Down-Regulation of LAMP3 Inhibits the Proliferation and Migration of HSCC

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Abstract

Background: Lysosomal-associated membrane glycoprotein 3 (LAMP3) has been shown to be highly expressed in various types of tumors. It is associated with their poor prognosis, proliferation, invasion, and metastasis, respectively. However, the role of LAMP3 in hypopharyngeal squamous cell carcinoma (HSCC) is unclear.

Objective: This article aims to investigate the role of LAMP3 in the proliferation and metastasis of HSCC.

Method: Detection of the expression of LAMP3 in clinical HSCC and paired adjacent healthy tissue samples by using immunohistochemistry. Furthermore, LAMP3 was knocked out of the HSCC cell line, FaDu, using a lentivirus vector and the in vitro biological effects of LAMP3 knockdown in FaDu cells were studied.

Results: Immunohistochemistry results showed that LAMP3 was highly expressed in HSCC. Additionally, it was verified at the mRNA and protein levels that lentiviral transduction effectively down-regulated the expression of LAMP3 in FaDu cells. Cell colony formation assays and CCK8 proliferation assays showed that down-regulation of LAMP3 on FaDu cells inhibited cell proliferation and growth rate. Wound healing experiments and transwell experiments showed that significant down-regulation of LAMP3 on FaDu cells inhibited cell migration.

Conclusion: Collectively, our results suggest that LAMP3 is a useful therapeutic target for the treatment of hypopharyngeal cancer.

Background

In developing countries, the incidence of HSCC is increasing and the prognosis is poor as the 5-year overall survival rate is only 30%. [1, 2] Recent studies have identified factors contributing to HSCC, such as smoking and HPV infection. [3, 4] Radiotherapy, chemotherapy, and surgery have all successfully been used for HSCC treatment;[5] however, the 5-year overall survival rate of HSCC remains low. [2, 6] Furthermore, the pharynx has unique anatomical features; therefore, patients with postoperative hypopharyngeal cancer suffer from damage or loss of hypopharyngeal function, which can significantly impact the patients' quality of life.[7] Therefore, the molecules involved in HSCC require further study to enable the development of new treatments to improve the prognosis of patients.

LAMPs, collectively referred to as lysosomal-associated membrane proteins, represent a family of glycosylated proteins present on lysosomal membranes that are expressed differently between different tissues.[8] LAMPs can participate in and influence cellular metabolic processes such as autophagy, phagocytosis, lipid transport, and senescence. [8, 9] Current research indicates that the LAMP family contains the following five members: LAMP1 / CD107a, LAMP2 / CD107b, LAMP3 / DC-LAMP, LAMP4 / Macrosialin / CD68 and LAMP5 / BAD-LAMP. [8, 9] LAMP1 and LAMP2 are ubiquitously expressed in human tissues and cell lines, while LAMP3, LAMP4 and LAMP5 are only expressed under specific

conditions in specific tissues.[9] LAMP3 not only plays a role in the maturation of dendritic cells, but also a marker of transformed type II alveolar cells.[10, 11] There is increasing evidence that LAMP3 is highly expressed in various types of primary tumors in humans, including esophageal cancer,[12] cervical cancer,[13] breast cancer, [14, 15] colon cancer, fallopian tube cancer, ovarian cancer, and liver cancer.[16] To date, no studies have analyzed the expression of LAMP3 in HSCC.

In this study, we observed high expression of LAMP3 in HSCC by immunohistochemistry. LAMP3 was shown to regulate proliferation and migration of HSCC FaDu cells following lentiviral knockdown. Our results provide new clinical strategies for the treatment of HSCC patients.

Methods

Immunohistochemistry (IHC)

Thirty-five pairs of formalin-fixed, paraffin-embedded HSCC tissue specimens were collected. Specimens were obtained from the Department of Pathology, Affiliated Hospital of XXXX University. The whole project was approved by the ethics committee of the affiliated hospital of XXXX University(2017-L052). Following tissue sectioning, the slices were dewaxed and dehydrated with xylene and ethanol. Antigen retrieval was performed in an EDTA buffer (pH = 9.0). Endogenous peroxidase was blocked with 3% H_2O_2. Non-specific binding was blocked by treatment with blocking reagents, and then slides were incubated with anti-LAMP3 antibody (1:100, Sangon Biotech, China) at 4 °C overnight. Then, at room temperature, the slides followed by incubation with a secondary antibody (Sangon Biotech, China) for 30 minutes. The immunolabel was then displayed using a DAB detection kit (Sangon Biotech, China) to visualize the reaction. LAMP3 protein expression levels were analyzed by using Image-Pro Plus 6.0.

shRNA design and construction of recombinant lentiviral vectors

The small interfering RNA (siRNA) sequence (NM_ 27074) (CCGGTCAGAAGCCTGTTCA) targeting LAMP3 was transformed into short hairpin RNA (shRNA) (stem-loop-stem structure) and cloned into a PGIPZ vector (Asian, Shanghai China). To exclude possible off-target effects of shRNA, comparable results were obtained using two other constructs shRNA-1 (ACGATGGCAGTCAAATGAG) and shRNA-3 (GGAAGCAGACTCTGTATAA) against LAMP3. Interference efficiency was screened for using RT-PCR. Recombinant lentiviral vectors were generated by co-transfecting HEK293T cells with lentiviral expression vectors and packaging plasmid mixtures using Lipofectamine™ 2000. Infectious lentiviral particles were harvested 48 hours after transfection, centrifuged to remove cell debris, and then filtered, centrifuged, and the virus concentrated. The titer of recombinant lentivirus was determined by serial dilution on 293T cells.

Recombinant lentiviral transduction in FaDu cells and screening stable strains

For lentiviral transduction, FaDu cells were subcultured into 6-well culture plates at ($1–2 \times 10^6$) cells/well. After growth to 70% confluence, cells were transduced with a lentivirus expressing LAMP3 shRNA at a multiplicity of infection (MOI) of 10. An empty vector was used as a control. The cells were harvested after 48 hours and the transduction efficiency was assessed by counting the percentage of GFP-positive cells. Stably transformed strains were screened for drug resistance. The drug for screening drug resistance is puromycin. The cultures were expanded and the interference efficiency was verified by RT-PCR and western blotting.

Cell culture

The human hypopharyngeal carcinoma cell line FaDu was purchased from Zhong Qiao Xin Zhou Biotechnology (Shanghai, China). The media used to culture the cells was supplemented with 10% high quality fetal bovine serum (FBS) and 1% Penicillin-Streptomycin solution. The culture conditions were carried out in a 5% CO_2 37 ^{O}C incubator.

Western blot analysis

The cells were collected and the protein was extracted using a protein lysis buffer (RIPA: PMSF = 100:1) according to the manufacturer's recommendations. The protein concentration was determined using BCA working solution (Beyotime, Shanghai, China). The protein sample was loaded (20 μg per well) and separated by electrophoresis on a 10% SDS-polyacrylamide gel. Samples were then transferred to a polyvinylidene fluoride film (Millipore, Billerica, MA, USA) and the membrane was blocked in 5% skim milk for two hours. The membrane was incubated with primary antibody at 4 °C overnight (anti-LAMP3 antibody, 1:800, Sangon Biotech, China), (anti-GAPDH antibody, 1:1000, Sangon Biotech, China). After washing with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) for 1.5 hours. Finally, the signal was developed using a chemiluminescent agent (NCM Biotech, Suzhou, China). Images were captured and Image J software was used to compare and calculate gray values.

qRT-PCR

Total RNA from cells was extracted using TRIzol reagent (Ambion, Life Technologies, USA) according to the manufacturer's protocol. The concentration of RNA was measured using a SmartSpec Plus spectrophotometer (Bio-Rad). RNA was reverse transcribed into cDNA. Primers were designed as follows: h-LAMP3 sense: 5'-ACTACCCCAGCGACTACAAAA-3' and antisense 5'-CTAGGGCCGACTGTAACTTCA-3'. GAPDH sense: 5'-GGAGCGAGATCCCTCCAAAAT-3', antisense: 5'-GGCTGTTGTCATACTTCTCATGG-3'. The primers were designed by Asia-Vector Biotechnology (Shanghai) Co., Ltd. GAPDH levels were used to normalize the relative expression levels of LAMP3 mRNA using the DDCt method.

Scratch-wound experiments

The FaDu cells were seeded in a six-well plate and grown until the cells were at 80% confluence. A scratch was made along the surface of the cells with the tip of a 10 μl pipette. The cells were washed three times

with PBS and the culture media was replaced with serum-free basal culture media. Photographs were taken at 0 h and 24 h to measure the width of the scratch.

Cell migration assay

A single cell suspension with a density of 5×10^4 / ml was prepared using serum-free media. The cell suspension (100 μl) was added to the upper chamber of each transwell chamber and 600 μl of 20% FBS medium was added to the lower chamber as a chemical attractant. Three sub-wells were set up in each group. The 24-well plates were incubated at 37 °C for 24 hours in a 5% CO_2 incubator. Non-migrating cells on the chamber membrane were removed with a cotton swab. The cells under the chamber were fixed in paraformaldehyde for 30 minutes and stained with 0.1% crystal violet for 10 minutes. Photographs were taken (magnification, × 100) and the cells counted.

Cell proliferation assay

To the wells of a 96-well plate, 2000 cells in 100 μl were added and incubated at 37 °C for 24 h. Cell viability was determined using a CCK8 kit (Sangon Biotech, China) according to the manufacturer's procedures. The number of viable cells was evaluated by measuring the absorbance at 450 nm using a microplate reader at 0.5 h, 1 h, 2 h, and 4 h.

Colony formation assay

FaDu cells (600 per well) were seeded into a six-well plate. After incubation for 14 days at 37 °C, a cell mass visible to the naked eye was formed and removed. The cells were fixed with 4% paraformaldehyde for 30 minutes and stained with crystal violet for 10 minutes. The number of cell pellets was calculated using an optical microscope.

Statistical analysis

Data are expressed as mean ± standard deviation. Statistical analysis was performed using IBM SPSS Statistics 20.0 software (IBM, Armonk, NY, USA). $P < 0.05$ was considered statistically significant.

Results

High expression of LAMP3 in HSCC

To investigate the expression of LAMP3 in HSCC, we selected 35 pairs of paraffin-embedded HSCC tissues and their matched adjacent healthy tissues for immunohistochemical staining (Fig. 1). Immunohistochemical staining showed that LAMP3 protein was mainly expressed in the cytoplasm of the HSCC samples as shown by brownish yellow staining (Fig. 1AB). Furthermore, optical density analysis identified increased expression of LAMP3 protein in cancer tissues compared with adjacent tissues. This difference was statistically significant (Fig. 1E) ($P < 0.01$).

Screening of LAMP3 shRNA, lentiviral-mediated shRNA inhibition of LAMP3 expression in FaDu cells

To identify whether LAMP3 could be used as a therapeutic target for HSCC, we used RNA interference (shRNA) to inhibit the expression of LAMP3 in the HSCC cell line FaDu. To ensure interference efficiency, three shRNAs were designed, named sh1, sh2, and sh3. The shRNA was screened using HEK293T cells. We screened the most efficient shRNA by real-time quantitative RT-PCR for subsequent experiments (Fig. 2C). Compared with the negative control group, the mRNA level of LAMP3 in the transfected shLAMP3 interference plasmid group was decreased, and the difference was statistically significant (***$P < 0.001$). The most effective shRNA tested was LAMP3-sh2.

We then used the HEK293T cells to package the lentiviral vector and examined GFP expression by fluorescence microscopy after 48 hours (Fig. 2AB). Sequential dilutions explored the appropriate viral titers. The dilution ratio of the mock transfected conditioned control group was 10^{-5} and the dilution ratio of the experimental group was 10^{-6}.

FaDu cells had an MOI value of 10 with the highest infection efficiency. The FaDu cells were successfully infected with packaged lentivirus, and the stably transformed strain was screened using puromycin resistance and examined by fluorescence microscopy (Fig. 2DE). The interference efficiency was verified by RT-PCR (Fig. 2F, $p < 0.01$) and western blot (Fig. 2G, $p < 0.01$).

Down-regulation of LAMP3 inhibits the proliferation of FaDu cells

The effect of LAMP3 down-regulation on the proliferation of FaDu cells was assessed by CCK8 assay and colony formation assay. Cellular growth of mock transfected conditioned control cells (FaDu-NC) and the experimental group (FaDu-shLAMP3) was measured at five time points. The results showed that the proliferation of the FaDu-shLAMP3 group was significantly lower than the control group. (Fig. 3B, $P < 0.05$). Furthermore, the number of colonies formed in the experimental group was significantly lower than in the control group and the FaDu cell only group ($P < 0.05$). This is consistent with the results seen in the CCK8 experiments. (Fig. 3A). These results indicate that down-regulation of LAMP3 inhibits the proliferation of FaDu cells.

Down-regulation of LAMP3 inhibits FaDu cell migration

We analyzed the effects of down-regulation of LAMP3 on FaDu cell migration by scratch-wound experiments and transwell migration assays. The scratch-wound experiments showed that the migration ability of FaDu cells was inhibited in the LAMP3 experimental group compared with the control group (Fig. 4A, $p < 0.05$). The results of the cell migration test were consistent with the scratch-wound experiments, whereby, the migration ability of the experimental group was lower than the control group (Fig. 4B, $p < 0.01$). These results indicate that down-regulation of LAMP3 inhibits the migration of FaDu cells.

Discussion

LAMP3 is a protein encoded by the LAMP3 gene in humans.[17] LAMP3 is highly expressed in differentiated and mature dendritic cells,[18] as well as many cancer cell types, including esophageal cancer, cervical cancer, and breast cancer.[12–15] Reportedly, LAMP3 has a number of diverse functions including hepatic lipid metabolism [19] and is associated with Bipolar disorder and Schizophrenia.[20] Recently, LAMP3 has been reported as a specific tumor protein.[13] For example, Liao et al. showed that the expression of LAMP3 in the epithelium is directly related to the prognosis of esophageal squamous cell carcinoma.[12] Furthermore, Ishigami et al. demonstrated that the presence of LAMP3 positive tumor-infiltrating mature dendritic cells has prognostic value for gastric cancer.[20] This evidence suggests that the biological functions of LAMP3 are diverse; however, the mechanisms of actions of LAMP3 in HSCC have not been studied. In the present study, we identified that LAMP3 had an inhibitory effect on the proliferation, migration, and invasion ability of FaDu cells.

We determined the expression of LAMP3 in HSCC by immunohistochemical analysis and found that the expression of LAMP3 in tumor tissues was higher than that in adjacent healthy tissues. We hypothesized that LAMP3 was involved in the development of HSCC; therefore, we used the FaDu cell line to analyze the silencing of LAMP3 in vitro.

After silencing LAMP3, FaDu cells showed a significant decrease in proliferative capacity and colony forming ability. Consistent with our study, Liu et al found that knockdown of LAMP3 significantly inhibited osteosarcoma cell viability and promoted apoptosis.[21] Moreover, studies have found that cell proliferation is significantly inhibited after LAMP3 knockdown in laryngeal carcinoma cell lines, with a concurrent increase in apoptosis. Furthermore, after complementation with LAMP3, the inhibition of cell proliferation is diminished and apoptosis is also reduced.[22] These data indicate that LAMP3 promotes the proliferation and growth of the HSCC cell line FaDu.

The scratch-wound experiments and cell migration assay showed that the migration ability of the FaDu-shLAMP3 was significantly lower than the FaDu-NC and FaDu cell only groups. Kanao et al found that the migration of cervical cancer cells overexpressing LAMP3 was significantly higher than control cervical cancer cells during in vitro invasion assays.[13] Recent studies have shown that HAGLR acts as a competitive endogenous RNA to miR-143-5p to increase the expression of LAMP3, thereby promoting proliferation, invasion, and migration in esophageal cancer cells.[23] These are consistent with our findings, suggesting that LAMP3 may contribute to the invasion and migration of the HSCC cell line FaDu.

There are some limitations to our experiments, namely the low number samples used for tissue protein expression detection (n = 35); thus, protein expression levels could not be used for survival analysis.

Conclusion

we identified increased expression of LAMP3 in HSCC samples compared with healthy tissues and demonstrated that LAMP3 is closely related to the development of HSCC. These findings suggest that LAMP3 could be a useful therapeutic target for the treatment of HSCC; however, the molecular mechanisms regulating LAMP3 expression remain to be analyzed.

Abbreviations

HSCC
Hypopharyngeal squamous cell carcinoma cells.

Declarations

Ethics approval and consent to participate:

My Title of the manuscript Down-regulation of LAMP3 inhibits the proliferati

on and migration of hypopharyngeal squamous cell carcinoma cells. I promise

that the study was performed according to the international, national and institu

tional rules considering animal experiments, clinical studies and biodiversity rig

hts. The study protocol was approved by Nantong University Affiliated Hospita

l Ethics Committee.

Consent for publication:

Not applicable.

Availability of data and materials:

The datasets used and/or analyzed during the current study are avilable from the corresponding author on reasonable request.

Competing interests:

The authors declare that they have no competing interests.

Funding:

Not applicable.

Authors' contributions:

JJL,KKY and XXQ conceived and designed research; SJ,YY,LJB,XRH and YS collected data and conducted research,JJLand KKY wrote the initial paper, ZXZ revised the paper,XXQ had primary responsibility for final content,All authors read and approved the final manuscript.

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Figures

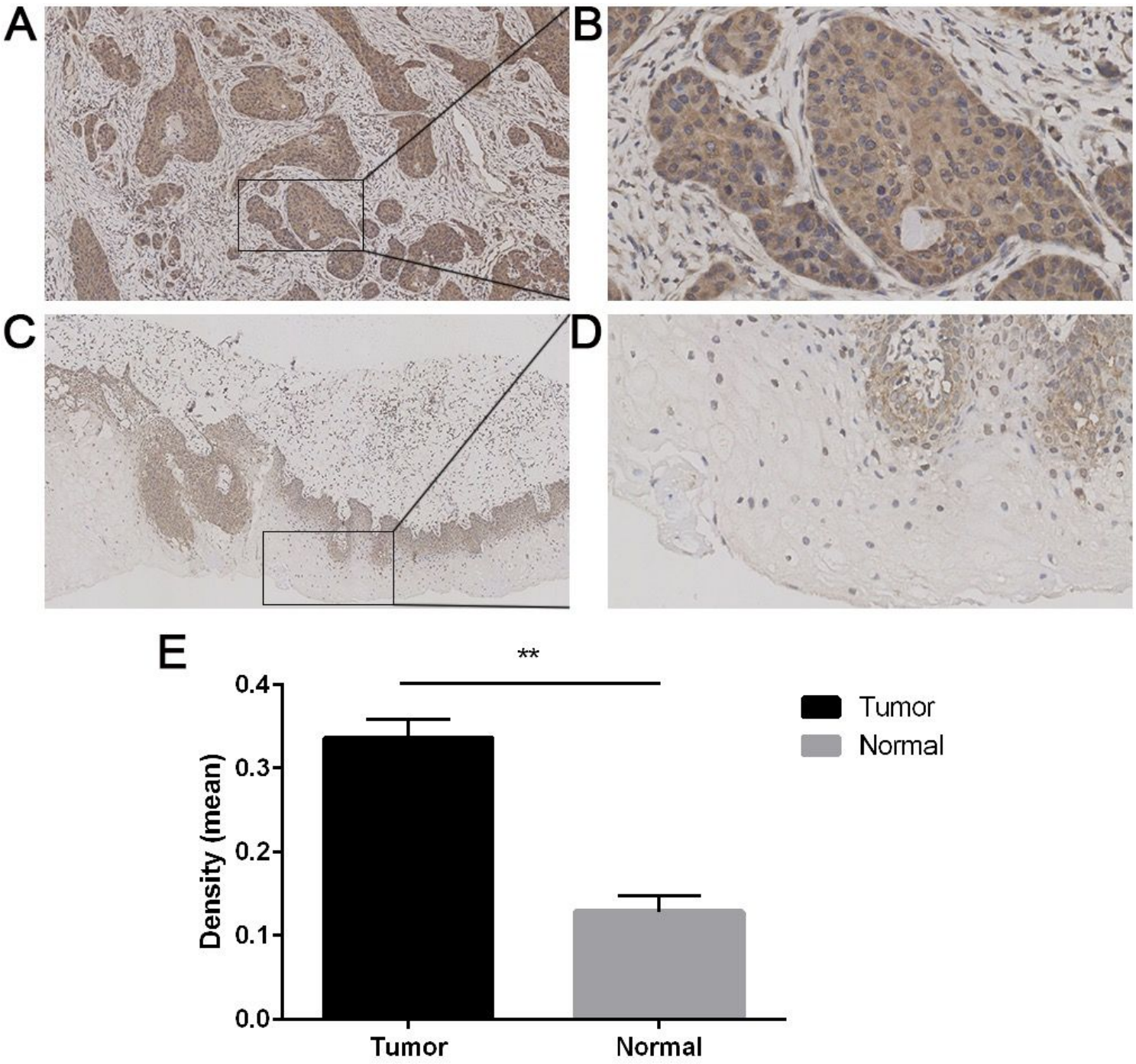

Figure 1

Determination of LAMP3 expression in hypharyngeal squamous cell carcinoma tissues and adjacent non-tumor tissues by IHC analysis. (A ,B)HSCC tissues showd high expression of LAMP3. (C ,D) Low expression level of LAMP3 was observed in adjacent non-tumor tissues. (A,C) Original magnification ×100. (B,D) Original magnification ×400. (E) Quantitative analysis of immunohistochemistry. Data were expressed as mean ± SD. **P < 0.01 represents statistical difference.

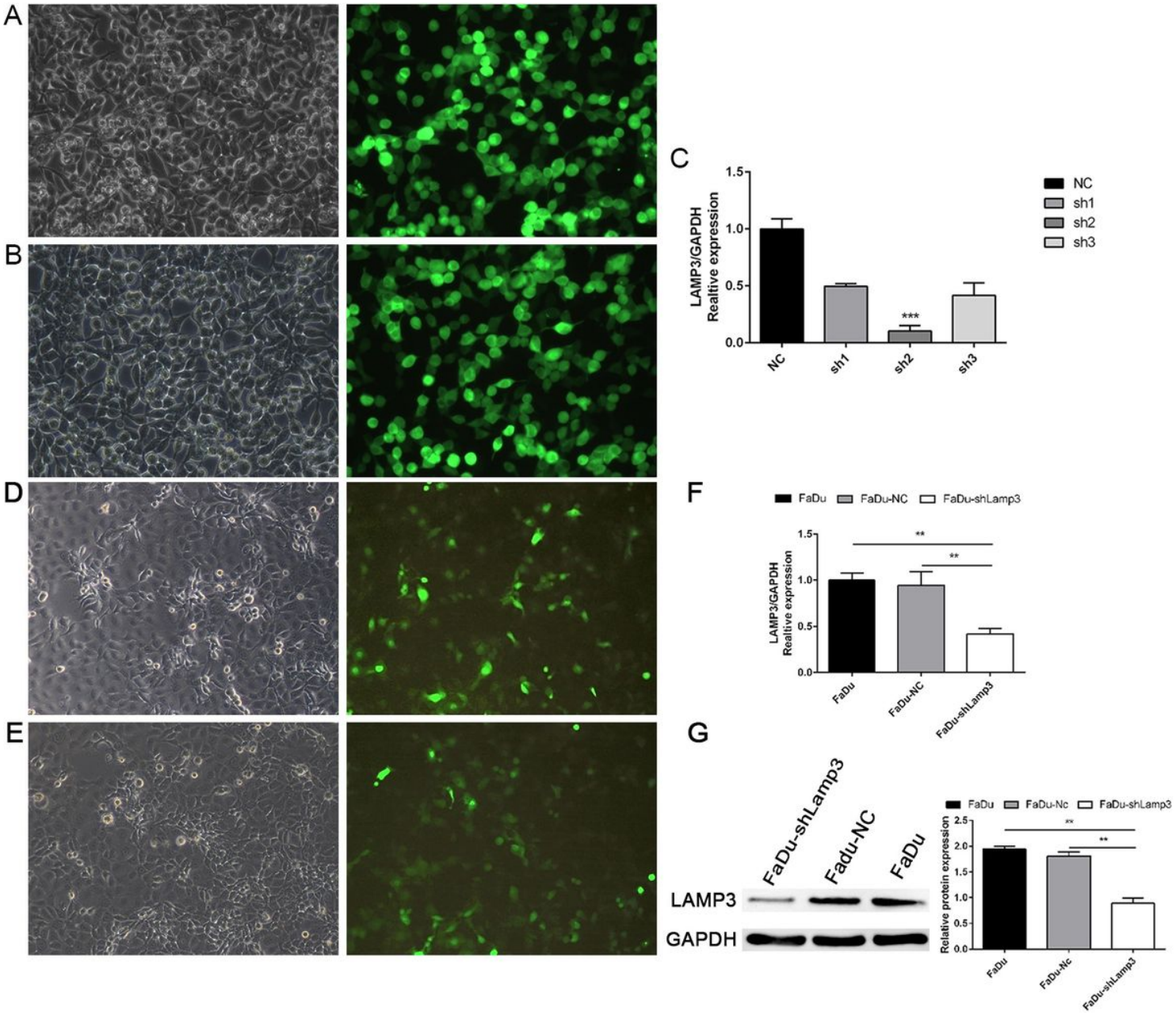

Figure 2

Screening of LAMP3 shRNA Lentivirus-mediated shRNA inhibited LAMP3 expression in FaDu cells. A. Transfection of Lenti-NC plasmid into 293T cells for 48h. (Original magnification ×100). B. Lenti-shLamp3 plasmid transfected 293T cells for 48h lesions. (Original magnification ×100) C. In HEK293T cells, compared with the negative control group, the mRNA level of LAMP3 in the transfected shLAMP3 interference plasmid group was decreased, and the difference was statistically significant (***P<0.001). The most effective one was LAMP3-sh2. D. FADU-NC bright field and fluorescent infection rate. (Original magnification ×100). E. FADU-shLamp3 bright field and fluorescent infection rate. (Original magnification ×100). F. The expressions of LAMP3 in the FaDu cell were detected by using qRT-PCR. G. The expression of LAMP3 was detected by western blotting assay. Datawere expressed as mean ± SD. *P < 0.05, **P < 0.01,***P<0.001 represents statistical difference

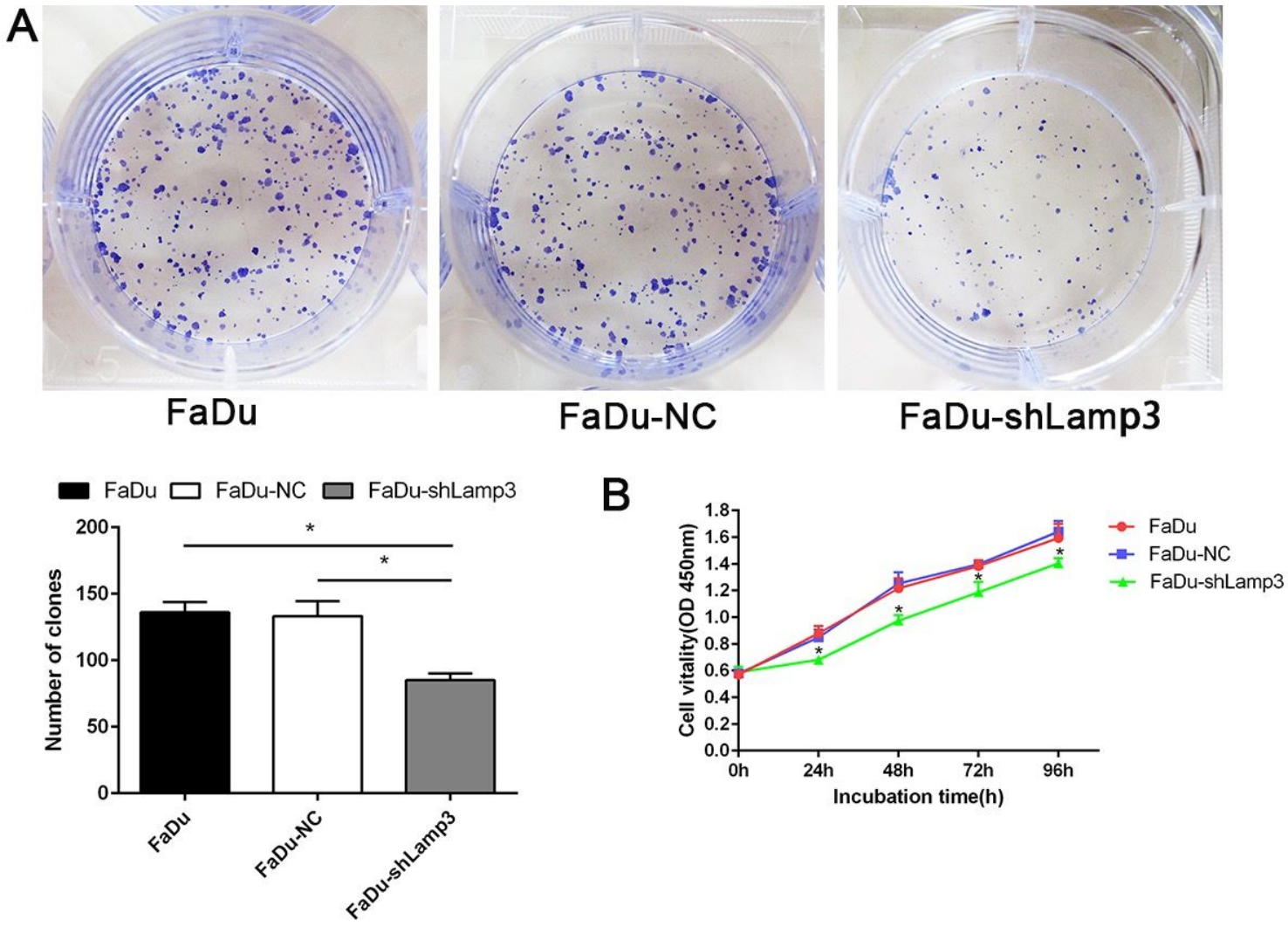

Figure 3

Effect of downregulation of LAMP3 on cell proliferation in FaDu cells A. Colony formation was performed to analyze cell proliferation. B. The FaDu cell growth was measured by CCK8 assay Data were expressed as mean ± SD. *P < 0.05, **P < 0.01 represents statistical differe

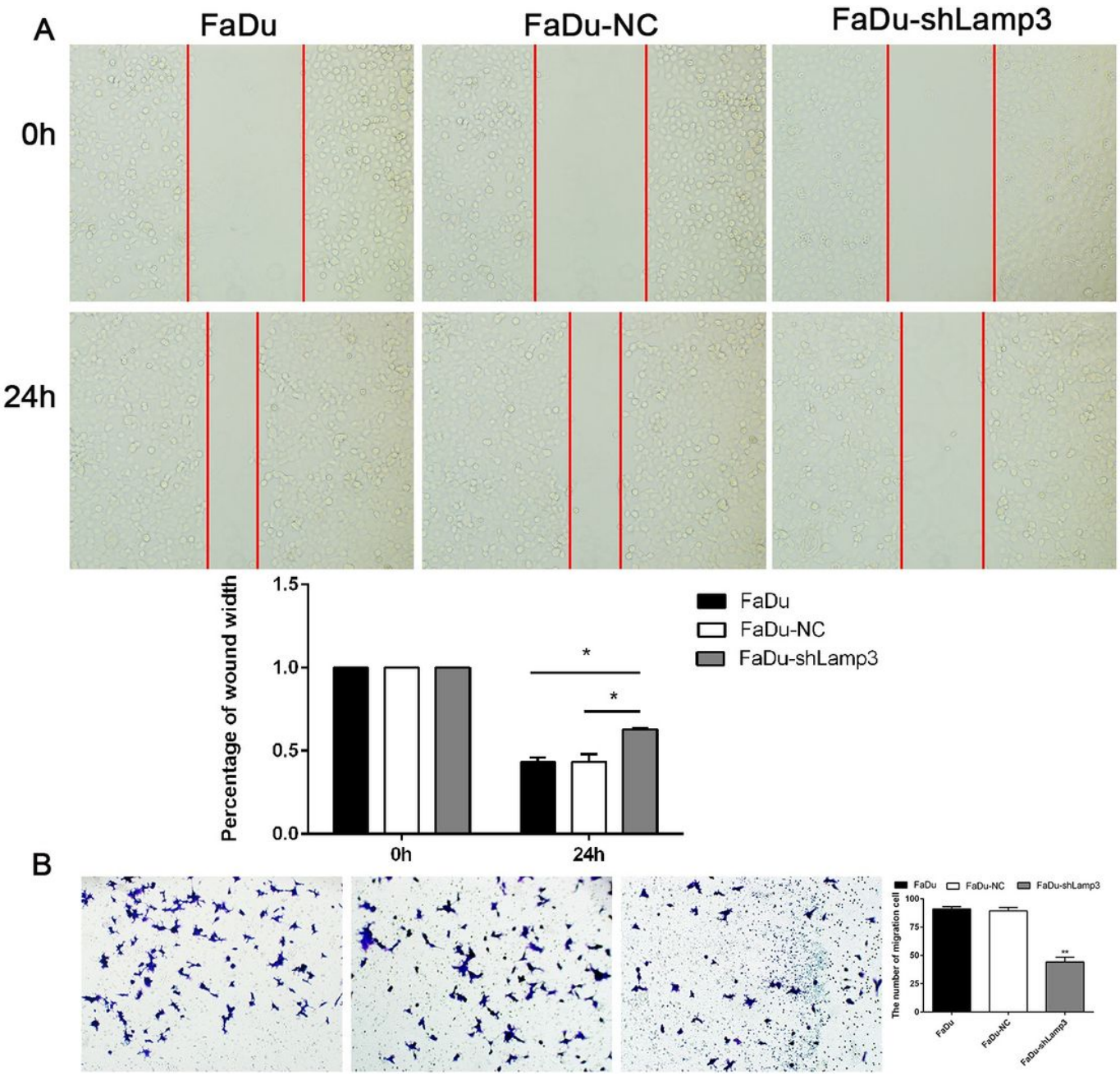

Figure 4

Effect of downregulation of LAMP3 on cell migration in FaDu cells A. Scratch-wound experiments showed cell migration in Fadu-shLamp3 B. Transwell migration assays were performed to measure cell migration. (Original magnification ×100). Data were expressed as mean ± SD. *P < 0.05, **P < 0.01 represents statistical difference.