that of the control vector group (P<0.05). At the end of the experiment, mice lungs were fixed with formalin and stained with H&E staining. In the control and vector groups, pulmonary nodules were observed at a higher percentage, while in the *dll4*-shRNA group pulmonary nodules were found in 66.6% of mice. In addition, we counted the number of large foci which were 6 in the Eca109 $^{dll4\text{-shRNA}}$ group, and 19 and 21 in the control and vector group, respectively (Fig. 5C). Finally, we observed that at the end of experiment, ~50% of the nude mice survived in the Eca109 $^{dll4\text{-shRNA}}$ group (Fig. 5D), which indicated that mice lifespan was prolonged in Eca109

dll4-shRNA group. Conversely, the nude mice in the control and vector group all died at the end of the experiment. The overall survival rate in the Eca109 *dll4*-shRNA group was higher than that in the control and vector group and the difference was statistically significant ($P<0.05$).

Discussion

At the early stages of esophageal cancer, certain changes in cell-cell and cell-matrix interactions lead to abnormal cell behavior and result in invasive and malignant transformation. In the late stages of tumor development, metastasis is the predominant complication and makes esophageal cancer difficult to control, which has been identified as the main cause of high mortality (17).

DLL4 plays a significant role in normal vascular development, which has been hypothesized as a potential target for anti-angiogenic therapy (18). In a previous study (19) it was observed in a mouse model of tumor-xenograft, that YW152F (DLL4 mAb) significantly increased vascular density, followed by a decrease in tumor growth rate, which clearly established the potential role of DLL4 in inhibiting tumor growth. However, the molecular mechanism through which DLL4 regulates vessel patterning remained unclear until recently. Despite extensive studies on vascular endothelial growth factor (VEGF) and DLL4 signaling, little is known on the role of DLL4 on cancer metastasis (20). In order to further elucidate the above-mentioned observations, an shRNA lentiviral vector was constructed to knockdown the expression of DLL4 in the Eca109 cells. Subsequently, the migration and invasion ability of *dll4*-shRNA1 cells downregulated significantly. The benefit of the lentiviral vector transfection is that the induced cells have heritable changes characteristics for subsequent study. Gene silencing caused by siRNA interference methods is unstable, which leads to genetic trait recovery. Of course, there are also some defects in the lentiviral technique. It could be time-consuming in the process of puromycin screening in requiring the target cells. It would be better if the upstream promoter of DLL4 was modified, which may be related to an inducible function of tetracycline, to make the process of gene silencing more credible.

The existing studies on DLL4 are mostly focusing on the role of DLL4 in regulating angiogenesis. Few studies aimed to investigate DLL4 in tumor metastasis and tumor growth. Previous research on cervical cancer indicated that DLL4 expression at both the mRNA and protein level in cervical cancer tissues was significantly higher than that in normal cervical tissues. Univariate and multivariate logistic regression analyses demonstrated that overexpression of DLL4 was strongly associated with lymph node metastasis. Furthermore, survival analysis revealed that the expression of DLL4 was an independent factor of an unfavorable overall survival. The study indicated that DLL4 may be a potential clinical diagnostic marker for patients with early stage cervical cancer (21). In contrast, evidence showed that Notch signaling drives a cancer stem cell phenotype by regulating genes that establish stemness. Using patient derived xenograft models the authors demonstrated that inhibition of the Notch signaling cascades is efficacious in decreasing tumor growth. Furthemore, the Notch activity in a patient EUS-derived biopsy was also found to predict the outcome of chemotherapy (22).

In the present study, we evaluated the role of DLL4 in the esophagus cancer cell line Eca109.

The scratch and Transwell assays demonstrated that after the *dll4* silencing, the invasive and migration ability of the Eca109 cells significantly decreased, with attenuation of the Akt signaling pathway and increase of the expression E-cadherin. Furthermore, *in vivo* studies, our results indicated that the ability of *dll4*-shRNA cells in migration and invasion significantly decreased compared with the control and vector control. the expression of both DLL4 and p-Akt was significantly lower in the *dll4* knocked-down cells. However, the present study still has some limitations; for instance, the relationship between DLL4/Notch/Akt signaling cascades has not been identified. Future studies should focus on the exact molecular signaling pathway that plays a role in regulating the apoptosis and metastasis of esophageal carcinoma. Finally, in the present study we used one cell line, Eca109, to explore the role of DLL4 in the progression of esophagus cancer, which may be a limitation of this study. These data are preliminary and further research is required to verify them in other esophageal cancer cell lines. Furthermore, the underlying mechanism could be further identified and elucidated in future studies.

In conclusion, the present study indicated that DLL4 could be viewed as a novel target on esophageal carcinoma treatment and targeted therapy. Further understanding focusing on the molecular pathway and the protein which DLL4 targeted would be performed to better understand the role of DLL4 in the progress of esophagus cancer.

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Availability of data and materials

We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality.

Authors' contributions

XG, YD and X Y contributed the central idea, analysed most of the data, and wrote the initial draft of the paper. The remaining authors contributed to refining the ideas, carrying out additional analyses and finalizing this paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were approved by the Institutional Review Board of the Department of Laboratory Animal Science of Fudan University Pudong Medical Center (Shanghai, China).

Consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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