

Original Article

# Tumor suppressor in lung cancer-1 (TSLC1) mediated by dual-regulated oncolytic adenovirus exerts specific antitumor actions in a mouse model

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**Aim:** The tumor suppressor in lung cancer-1 (TSLC1) is a candidate tumor suppressor of lung cancer, and frequently inactivated in primary non-small cell lung cancer (NSCLC). In this study, we investigated the effects of TSLC1 mediated by a dual-regulated oncolytic adenovirus on lung cancer, and the mechanisms underlying the antitumor actions.

**Methods:** The recombinant virus Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 was constructed by inserting the TSLC1 gene into the dual-regulated Ad-sp-E1A<sub>(Δ24)</sub> vector, which contained the survivin promoter and a 24 bp deletion within E1A. The antitumor effects of Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 were evaluated in NCI-H460, A549, and H1299 lung cancer cell lines and the normal fibroblast cell line MRC-5, as well as in A549 xenograft model in nude mice. Cell viability was assessed using MTT assay. The expression of TSLC1 and activation of the caspase signaling pathway were detected by Western blot analyses. The tumor tissues from the xenograft models were examined using H&E staining, IHC, TUNEL, and TEM analyses.

**Results:** Infection of A549 lung cancer cells with Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 induced high level expression of TSLC1. Furthermore, the Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 virus dose-dependently suppressed the viability of NCI-H460, A549, and H1299 lung cancer cells, and did not affect MRC-5 normal fibroblast cells. Infection of NCI-H460, A549, and H1299 lung cancer cells with Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 induced apoptosis, and increased activation of caspase-8, caspase-3 and PARP. In A549 xenograft model in nude mice, intratumoral injection of Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 significantly suppressed the tumor volume, and increased the survival rate (from less than 15% to 87.5% at d 60). Histological studies showed that injection of Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 caused tumor cell apoptosis and virus particle propagation in tumor tissues.

**Conclusion:** The oncolytic adenovirus Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 exhibits specific antitumor effects, and is a promising agent for the treatment of lung cancer.

**Keywords:** lung cancer; tumor suppressor in lung cancer-1; oncolytic adenovirus; survivin; apoptosis; caspase signaling pathway; tumor xenograft model

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## Introduction

Although major progress has been made in the diagnosis and treatment of lung cancer, the five-year survival rate is only 14%, which is largely because of the failure of cancer debulking surgery and systemic chemotherapy<sup>[1]</sup>. According to the Cancer Statistics Review, which was conducted between 1975

and 2009 by the SEER (Surveillance, Epidemiology and End Results) group at the National Cancer Institute in the USA, 219 440 patients were estimated to have primary lung and bronchial tumors in 2009, and 158 350 were estimated to have died as a result. Lung and bronchial tumors ranked the highest of all cancers in both the number of cases and the number of deaths. Currently, lung cancer is the most common primary malignant tumor, with over 1 million deaths worldwide occurring annually; this may in part be due to the increase in the population of smokers and environmental pollution. Thus, novel treatment methods for lung cancer are urgently needed,

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and these treatments must be highly effective and safe.

The Cancer Targeting Gene-Viro-Therapy (CTGVT) strategy developed by our group is one of the most promising approaches for cancer therapy<sup>[2]</sup>. The strategy combines the advantages of both gene therapy and virotherapy by using an oncolytic adenoviral vector (OA) harboring an anti-cancer gene. Currently, two main strategies are applied to construct the viral vector with oncolytic ability, which can specifically infect tumor cells but rarely infects normal cells<sup>[3]</sup>. The first strategy is to delete the adenoviral genes that are necessary for viral replication in normal cells but not in tumor cells. For example, the ONYX-015 and ZD55 oncolytic viruses were constructed by deleting the *E1B* gene, which functions in late viral RNA export<sup>[4]</sup>. The second strategy involves transcription targeting through the use of tumor- or tissue-selective promoters, which can control the expression of early viral genes such as E1A and/or E1B that are essential for replication. Our previous studies have shown that CTGVT exhibits greater antitumor effects than gene therapy or virotherapy alone<sup>[3, 5, 6]</sup>.

The tumor suppressor in lung cancer-1 (TSLC1) was originally identified as a putative tumor suppressor for non-small-cell lung cancer (NSCLC) and was the first named tumor suppressor in lung cancer<sup>[7]</sup>. It is expressed in a variety of tissues and organs in the human body, particularly in the normal lung, brain, liver and skin<sup>[8]</sup>. The downregulation of the TSLC1 gene was frequently detected in various human cancers including gastric cancer<sup>[9-11]</sup>, hepatocellular carcinomas<sup>[12]</sup>, cervical cancer<sup>[13]</sup>, nasopharyngeal cancer<sup>[14]</sup>, breast cancer<sup>[15]</sup>, prostate cancer<sup>[16]</sup>, and pancreatic cancer<sup>[17]</sup>. TSLC1 is a transmembrane adhesion molecule that belongs to the immunoglobulin superfamily<sup>[18]</sup>, and it consists of an extracellular domain (EC), a transmembrane domain (TM) and a cytoplasmic domain (CP). The EC of TSLC1 mediates the formation of TSLC1 homodimers or heterodimers with other cell adhesion molecules, such as Necl-1, CRTAM, and Nectin-3, to regulate cell-cell adhesion. The CP interacts with DAL-1, another tumor suppressor gene, and membrane-associated guanylate kinase (MAGuK) homologs such as MPP3. The CP is able to regulate the activation of small Rho GTPases, thus acting as a vital connection between extracellular adhesion and intracellular signaling cascades. Furthermore, the possible molecular mechanisms of TSLC1 include the suppression of tumor formation, modulation of the cell cycle, pro-apoptotic activity and regulation of the epithelial-mesenchymal transition (EMT)<sup>[19]</sup>.

Human survivin, the smallest member of the inhibitor of apoptosis protein (IAP) family, plays a key role in both the regulation of cell division and in the inhibition of apoptosis<sup>[20, 21]</sup>. Of significance, survivin has aberrantly high expression in cancer cells such as lung cancer but little expression in most normal tissues, making survivin an attractive anticancer target<sup>[22, 23]</sup>. Recent studies have shown that a designed oncolytic adenovirus driven by the survivin promoter exhibited a tumor-selective antitumor effect *in vitro* and *in vivo*<sup>[3, 24, 25]</sup>, suggesting that the survivin promoter is a good candidate for cancer therapy. To improve the OA tumor-specificity, a 24 bp region within the E1A conserved region 2 (CR2), which

is responsible for binding the retinoblastoma (Rb) protein, was deleted. This deletion restricts viral replication to dividing cells or Rb-inactive and arrested cells<sup>[3]</sup>. In this study, the dual-regulated Ad-sp-E1A<sub>(Δ24)</sub> oncolytic virus contained the 24 bp deletion within E1A and was driven by the survivin promoter. Previous studies demonstrated that TSLC1, a candidate tumor suppressor in lung cancer, was depleted or not expressed in lung cancer cells<sup>[7, 26]</sup>. Thus, it was inserted into the Ad-sp-E1A<sub>(Δ24)</sub> OA, yielding the Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 construct. Our data indicated that Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 specifically induces dramatic cytotoxicity in lung cancer cells *in vitro* and effectively suppresses xenografted lung cancer in nude mice, suggesting that Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 may be a promising therapeutic agent for lung cancer.

## Materials and methods

### Cell lines and culture conditions

HEK293 (human embryonic kidney cell line containing the E1A region of Ad5) cell was obtained from Microbix Biosystem Inc (Toronto, Canada). All of the lung cancer cell lines (A549, NCI-H460, and H1299) and the normal lung cell line MRC-5 were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) or Shanghai Cell Collection (Shanghai, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mmol/L glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin. All cell lines were cultured at 37°C in 5% CO<sub>2</sub>.

### Plasmids

The pcDNA3-hygro-TSLC1 plasmid was graciously provided by Dr R STEENBERGEN at the Vrije Universiteit Medical Center (Amsterdam, Netherlands). The pXC2 adenovirus shuttle vector, pMD-T, and the pBHGE3 adenoviral packaging vector were constructed in our laboratory. The pXC2-sp-E1A<sub>(Δ24)</sub> OA plasmid was previously constructed in our laboratory<sup>[3]</sup>. The TSLC1 cDNA sequence was first cloned between the *EcoR* I and *Xho* I sites in the pMD-T vector to yield pMD-T-TSLC1. Then, pXC2-sp-E1A<sub>(Δ24)</sub>-TSLC1 was constructed by inserting the entire TSLC1 expression cassette derived from pMD-T-TSLC1 into the *Sal* I site of the pXC2-sp-E1A<sub>(Δ24)</sub> vector. All plasmid constructs were confirmed by restriction enzyme digestion, PCR and DNA sequencing.

### Quantitative RT-PCR

Total RNA was isolated from prepared lung cancer cells or normal cells with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. For the analysis of Survivin and TSLC1 expression, cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen, USA) as described by the manufacturer. Quantitative real-time PCR was performed using a SYBR Green kit (TOYOBA, Japan). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used for normalization. The following primers were used: TSLC1 forward primer, 5'-CGGCT-

TCTGCTGTTGCTCTTCT-3'; TSLC1 reverse primer, 5'-AAATAAATGGTCTGCCTGTTGG-3'; survivin forward primer, 5'-GACCACCGCATCTC-3'; and survivin reverse primer, 5'-AAGTCTGGCTCGTTC-3'. The RT-PCR was performed on an ABI Prism 7500 Sequence Detector (Applied Biosystems, USA). All of the reactions were conducted in triplicate. The  $\Delta\Delta C_t$  method was used for relative quantification of gene expression to determine survivin and TSLC1 mRNA expression.

**Generation, identification, purification, and titration of adenovirus**  
Ad-sp-E1A<sub>( $\Delta 24$ )</sub>-TSLC1 and Ad-sp-E1A<sub>( $\Delta 24$ )</sub> viral vectors were generated by homologous recombination of pXC2-sp-E1A<sub>( $\Delta 24$ )</sub>-TSLC1 and pXC2-sp-E1A<sub>( $\Delta 24$ )</sub>, respectively, with the PBHGE3 adenoviral packaging vector in HEK293 cells. Individual plaques were selected and used to infect HEK293 cells. After observing cytopathic effects, the cell culture medium was collected and viral genomic DNA was extracted. Then, wild-type adenovirus and foreign gene expression cassettes were identified by PCR methods using primer pairs complementary to the E1A region or an exogenous gene. Recombinant adenoviruses were amplified in HEK293 cells and purified by cesium chloride gradient ultracentrifugation. Viral titers were determined by TCID<sub>50</sub> (median tissue culture infective dose) assays in HEK293 cells.

#### Cell viability assay

Cells were plated in 96-well plates and treated with different recombinant adenoviruses at the following MOIs: 0.5, 1, 2, 5, and 10 for 48 h. Then, 20  $\mu$ L of MTT (Sigma, USA) solution (5 mg/mL) was added to each well. Cells were incubated at 37°C for 4 h. The supernatant of each well was carefully removed, and an equal amount of DMSO (150  $\mu$ L) was added to each well and mixed thoroughly on a shaker for 10 min. The absorbance of each well was read at 595 nm with a DNA microplate reader (GENios model, Tecan; Maennedorf, Switzerland).

#### Cytopathic effect (CPE) assay

NCI-H460, A549, and H1299 lung cancer cell lines and the normal fibroblast cell line MRC-5 were grown to subconfluence and infected with adenoviruses at various MOIs as described above. Six days after infection, a 2% crystal violet solution in 20% methanol was added to cells for 15 min and then washed with distilled water and photographed.

#### Hoechst 33342 staining

To detect chromatin condensation and nuclear fragmentation, which are characteristics of apoptosis, nuclei were stained with Hoechst 33342. A549, H1299, NCI-H460, and MRC-5 cells were infected with Ad-sp-E1A<sub>( $\Delta 24$ )</sub> and Ad-sp-E1A<sub>( $\Delta 24$ )</sub>-TSLC1 viruses at an MOI of 10 for 72 h. cells were fixed with 4% paraformaldehyde and then stained with the Hoechst 33342 staining kit (Beyotime, Nantong, China) for 20 min as described in the manufacturer's protocol. Cells were then washed twice with PBS and visualized under a fluorescence

microscope. Uninfected cells served as a control.

#### Western blot analysis

Western blot analysis was performed using standard protocols to determine the expression of various proteins. Cells were trypsinized, harvested and resuspended in lysis buffer (62.5 mmol/L Tris-HCl [pH 6.8], 2% SDS, 10 mmol/L glycerol and 1.55% dithiothreitol). The total protein concentration was determined using the BCA™ Protein Assay kit (Pierce, Rockford, IL, USA) as described by the manufacturer. Then, protein samples were separated by 10%–15% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore, MA, USA). Membranes were blocked in a 5% BSA solution and incubated with primary antibodies. Proteins were detected using the appropriate secondary antibodies conjugated to fluorescent molecules and visualized with an Odyssey Infrared imaging system (LI-COR Biosciences Inc, Lincoln, NE, USA). Caspase-3 and caspase-8 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). TSLC1, E1A, and PARP antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The GAPDH antibody was purchased from Bioworld Technology (Bioworld, MN, USA).

#### Animal experiments

All of the animal experiments were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the rules of our institution. Female BALB/c nude mice (4–5 weeks old) were purchased from the Shanghai Experimental Animal Center (Shanghai, China). A549 cells were subcutaneously injected into the lower right flank of female nude mice, and a tumor xenograft model was established. Each group was composed of at least eight animals, and tumor growth was monitored and measured every 3 d with a Vernier caliper. Tumor volume ( $V$ ) was calculated according to the formula:  $V(\text{mm}^3) = 1/2 \times \text{length}(\text{mm}) \times \text{width}(\text{mm}^2)$ . When tumors reached an average volume of 100–130  $\text{mm}^3$ , mice were randomly allocated to six groups and given a daily dose of  $5 \times 10^8$  plaque-forming units (pfu) of various adenoviruses in 100  $\mu$ L of PBS or PBS alone. Adenoviruses or PBS alone were administered intratumorally for 4 d. Tumors were harvested 4 d after the last treatment for histopathologic and immunohistochemical (IHC) analyses.

#### Immunohistochemistry

For IHC analysis, tumors were harvested 4 d post-treatment and fixed in 4% paraformaldehyde, embedded in paraffin and cut into 4-mm sections. These sections were stained with goat monoclonal anti-adenoviral hexon antibody at a dilution of 1:200. Slides were then washed with PBS and incubated with the avidin-biotin-peroxidase complex (ABC) reagent (Vector Laboratories, Burlingame, CA, USA) and detected with diaminobenzidine tetrahydrochloride solution containing 0.006% hydrogen peroxide. Hematoxylin was used as a counterstain. Tissue sections stained without primary antibodies were used as negative controls.

**TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay**

The TUNEL method was used to detect apoptotic cells. The *in situ* cell apoptosis detection kit (Roche, Palo Alto, USA) was used. The staining was performed according to the manufacturer's instructions. Tissue sections in the PBS-treated group were stained and served as negative controls. The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis and discriminates apoptosis from necrosis and primary DNA strand breaks induced by apoptotic agents.

**Transmission electron microscopy (TEM) analysis**

For electron microscopy analysis, tumor samples (1 mm<sup>3</sup>) were fixed in a PBS mixture containing 2.5% glutaraldehyde overnight and then incubated in 1% osmium tetroxide for 1 h. Tissues were rinsed in ddH<sub>2</sub>O, dehydrated through a graded series of ethanol and propylene oxide and finally embedded in Epon 812 resin (Shell Chemicals, Houston, TX, USA). After examination of semithin sections, areas were selected and subjected to ultrathin sectioning. Sections collected on 200 mesh copper grids were contrasted with lead citrate and uranyl acetate, examined and photographed with a JEOL 100CX transmission electron microscope (JEOL, Akishima, Japan).

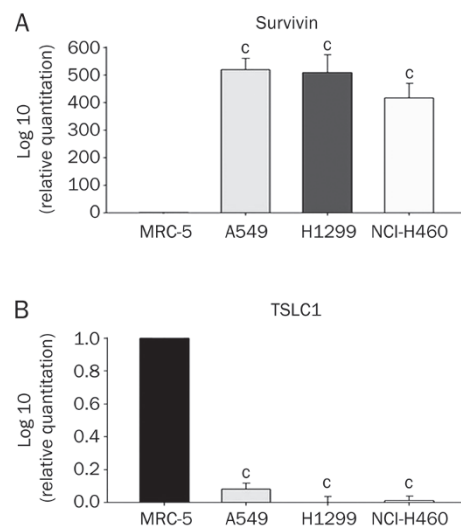
**Statistical analysis**

The statistical significance of experimental results was calculated by the analysis of variance (ANOVA) and Student's *t*-test. All data are expressed as the mean±standard deviation (SD). Results were considered statistically significant at *P*<0.05.

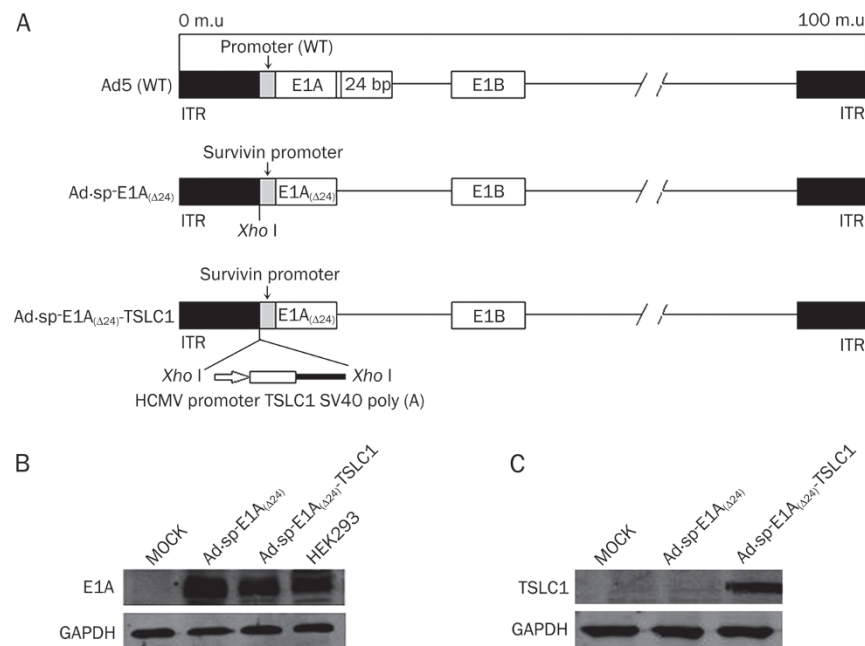
**Results****Characteristics of the oncolytic adenovirus Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1**

To investigate the expression level of survivin and the TSLC1 gene, we first performed quantitative PCR. The results dem-

onstrated that TSLC1 was significantly downregulated in several lung cancer cell lines (H1299, A549, and NCI-H460) compared to normal human fibroblast cells (MRC-5, Figure 1B). Conversely, survivin expression was cancer-specific and was detected in lung cancer cells (Figure 1A), which is consistent with previous reports<sup>[23, 24]</sup>. Based on these results, we constructed the dual-regulated Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 viral vector in which the antitumor gene TSLC1 was inserted into Ad-sp-E1A<sub>(Δ24)</sub>, which contains the survivin promoter and a 24 bp deletion within the E1A CR2 region (Figure 2A).



**Figure 1.** Relative expression level of survivin and TSLC1 in lung cancer cells. Survivin (A) and TSLC1 (B) mRNAs extracted from three lung cancer cell lines (H1299, A549, and NCI-H460) and human lung fibroblast cells line MRC-5 were subjected to real-time quantitative PCR. Mean±SD. *n*=3. \**P*<0.01.



**Figure 2.** Characterization of oncolytic adenovirus Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1. (A) Schematic diagram of recombinant oncolytic adenovirus structure. All viruses were constructed using the backbone of wild-type Ad5. ITR, inverted terminal repeat. Characterization of Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 was analyzed by Western blot. Lung cancer cell line A549 was infected with Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 at a multiplicity of infection (MOI) of 10 pfu/cell, and the E1A (B) and TSLC1 protein expression (C) was detected after 48 h by Western blotting analysis.

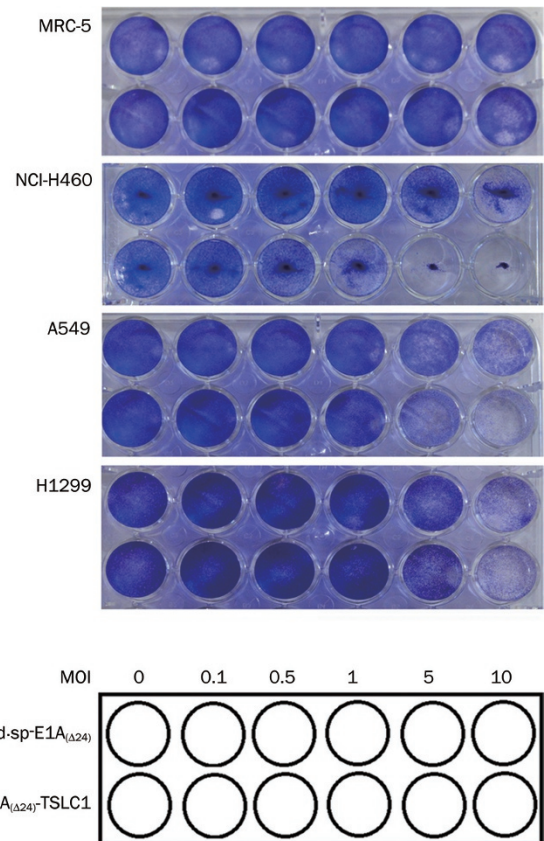


We detected the tumor-specific expression of adenovirus E1A and the TSLC1 transgene. The A549 lung cancer cell line was infected with Ad-sp-E1A<sub>(Δ24)</sub> and Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 at an MOI of 10 for 48 h, and the expression of E1A and TSLC1 was then detected. These results indicated that both Ad-sp-E1A<sub>(Δ24)</sub> and Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 induced strong E1A expression (Figure 2B), implying that they replicated well in lung cancer cells. Additionally, the TSLC1 construct strongly induced TSLC1 expression compared to the mock treatment and Ad-sp-E1A<sub>(Δ24)</sub> control virus (Figure 2C). These results demonstrate that the oncolytic virus can mediate TSLC1 expression in cancer cells.

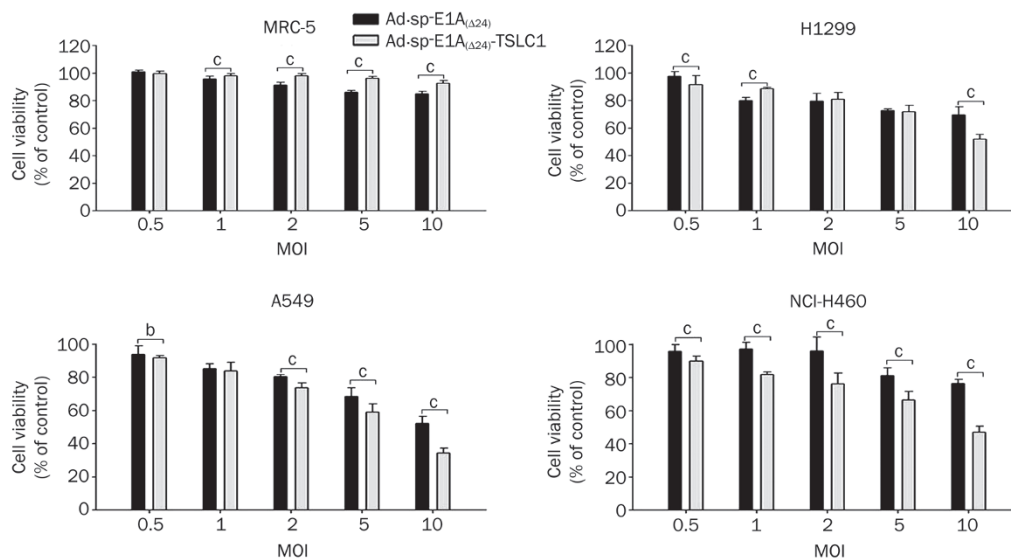
### Tumor cell-specific cytotoxicity mediated by Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 *in vitro*

Next, we investigated the effect of Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 on cell viability. The human lung cancer cell lines A549, NCI-H460, H1299, and the human normal fibroblast cell line MRC-5 were infected with Ad-sp-E1A<sub>(Δ24)</sub> and Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 at an MOI of 10, and cell proliferation was measured using the MTT assay. As shown in Figure 3, Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 induced cell death in approximately 48% to 65% of the infected cancer cells, and the tumor-killing effect of Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 was more effective than Ad-sp-E1A<sub>(Δ24)</sub> in a dose-dependent manner. In contrast, 90% of the MRC-5 cells were still viable after Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 infection. These results demonstrate the advantages of treating tumor cells with the dual-regulated oncolytic adenovirus.

Furthermore, the cytopathic effects induced by Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 infections were visualized by crystal violet staining. Similar results were obtained by conducting the MTT assay on cancer cell lines treated with the various OAs for 4 d. As shown in Figure 4, significant cytopathic effects were



**Figure 4.** Tumor-specific cytopathic effect induced by Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1. Three lung cancer cell lines (H1299, A549, and NCI-H460) and normal lung fibroblast cell lines MRC-5 were seeded in 24-well plates as a density of  $5 \times 10^4$  cells/well and infected with Ad-sp-E1A<sub>(Δ24)</sub> and Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 at the indicated MOIs. Six days later, cells were stained with crystal violet.



**Figure 3.** Suppression of tumor cell proliferation by Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 in tumor cells *in vitro*. The lung cancer cell lines (H1299, A549, and NCI-H460) and normal lung fibroblast cell lines MRC-5 were infected with Ad-sp-E1A<sub>(Δ24)</sub> and Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 at a MOI of 0.5, 1, 2, 5, and 10. Seventy-two hour later, cell viability rate was measured by MTT assay. Mean±SD.  $n=4$ . <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$ .

observed in lung cancer cells infected with Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1, which mediated more cytopathic effects than Ad-sp-E1A<sub>(Δ24)</sub>. Moreover, no obvious cytotoxicity was observed in normal cells under the same treatment conditions. Thus, the dual-regulated Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 oncolytic virus could replicate selectively in lung cancer cells and induced tumor-specific cytotoxic effects.

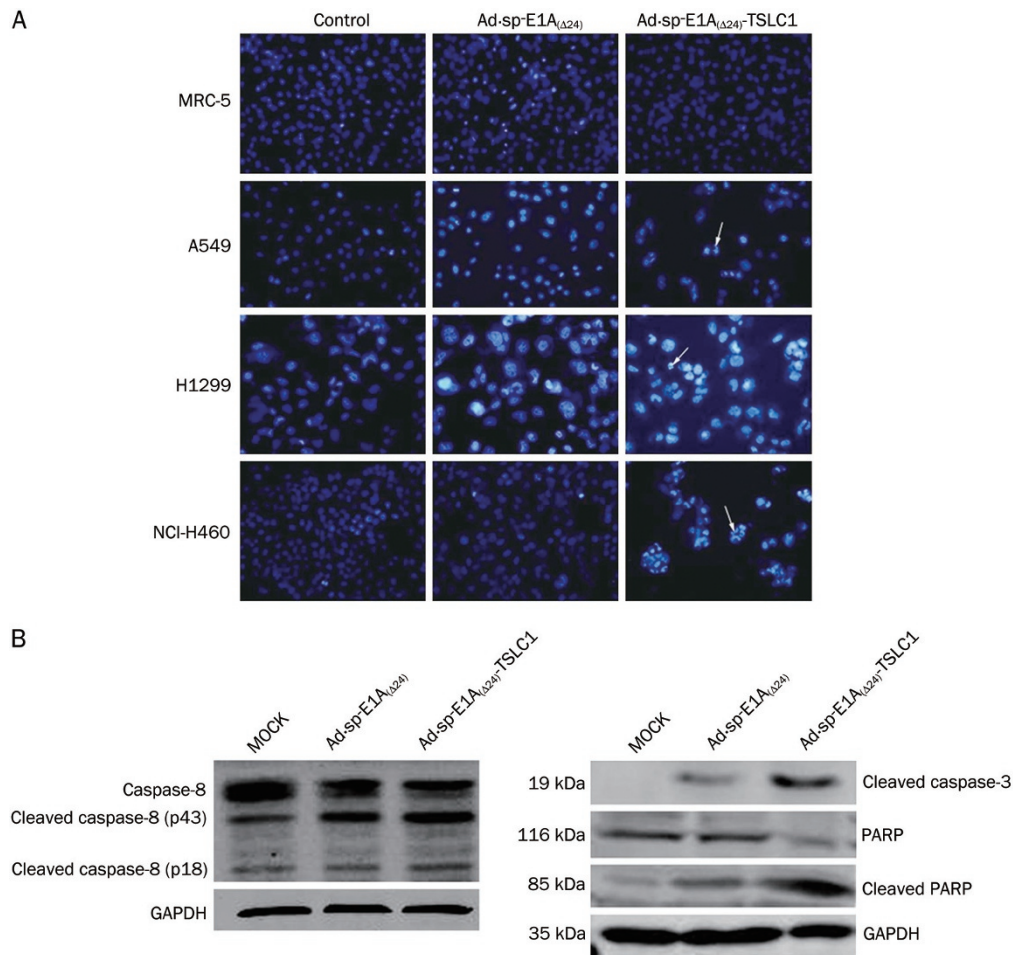
#### Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 selectively induces cell apoptosis *in vitro*

We also evaluated whether OA-mediated TSLC1 induces tumor-specific cell apoptosis in lung cancer cells. Treatment of cancer cells with Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 led to increased apoptosis, which featured chromatin condensation, nuclear fragmentation and apoptotic bodies (Figure 5A). To assess whether the mechanism of apoptosis involved the caspase signaling pathway, Western blotting analysis was performed to detect the expression of caspase cascade proteins. Consistent with the above findings, increased activation of caspase-8,

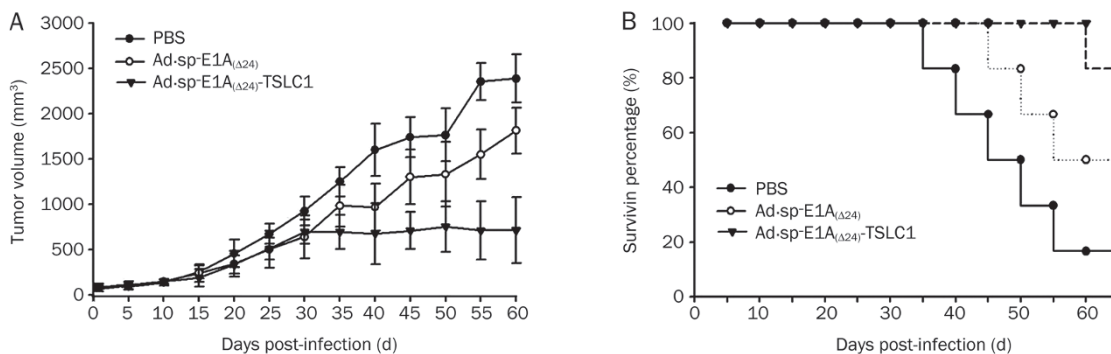
caspase-3 and PARP was detected in lung cancer cells treated with Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 compared to mock-treated or Ad-sp-E1A<sub>(Δ24)</sub>-treated cells (Figure 5B). These results suggest that TSLC1 induces tumor cell apoptosis via activation of the caspase pathway.

#### Antitumor activity of Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 *in vivo*

The *in vivo* antitumor effects of Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 were evaluated with a A549 xenograft model in nude mice. For all studies, mice with established tumors received percutaneous intratumoral injections of the viruses. Ad-sp-E1A<sub>(Δ24)</sub> and Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 were injected as single doses of  $5 \times 10^8$  pfu in a volume of 100  $\mu$ L. Injections were given daily for 4 d to a group of mice ( $n=8$ ). PBS was used as a control. Tumor growth curves were plotted to compare the antitumor effects. As shown in Figure 6A, Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 treatment significantly suppressed lung carcinoma development compared to PBS treatment ( $P<0.05$ ). Furthermore, Ad-sp-E1A<sub>(Δ24)</sub>-



**Figure 5.** Detection of tumor cell apoptosis induced by Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1. (A) Apoptosis detection by Hoechst 33342 staining. Cells were plated in 6-well plates and infected with Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1, and Ad-sp-E1A<sub>(Δ24)</sub> at a MOI of 10, uninfected cells served as control. Seventy-two hours later, cells were treated with Hoechst33343 staining at 1 mg/mL for 30 min, and then observed under the inverted fluorescence microscope. Original magnification,  $\times 200$ . (B) Activation of caspase signaling pathway by Adsp-E1A<sub>(Δ24)</sub>-TSLC1. The A549 cells were treated with the Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 at 10 MOI. Forty-eight hours later, cells were harvested and examined by Western blotting analysis. Activation of caspase-8, caspase-3, and the downstream apoptotic substrate protein poly (ADP-ribose) polymerase (PARP) was detected. GAPDH was used as the internal control.



**Figure 6.** Antitumor effect of Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 in xenograft nude mice. Female BALB/c nude mice were subcutaneously inoculated with A549 cells ( $5 \times 10^5$ ). When tumors reached 100–130 mm<sup>3</sup>, the animals were treated with PBS, Ad-sp-E1A<sub>(Δ24)</sub>, or Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 through intratumoral injection. (A) Tumor volume of various treatment groups was measured. (B) Survival rate of mice was shown by the Kaplan-Meier survival curves. A pair-wise log-rank test was used to analyze survival rates in the different groups. Mean $\pm$ SD.  $n=8$ .

TSLC1 exhibited greater antitumor activity than Ad-sp-E1A<sub>(Δ24)</sub> in nude mice, demonstrating that Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 is a potent antitumor agent *in vivo*.

Survival of xenografted nude mice was monitored with a Kaplan-Meier curve (Figure 6B). Only one of the eight mice treated with Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 died within the first 65 d. Conversely, PBS-treated mice gradually died after 35 d, and the survival rate of these mice was less than 15%. Furthermore, 50% of the Ad-sp-E1A<sub>(Δ24)</sub>-treated mice and 87.5% of the Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1-treated mice survived beyond the end of the experiment.

#### Pathological effects of Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 on tumor inhibition in nude mice

To detect cell death and the expression of TSLC1 and adenovirus hexon in tumor tissues, H&E staining and IHC analysis utilizing anti-TSLC1 and anti-hexon antibodies were performed following various treatments. H&E staining demonstrated that Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 resulted in more severe cytopathic effects than Ad-sp-E1A<sub>(Δ24)</sub> (Figure 7). IHC staining confirmed the strong expression of both TSLC1 and adenovirus hexon protein in the tumor tissues following treatment with Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 (Figure 7), suggesting that the expression of TSLC1 increased as the oncolytic virus replicated in the tumor cells.

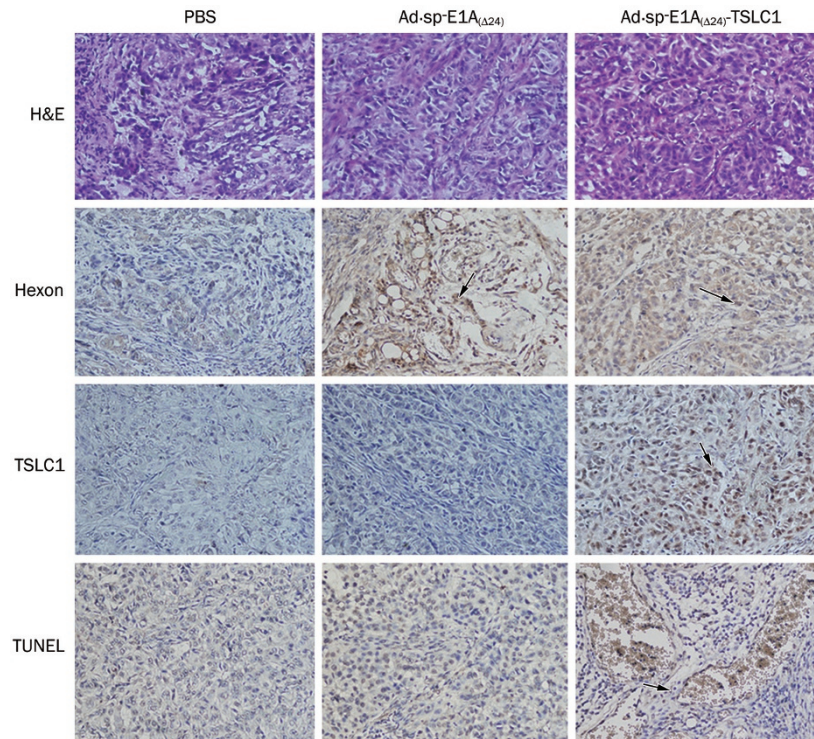
TUNEL assay results indicated that Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 treatment induced more extensive apoptosis in tumor tissue than Ad-sp-E1A<sub>(Δ24)</sub> or PBS treatment (Figure 7). Morphological changes in tumor masses were also observed by TEM analysis (Figure 8A). Characteristics of apoptosis, including nuclear collapse, nuclear envelope disappearance, an increased nuclear-to-cytoplasmic ratio, nuclear deformation, the presence of heterochromatin and chromatin condensation were observed in tumors treated with Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1. In addition, the presence and replication of Ad-sp-E1A<sub>(Δ24)</sub> and Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 were observed in tumor tissues (Figure 8B). These results suggest that specific propagation of oncolytic viruses is involved in the inhibition of tumor growth.

#### Discussion

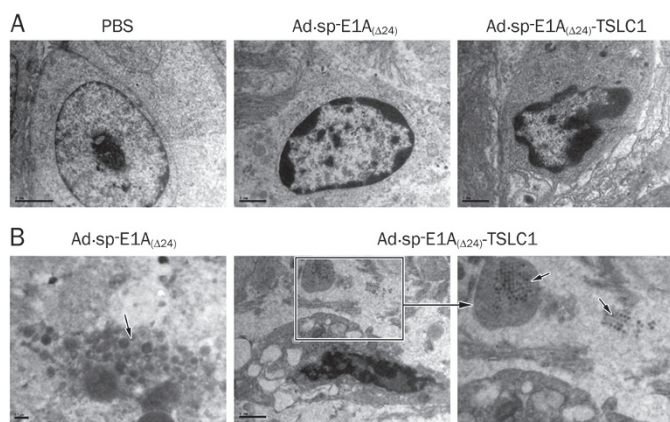
We developed the Cancer Targeting Gene-Viro-Therapy (CTGVT) strategy in 2001<sup>[27]</sup>, and this strategy has shown promising anticancer effects. Tumor-selective oncolytic viruses have been used as vectors to deliver the antitumor gene in CTGVT. The strategy improves cancer therapy through the dual action of the therapeutic gene and the oncolytic virus itself. The oncolytic poxvirus JX-594, which delivers granulocyte macrophage-colony stimulating factor (GM-CSF), exhibited outstanding antitumor results in a clinical trial via viral oncolysis and tumor-specific anti-cancer immunity<sup>[28]</sup>. JX-594 could selectively infect, replicate and express its transgene in cancer tissues but did not affect normal tissue; JX-594 was delivered into human metastatic solid tumors via intravenous infusion<sup>[29]</sup>.

The oncolytic adenovirus is the most customizable vector in clinical and preclinical studies for human cancer therapy due to several biological properties including ease of production, oncolysis ability and a large packaging capacity. Recently, additional modifications to the oncolytic virus were made to improve its targeting and application for cancer therapy. The early E1A protein is an essential factor for efficient adenoviral replication in host cells; thus, the modification of E1A is a preferred strategy. A 24 bp deletion within the E1A CR2 region allows the adenovirus to target the Rb pathway in cancer cells<sup>[30]</sup>. Moreover, the use of a tumor-specific promoter as a replacement for the E1A endogenous promoter improves the targeting of the oncolytic virus. The human telomerase reverse transcriptase (hTERT) promoter<sup>[31]</sup>, the  $\alpha$ -fetoprotein (AFP) promoter<sup>[32, 33]</sup> and the differential display code 3 (DD3) promoter<sup>[34]</sup> have been extensively utilized in targeted cancer therapy. Survivin is a cancer gene and could potentially be useful for cancer diagnosis and therapy<sup>[20, 21, 35]</sup>. A previous study demonstrated that the survivin promoter was superior for cancer targeting when compared to the hTERT promoter<sup>[24]</sup>. Thus, the Ad-sp-E1A<sub>(Δ24)</sub> dual-regulated adenoviral vector was constructed with the survivin promoter and the 24-bp-deleted E1A gene to induce cancer-specific cytotoxic effects.





**Figure 7.** Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 induced tumor cell death in *in vivo* lung cancer model by HE, IHC, and TUNEL assay. Mice xenograft A549 tumor tissues receiving various treatments were harvested and tumor sections were treated as described in Materials and methods. The most upper row is hematoxylin and eosin (HE) staining analysis. Tumor tissues treated with Adsp-E1A<sub>(Δ24)</sub>-TSLC1 showed more cell death than other groups. The middle two rows showed adenovirus hexon and TSLC1 expression by IHC analysis in tumor tissues, respectively. The lowest row is TUNEL assay for detecting apoptotic cells treated with different treatment. Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 induced significant apoptosis of tumor cells. The brown color represents the apoptotic cells (as arrows shown). Original magnification:  $\times 200$ .



**Figure 8.** Morphological observation of tumor tissues by TEM analysis. (A) Morphological observation of cell apoptosis. The more obvious apoptotic phenomenon treated with Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 was detected in tumor tissues than that of other treated groups, such as nuclear collapse, appearance of nucleus deformation, and the chromatin condensed in lumps *et al.* (B) Viral particles and replication (as arrows shown) in tumor tissues treated with Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1.

In this study, Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 specifically replicated in lung cancer cells and induced cytotoxicity in those cells but

not in normal cells (Figure 3, 4, and 6). Therefore, the survivin promoter-controlled E1A containing the 24 bp deletion may be useful for CTGVT in future lung cancer clinical trials.

Our results indicated that the expression of TSLC1 played a vital role in the cytotoxicity induced by Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1. TSLC1 is a cell adhesion molecule that potentially mediates the crosstalk between extracellular adhesion and intracellular signaling cascades through its interaction with additional tumor suppressors (*eg*, DAL4.1 and MPP1-3)<sup>[36]</sup>. Thus, TSLC1 mediates potential anti-cancer effects. Notably, our previous study reported that OA encoding TSLC1 exhibits significant antitumor effects on hepatocellular cancer cells<sup>[25]</sup>. The differences between these studies include the OA design and the cancer types investigated. The previous study utilized SD55-TSLC1, which contained TSLC1 inserted into the dual-regulated OA; this vector also encoded E1A driven by the survivin promoter and included an *E1B* gene deletion. This construct was used to explore the inhibitory effect of TSLC1 on liver cancer growth. In this study, the oncolytic adenoviruses Ad-sp-E1A<sub>(Δ24)</sub> and Ad-sp-E1A<sub>(Δ24)</sub>-TSLC1 suppressed lung tumors, but the latter exerted the strongest effect both *in vitro* and *in vivo* (Figure 3 and 6), indicating the powerful anti-cancer role of TSLC1. In addition, inactivating mutations in TSLC1 have been observed in human solid tumors. A mouse model containing a TSLC1 deletion was established, and these



mice exhibited increased tumorigenesis and died significantly faster than wild-type controls<sup>[37]</sup>. These results confirm that TSLC1 is indeed an optimal tumor suppressor gene candidate for cancer gene therapy. Furthermore, we detected the activation of apoptosis via the caspase-dependent pathway, which was induced by oncolytic virus-mediated TSLC1 expression in lung cancer cells (Figure 5). Tumor growth of lung cancer xenografts was restricted to a small volume 30 d after Ad·sp-E1A<sub>(Δ24)</sub>-TSLC1 was injected into the nude mouse (Figure 6A), suggesting that Ad·sp-E1A<sub>(Δ24)</sub>-TSLC1 can be used as a potential treatment for lung cancer. In this study, high levels of TSLC1 expression were obtained by our dual-regulated oncolytic virus, which replicated selectively in lung cancer cells. The survivin promoter was employed to control E1A expression. The antitumor effects mediated by TSLC1 in our dual-regulated oncolytic adenovirus were enhanced compared to the adenovirus-mediated TSLC1 that was previously reported for use in lung cancer<sup>[38]</sup>. This difference may be due to Ad·sp-E1A<sub>(Δ24)</sub>-TSLC1-induced apoptosis and oncolysis by selective replication in lung cancer cells (Figure 8B). A recent study reported that the interaction of TSLC1 with its ligand CRTAM enhances the anti-tumor immune response to trichosanthin in a murine Lewis lung cancer model<sup>[39]</sup>. The role of TSLC1 in the activation of the host immune response requires further investigation. More recently, studies have reported that the TSLC1 gene is suppressed by microRNA-216a and microRNA-10b, facilitating early carcinogenesis, invasion and migration in hepatocellular carcinoma<sup>[40, 41]</sup>. These other studies further validated the role of TSLC1 in cancer gene therapy.

In conclusion, we constructed a novel dual-regulated CTGVT oncolytic viral vector, Ad·sp-E1A<sub>(Δ24)</sub>-TSLC1, containing a survivin promoter to control the expression of the E1A gene containing a 24 bp deletion. Ad·sp-E1A<sub>(Δ24)</sub>-TSLC1 displayed excellent antitumor effects in both lung cancer cells and in a nude mouse model. This report may provide a new strategy for the treatment of lung cancer.

### Abbreviations

TSLC1, tumor suppressor of lung cancer 1; CTGVT, Cancer Targeting Gene-Viro-Therapy; SEER, Surveillance, Epidemiology and End Results; OA, oncolytic adenovirus; NSCLC, non-small-cell lung cancer; EMT, epithelial-mesenchymal transition; MOI, multiplicity of infection; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CRTAM, Class I-restricted T cell-associated molecule.

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### Author contribution

Yi-gang WANG designed the study; Wen LEI, Hong-bin LIU,

Bu-yun MA, and Yu-long XIA performed the research; Shi-bing WANG, Xiu-mei ZHOU, and Shui-di ZHENG contributed new reagents and analytic tools; Ke-ni GUO, Wen-song TAN, and Xin-yuan LIU analyzed data; Wen LEI and Yi-gang WANG wrote the paper.

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