Oncolytic adenovirus-mediated dual knockdown of survivin and OCT4 improves therapeutic efficacy in esophageal cancer

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Background: Survivin and octamer-binding transcription factor 4 (OCT4) are reportedly up-regulated in esophageal cancer (EC) and have been correlated with high tumor proliferative activity and poor prognosis. Oncolytic viruses encoding specific transgenes have been considered as therapeutic methods to increase therapeutic efficacy in a variety of solid tumors.

Methods: In this study, an oncolytic adenovirus carrying short hairpin RNA (shRNA) of survivin (shSRVN) and OCT4 (shOCT4) was constructed to achieve dual knockdown of survivin and OCT4 and to explore the potential effect of the oncolytic adenovirus in EC.

Results: The oncolytic adenovirus replicated abundantly in human EC cells, with the replication multiplying by up to 192,085 and 620,055 times in esophageal carcinoma (Eca)-109 cells transfected with purified and completed recombinant adenoviruses called AdSProE1a-dual shRNA (shSRVN + shOCT4) and TE1 cells transfected with AdSProE1a-survivin shRNA (shSRVN) 96 hours after infection, respectively. The shRNAs targeting survivin and OCT4 significantly downregulated the expression levels of survivin and OCT4 in cells, thereby inhibiting the proliferative activity of cancer cells. Furthermore, E-cadherin and vimentin, which are both considered epithelial mesenchymal transition (EMT) markers, were found to be upregulated and downregulated, respectively, in cancer cells after exposure to the viral infection. The interference of survivin and OCT4 also contributed to cell cycle arrest and apoptosis, the half maximal inhibitory concentrations (IC50s) of oncolytic adenovirus loaded with AdSProE1a-shSRVN + shOCT4 in the Eca109 cells and the TE1 cells were 0.7271 and 0.1032 pfu/mL, respectively. Xenograft experiments *in vivo* showed that oncolytic adenovirus-mediated dual knockdown of survivin and OCT4 effectively inhibited the growth of xenografts and induced cancer cell apoptosis. We concluded that therapies targeting survivin and OCT4 have great potential for improving the therapeutic efficacy in EC.

Conclusions: The dual target design strategy ensured the efficacy and safety of the treatment system and provided a novel and effective adjuvant target therapy for EC.

Keywords: Esophageal cancer (EC); oncolytic virus; survivin; octamer-binding transcription factor 4 (OCT4); xenograft

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Introduction

According to the International Agency for Research on Cancer (IARC), 19.3 million new cancer cases and nearly 10 million cancer deaths were reported worldwide in 2020, of which 604,100 were esophageal cancer (EC) cases and 544,000 were EC patient deaths (1). The incidence of EC in China ranks first in the world, accounting for 60% of new EC cases (2). Survivin has a full-length of 14.7 kb, is located on chromosome 17q25, and contains 4 exons and 3 introns. It is regarded as the smallest member of the inhibitor of apoptosis protein (IAP) family. The IAP family generally contains 2–3 tandem baculoviral IAP repeats (BIR), and the adjacent carboxyl terminus contains a ring-finger structure. Survivin has been found to be widely expressed in embryonic tissues and a few vigorously dividing mature tissues. Although rarely observed in well-differentiated tissues, it has been seen as highly expressed in tumor tissues which indicate its promotional role in tumorigenesis (3,4). Previous studies have revealed that survivin may act as an oncogene in lung cancer (5), colon cancer (6), pancreatic cancer (7), prostate cancer (8), breast cancer (9), lymphoma (10), and EC (3). Vallböhmer *et al.* demonstrated that survivin was significantly upregulated in esophageal adenocarcinoma compared to the adjacent normal tissues. Correlation analysis verified that survivin was not only related to the pathological stage and lymph node metastasis of EC, but was also implicitly related to 5-year overall survival (OS), with the OS of survivin-positive patients significantly lower than that of those who were survivin-negative (11). Our previous research results suggested that the expression of survivin protein in cancer cells was regulated by the p16 protein. In previous research, cancer cells with inactive p16 expressed higher levels of survivin, and upregulated p16 was shown to inhibit the expression of survivin and reduce the proliferation ability of cancer cells (12).

Meanwhile, octamer-binding transcription factor 4 (OCT4)-positive cancer cells with tumor stem cell characteristics have been observed in EC. OCT4 is known to positively upregulate survivin and maintain cell pluripotency. The possible mechanism involves directly upregulating the expression of the cyclin, CCND1, thereby promoting the proliferation of and indirectly activating the expression of survivin (13,14). As one of the members of the Pit-Oct-Unc (POU) transcription factor family, OCT4 activates its target genes by binding to its ATGCAAAT sequence. In stem cells, the promoter activity of 623 protein-coding genes and 5 messenger RNA (mRNA) genes is regulated by OCT4. An increasing number of evidence supports that survivin and OCT4 act as oncogenes in the occurrence and progression of EC, and they play an important role in maintaining the stemness of cancer cells. Therapeutic strategies targeting survivin and OCT4 are likely to be crucial in improving the clinical efficacy of EC treatments.

Immunotherapy has become an important part of a comprehensive tumor strategy. Oncolytic virus therapy, an immunotherapy method, is also receiving increasing attention (15,16). Oncolytic virus therapy not only bestows safety on treatment but also releases a variety of tumor-specific antigens and viral proteins when exerting an oncolytic effect and stimulating an immune response to produce specific or non-specific antibodies. Oncolytic viruses can also be used as carriers for gene therapy, with the specific replication of the virus in tumor cells and the copy number and expression of anti-cancer genes greatly increased (17,18). In this study, we used an oncolytic adenovirus carrying short hairpin RNA (shRNA) of survivin and OCT4 to inhibit the expression of OCT4 and survivin in the esophageal squamous cell carcinoma (ESCC) cell lines Eca109 and TE1, and then evaluated their roles in affecting cell proliferation, cell cycle, and xenograft growth. We aimed to explore whether dual-targeted therapy strategy targeting OCT4 and survivin expression could be an effective treatment method for ESCC. We present the following article in accordance with the ARRIVE reporting

Highlight box

Key findings

- Dual-knockdown of survivin and OCT4 significantly inhibited the proliferative activity of ESCC cells and contributed to cell cycle arrest and apoptosis *in vivo* and *in vitro*.
- Dual-knockdown of survivin and OCT4 could reverse the process of EMT.

What is known and what is new?

- Survivin and octamer-binding transcription factor 4 (OCT4) are reportedly up-regulated in esophageal cancer (EC) and have been correlated with high tumor proliferative activity and poor prognosis.
- Oncolytic viruses containing shRNAs of survivin and OCT4 were found to perform a therapeutic effect in ESCC.

What is the implication, and what should change now?

- The dual target design strategy provided a novel and effective adjuvant target therapy for ESCC and should be profoundly explored.

Table 1 Sequences of shRNA and survivin primers

Primer	Primer sequence
shSRVN	5'-GAAAGTGCGCCGTGCCATC-3'
shOCT4	5'-CCCTCACTTCACTGCACTG-3'
shNC	5'-GACTTCATAAGGCGCATGC-3'
Survivin upstream primer	5'-CGGCTAGCCATAGAACCAGAG-3'
Survivin downstream primer	5'-GAAGATCTGCCGCCGCCGCCACCT-3'

shRNA, short hairpin RNA.

checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-4499/rc).

Methods

Construction of shRNA vectors and survivin promoter-regulated adenoviruses

Plasmids expressing specific shRNA, including survivin-shRNA (shSRVN), OCT4-shRNA (shOCT4), dual-shRNA (shSRVN + shOCT4), and control shRNA (shNC), were synthesized by Wuhan Genesil Biotechnology Co., Ltd. (Wuhan, China). The corresponding interference sequences of genes are shown in *Table 1*. The shRNA structure was composed of U6 promoter, 19 nucleotides-sense DNA, loop (TTCAAGACG), antisense DNA, and tail (TTTTTT), and the dual-shRNA was arranged in a "foot-to-foot" structure.

Based on the type 5-adenovirus (Ad5) backbone pBHGloxdeltaE13Cre (Microbix Biosystems; Ontario, Canada) with the deletion of the E1 and E3 regions, the aforementioned shRNAs were inserted into the E3 region to construct the pAd5-shRNA vector. Survivin upstream and downstream primers were used to amplify the survivin promoter sequence in the pSRVN-Luc plasmid and replace the mCMV promoter sequence in pDC315 (Microbix Biosystems) with the amplified product used to construct pDC315Spro. The E1a full-length complementary DNA (cDNA) sequence of Ad5 was synthesized, the Kozak consensus sequence was added, and the downstream section of the survivin promoter sequence in pDC315SPro was inserted to construct pDC315SPro-E1a. The luciferase plasmid, pSRVN-Luc, in which luciferase expression was under the control of the survivin promoter (nucleotides 1824-2800, GenBank U75285), was kindly provided by Himanshu Garg (Center of Excellence for Infectious Disease, Texas Tech University Health Sciences Center, TX, USA). The 293T cells were co-transfected with pDC315SPro-E1a and pAd5-shRNA. After intracellular recombination, the recombinant adenoviruses were purified and completed, and named AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, and AdSProE1a-shNC, respectively. In addition, the replication control adenovirus, AdSProE1a-EGFP, and the replication-deficient control adenoviruses, Ad-shSRVN + shOCT4 and Ad-EGFP, were constructed with enhanced green fluorescent protein (EGFP) instead of the shRNA expression sequence (*Figure 1A*).

Cell culture

Human esophageal squamous cell cancer (ESCC) lines, Eca109, TE1, and human embryonic lung fibroblasts MRC-5, were purchased from the Chinese Academy of Sciences (Shanghai, China). All the cell lines were authenticated by short tandem repeat DNA fingerprinting. The Eca-109 cells were cultivated in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution.

Western blot and immunofluorescence staining

Western blotting was used to detect the expression levels of survivin, OCT4, E1a, and epithelial-mesenchymal transition (EMT) markers, including E-cadherin and vimentin. The cells were plated in a 24-well plate with 5×10^4 cells/well. The FBS medium was replaced with the serum-free medium after cells had attached, and the viruses AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, and AdSProE1a, were diluted with serum-free medium. The short hair pin negative control (shNC) group and the Ad-shSRVN + shOCT4 group were used to infect cells at multiplicity of infection (MOI) =5 pfu/cell for 2 hours, and then culture medium containing 5% serum was used for 72 hours. A total of 30 μg protein was extracted from cells and separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane via electroblotting. The membrane was blocked with 5% w/v nonfat, dry milk for 1 hour, and the antibodies were incubated at 4 ℃ with gentle shaking, overnight. Next, goat anti-mouse or anti-rabbit antibody was incubated for 1 hour at room temperature. Target proteins were then visualized

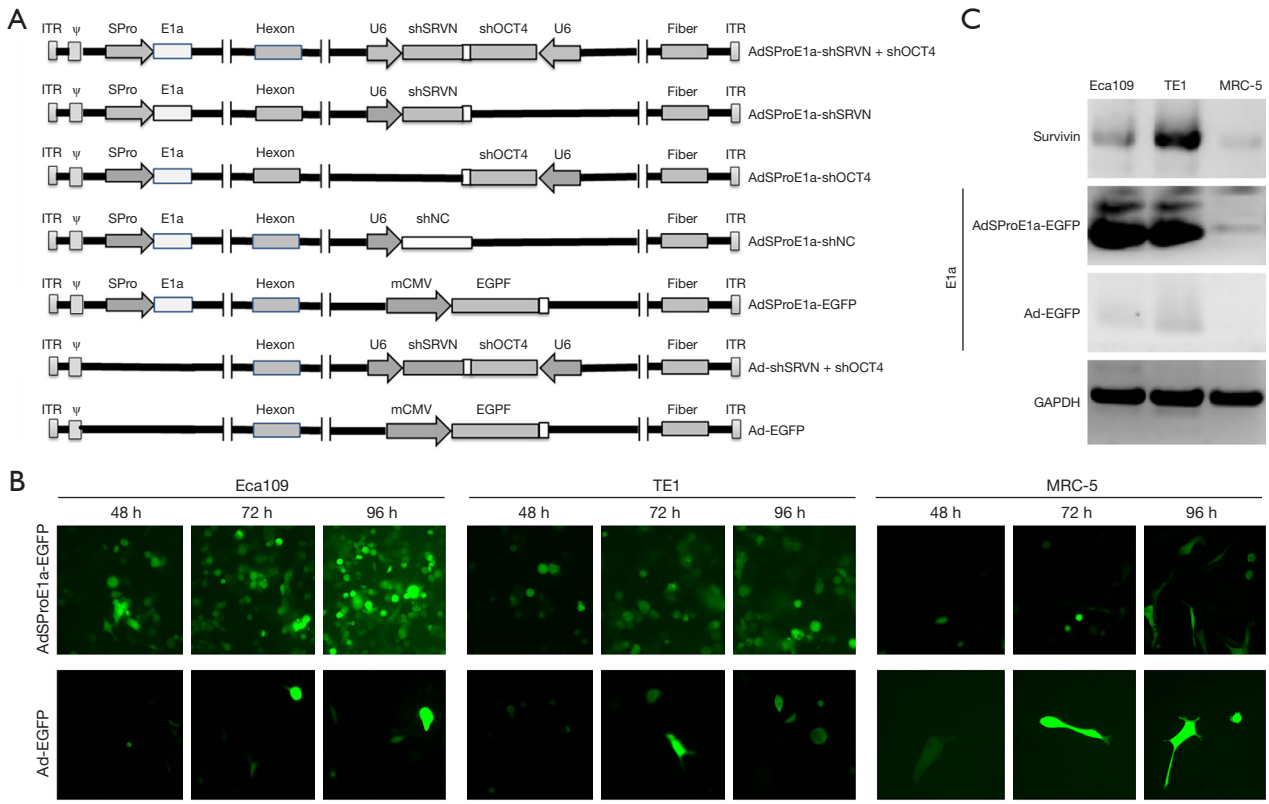

Figure 1 The oncolytic adenovirus specifically replicated and mediated E1a and EGFP expression in ESCC cancer cells. (A) Schematic diagram of experimentally constructed viral vectors. (B) Replicative and non-replicative adenoviruses mediated the expression of EGFP. Experimental cells were seeded in 24-well plates at 5×10^4 cells/well and infected with replicating virus AdSProE1a-EGFP and replication-deficient virus Ad-EGFP at MOI =5 pfu/cell, respectively, and cultured for 48, 72, and 96 h. EGFP expression was observed by fluorescence microscopy at 3 time points. Original magnification: 100×. (C) Detection of survivin and E1a expression. After 96 h in the above culture system, cells were collected, and the expression levels of survivin and E1a were detected by western blotting. GAPDH was used as an internal reference for loading. ITR, terminal reverse sequence repeat; ψ, adenovirus packaging signal; SPro, survivin promoter; shSRVN, survivin shRNA; shOCT4, OCT4 shRNA; shNC, control shRNA; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOI, multiplicity of infection; ESCC, esophageal squamous cell carcinoma.

with an enhanced chemiluminescent reagent.

Furthermore, the localization and relative expression of survivin and OCT4 in cells were detected by immunofluorescence staining. The cells were harvested 72 hours after transfection. Cells were transferred to a Lab-Tek chamber at a concentration of 1×10^4 cells/well, and fixed with 4% formaldehyde for 30 minutes. Survivin and OCT4 monoclonal antibodies were used and incubated overnight at 4 ℃. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG or tetramethylrhodamine (TRITC)-conjugated anti-mouse IgG were then added and incubated for another 1 hour at room temperature. The intensity of gene expression was observed under a laser confocal microscope.

Tumor-specific identification of oncolytic adenovirus replication

The cells were plated in a 24-well plate with 5×10^4 cells/well. After the cells had adhered, the serum-free medium was used, and the replication-type virus, AdSProE1a-EGFP, and the replication-defective virus, Ad-EGFP, were diluted with the serum-free medium, according to MOI =5 pfu/cell infected cells for 2 hours. They were then changed to medium containing 5% serum and continued in culture for 48, 72, and 96 hours. The expression of EGFP was observed by fluorescence microscope at 3 time points, and the proportion of positive cells was counted; the cells were collected at 96 hours, and the expression of E1a was

detected by western blotting method.

Cells were plated in 96-well plates with 1×10^4 cells/well. After the cells had attached to the wall, a serum-free medium was used for transfection. Then, AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, and AdSProE1a-shNC were diluted with serum-free medium. The Ad-shSRVN + shOCT4 was added to infect the cells at MOI =5 pfu/cell for 2 hours, and medium containing 5% FBS was applied and the culture continued for 0, 24, 48, 72, and 96 hours. Cells were collected at 5 time points and virus titers were determined by the 50% Tissue Culture Infectious Dose (TCID50) method.

Cell Counting Kit-8 (CCK-8) assay and flow-cytometry

Cells were plated in 96-well plates with 1×10^4 cells/well, and serum-free medium was used after cells had attached to the wall. Then, AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, and AdSProE1a-shNC were diluted with serum-free medium. The Ad-shSRVN + shOCT4, with concentrations of MOI =0, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 pfu/cell, was added to infect the cells for 2 hours, and then cells were cultured in medium containing 5% FBS for 7 days. A total of 8 replicate wells were set corresponding to each MOI value. The CCK-8 (Dojindo Laboratories, Kumamoto, Japan) was used according to the manufacturer's instructions to detect cell viability, and the IC50 value of each group was calculated and compared.

Cells were plated in 6-well plates with 1×10^5 cells/well. Then, AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, and AdSProE1a-shNC were diluted with serum-free medium and were added to infected cells for 2 hours at a concentration of MOI =5 pfu/cell. The medium was replaced and culturing continued for 72 hours. Cells were collected, washed twice with PBS, and then fixed with 75% ethanol overnight at 4 ℃. A total of 5 μL propidium iodide (PI) was added and incubated for 30 minutes in the dark, and the cell cycle was detected by flow cytometry.

Xenograft experiment

Animal experiments were approved by the Ethics Committee of Shanghai Chest Hospital, in compliance with guidelines for the care and use of animals of Shanghai Jiao Tong University School of Medicine. A total of 50 5-week-old male BALB/c nude mice were used to conduct a xenograft experiment. We subcutaneously injected 5×10^6 cells under the forelimb in each mouse. After tumors had formed in all mice after 14 days, the 4 larger and the 4 smaller tumors were excluded and the remaining 42 animals were randomly divided into 7 groups (AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, AdSProE1a-shNC, Ad-shSRVN + shOCT4, Ad-EGFP, and negative control). Mice in each group were given an intra-tumoral injection of the therapeutic virus, 2×10^8 pfu each time, once every other day, 5 consecutive times. The tumor size was measured routinely. The animal welfare guidelines for the care and use of laboratory animals were approved. The experiment was terminated when the average tumor volume in any group exceeded 2,500 mm^3.

At the end of the observation period, the mice were sacrificed, and tumor specimens were removed, fixed in 10% formalin overnight, and then paraffin-embedded for sectioning. The expressions of adenovirus protein E1a, OCT4, survivin, E-cadherin, and vimentin were measured by immunohistochemical (IHC) staining. The apoptosis ratio was evaluated by the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) method.

Statistical analysis

Differences between the treated and control groups were analyzed using the Student's *t*-test or one-way analysis of variance (ANOVA) if they followed a normal distribution, otherwise, the Mann-Whitney test was adopted. All statistical analyses were performed using the software SPSS 24.0 (IBM Corp., Armonk, NY, USA). A value of $P<0.05$ was considered statistically significant.

Results

Oncolytic adenovirus replicated stably in tumor cells

In order to achieve dual knockdown of survivin and OCT4, an oncolytic adenovirus carrying survivin-shRNA (shSRVN) and/or OCT4-shRNA (shOCT4) was constructed and transferred to ESCC cells (*Figure 1A*). We first detected the intracellular replication viability of different viruses, and the results revealed that the oncolytic adenovirus, AdSProE1a-EGFP, effectively infected cancer cells, and the number of copies of virus increased over time according to epidermal growth factor receptor (EGFR) expression, whereas EGFP did not increase over time in MRC-5 cells. After the replication-deficient adenovirus Ad-EGFP infected cancer

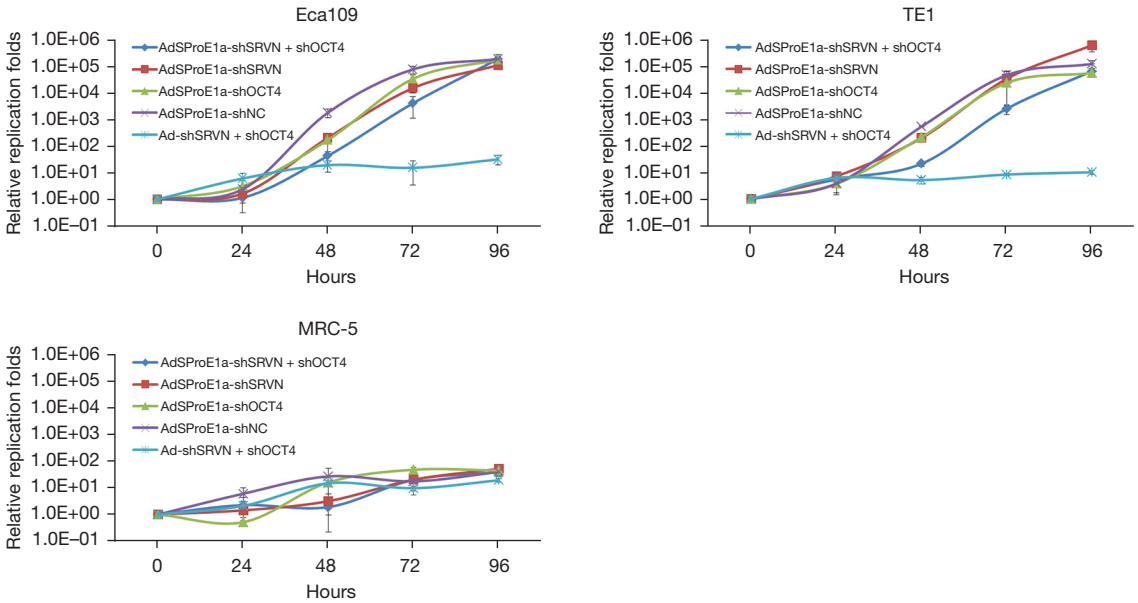

Figure 2 Identification of specific replication of oncolytic adenovirus in ESCC cancer cells. Cultured cells were plated in 96-well plates at 1×10^4 cells/well and infected with AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, AdSProE1a-shOCTNC, and Ad-shSRVN + shOCT4 at an intensity of MOI =5 pfu/cell. Culture was continue for 0, 24, 48, 72, and 96 h. Cells were collected at 5 time points, and the virus was determined by TCID50 assay. The virus replication fold at other time points was calculated using the standard of 0 h. shSRVN, survivin shRNA; shOCT4, OCT4 shRNA; shNC, control shRNA; ESCC, esophageal squamous cell carcinoma; MOI, multiplicity of infection; TCID50, 50% tissue culture infectious dose.

cells, there was no significant up-regulation of EGFP at 48, 72, and 96 hours (*Figure 1B*). By detecting the expression of survivin and E1a in cells after virus infection, we found E1a was strongly expressed in cancer cells after AdSProE1a-EGFP infection, while it was barely expressed in MRC-5 cells. The expression of survivin was consistent with E1a, which was up-regulated after transfection in cancer cells and with no significant change in MRC-5 cells. Neither E1a nor survivin was expressed in cells infected with Ad-EGFP (*Figure 1C*).

The virus titer was determined by TCID50 assay to evaluate the replication viability of the virus in different cells. The results showed that the oncolytic adenoviruses, AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, and AdSProE1a-shNC, could all replicate in cancer cells, and replication activity gradually increased over time and started to drop at 96 hours after transfection. The oncolytic adenovirus containing AdSProE1a-shSRVN + shOCT4 had the highest replication of 192,085 times in Eca109 cells, and the oncolytic adenovirus carrying AdSProE1a-shSRVN had the highest replication of 620,055 times in TE1 cells; however, none of the oncolytic viruses had significant replication in MRC-5 cells. The replication-deficient adenovirus, Ad-shSRVN + shOCT4, had no obvious replication activity in both cancer cells and normal cells (*Figure 2*).

Effects of oncolytic adenovirus on gene expression in ESCC cells

Western blotting detected the expression of survivin, OCT4, E-cadherin, and vimentin. The results indicated that AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, and Ad-shSRVN + shOCT4 could reduce the expression of survivin and vimentin in Eca109 and TE1 cells. At the same time, E-cadherin was up-regulated, especially in cells infected with oncolytic virus containing AdSProE1a-shSRVN + shOCT4. It was shown that AdSProE1a-shSRVN + shOCT4, AdSProE1a-shOCT4, and Ad-shSRVN + shOCT4 could down-regulate the expression of OCT4, especially AdSProE1a-shSRVN + shOCT4; AdSProE1a-shSRVN did not affect the expression of OCT4.

In MRC-5 cells, survivin was barely detected, and OCT4

was negatively expressed. All of AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, and AdSProE1a-shOCT4 could down-regulate the expression of survivin in MRC-5 cells yet survivin was completely negative in MRC-5 cells transfected with oncolytic virus containing AdSProE1a-shSRVN + shOCT4. All oncolytic viruses had no significant effect on the expression of E-cadherin and vimentin in MRC-5 cells (*Figure 3A*). Confocal immunofluorescence staining was used to locate and quantify survivin and OCT4. Compared to the control group, AdSProE1a-shSRVN + shOCT4 significantly reduced the expression of survivin and OCT4 in Eca109 cells, while AdSProE1a-shNC and Ad-shSRVN + shOCT4 had no significant effect on the expression of survivin and OCT4 in cells (*Figure 3B*).

The effect of oncolytic adenovirus on the proliferation and cell cycle of ESCC cells

Cell proliferation ability after transfection was detected by the CCK-8 method. The cell survival rate was 100% at the starting point of culture (0 h), and the viability of Eca109 and TE1 cells in each group gradually decreased with the increase in the MOI value, while MRC-5 cells had no apparent response to the oncolytic virus. The IC50 value was used to evaluate the cytotoxicity of the oncolytic virus, and it was found that the knockdown of survivin or OCT4 significantly reduced the IC50 value. The IC50 of cancer cells transfected with AdSProE1a-shSRVN decreased significantly more than in any other group, except for the dual knockdown group. The cytotoxicity of AdSProE1a-shNC on cells originated from the oncolysis of viral replication, hence the inhibitory effect mediated by the replication-type oncolytic adenovirus was stronger than that mediated by the replication-deficient adenovirus (*Figure 4A*). Both replication-type oncolytic adenoviruses and replication-deficient adenoviruses have high IC50 values for MRC-5 inhibition. This study confirmed that the dual knockdown of survivin and OCT4 mediated by replicative oncolytic adenovirus not only strengthened the cytotoxicity of the cancer cells, but also improved the specificity of therapy against the cancer cells. Cell cycle identification showed that in Eca109 and TE1 cells, knockdown of OCT4 with AdSProE1a-shOCT4 mainly caused G0/G1 phase arrest, and AdSProE1a-shSRVN knockdown of survivin mainly caused G2/M phase arrest. The double knockdown of survivin and OCT4 by AdSProE1a-shSRVN + shOCT4 was still characterized by G0/G1 arrest (*Figure 4B*).

Oncolytic adenovirus-mediated dual knockdown of survivin and OCT4 inhibited xenograft growth in vivo

A xenograft model was established by subcutaneous injection of Eca109 cells followed by intratumoral injection of the virus. As a result, compared to the control group, the tumor inhibition rates of the AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, AdSProE1a-shNC, Ad-shSRVN + shOCT4, and Ad-EGFP groups were 89.93% ($P<0.0001$), 81.43% ($P<0.0001$), 51.62% ($P<0.0001$), 35.94% ($P<0.0001$), 18.22% ($P=0.0209$), and 4.86% ($P=0.5936$), respectively. The results showed that the inhibitory effect of dual knockdown was significantly better than that of single-gene intervention, and the therapeutic effect mediated by replication-type oncolytic adenovirus was significantly stronger than that of a replication-deficient adenovirus vector, confirming that the oncolytic adenovirus with survivin and OCT4 dual knockdown could produce significant anticancer effects and improve ESCC treatment effects (*Figure 5A*).

In addition, tumor specimens were taken for IHC examination. It was revealed that E1a was strongly expressed in AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, and the AdSProE1a-shNC group and negative expression was observed in Ad-shSRVN + shOCT4, Ad-EGFP, and the control group. Survivin decreased in AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, and in the Ad-shSRVN + shOCT4 group; OCT4 decreased in the AdSProE1a-shSRVN + shOCT4 and the AdSProE1a-shOCT4 group. However, E-cadherin and vimentin in tumors from AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, and the AdSProE1a-shOCT4 group were up-regulated and down-regulated, respectively. The results of TUNEL revealed that except for the Ad-shSRVN + shOCT4 and Ad-EGFP group, the apoptosis rate of cancer cells in all groups, especially the AdSProE1a-shSRVN + shOCT4 group, significantly promoted apoptosis in cancer cells compared to the control group (*Figure 5B*).

Discussion

Our previous study found that survivin was expressed in 62% of ESCC tissues and 8% of adjacent normal esophageal tissues, suggesting that survivin might regulate the malignant biological behavior of ESCC cells and could be negatively correlated with the prognosis. Besides, inhibition of survivin could induce apoptosis, lead to cell

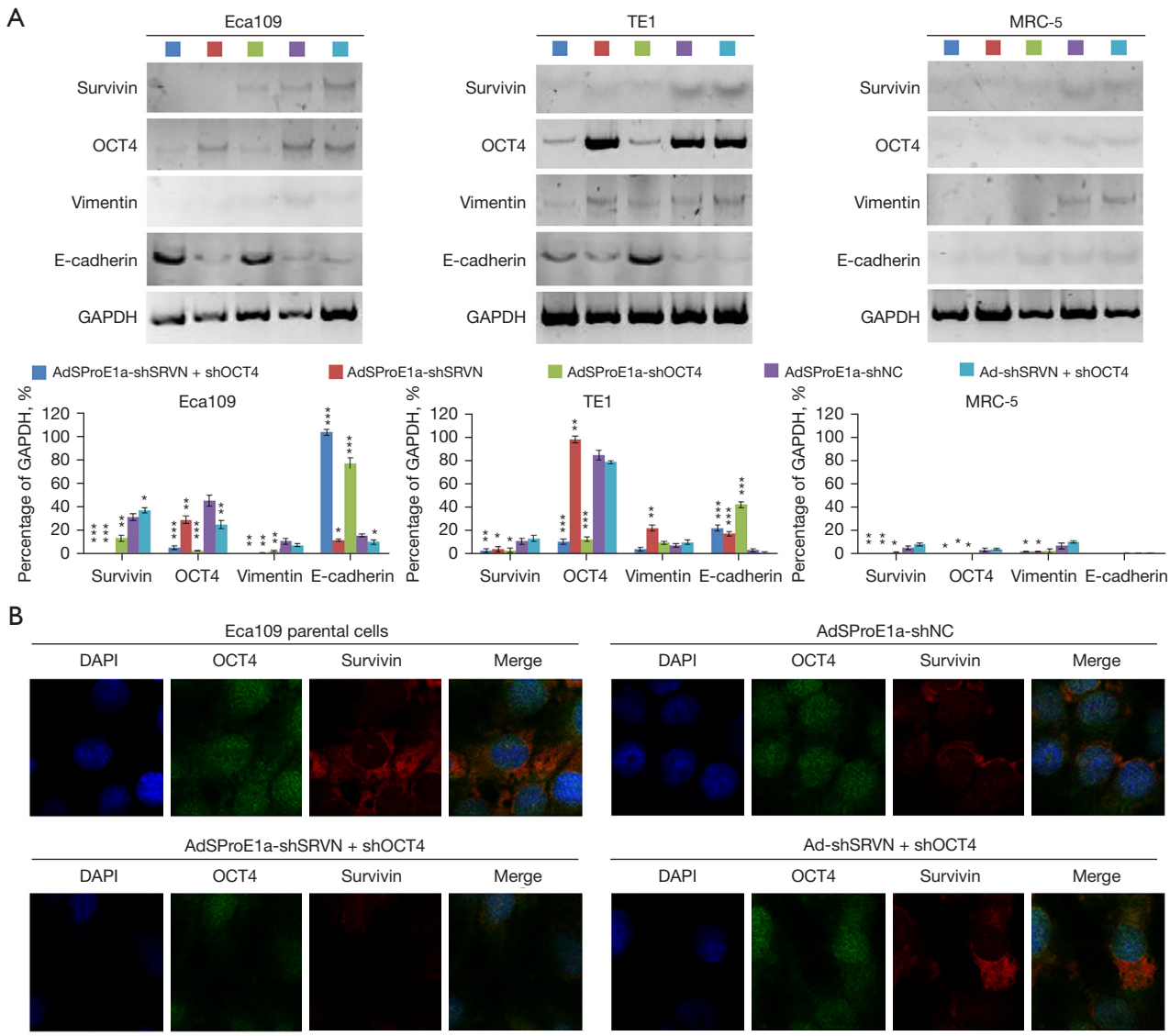

Figure 3 Effect of oncolytic adenovirus on gene expression in ESCC cells. (A) The expression levels of survivin, OCT4, vimentin, and E-cadherin in the cells were detected by western blotting. Cultured cells were plated in 24-well plates at 5×10^4 cells/well and infected with AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, AdSProE1a-shNC, and Ad-shSRVN + shOCT4 at an MOI =5 pfu/cell. The culture was continued for 72 h. The cells were collected, and total cellular protein was extracted by the TRIzol method and loaded for detection. GAPDH was used as an internal reference for loading. Compared with the shNC virus group, *P<0.05, **P<0.01 and ***P<0.001. (B) The expression levels of survivin and OCT4 were detected by immunofluorescence staining markers. The cells at 72 hours after virus infection were collected and inoculated into Lab-Tek culture plates at a concentration of 1×10^4 cells/well. After cell attachment, the cells were fixed in 4% formaldehyde for 30 min, washed with PBS (pH 7.2), incubated with survivin and OCT4 antibodies overnight at 4 ℃, and then incubated with FITC and TRITC conjugated bridging antibodies for 1 hour. The intensity of gene expression was observed under a confocal laser scanning microscope. DAPI was used to stain nuclei. Original magnification: 400×. OCT4, octamer-binding transcription factor 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; shSRVN, survivin shRNA; shOCT4, OCT4 shRNA; DAPI, 4',6-diamidino-2-phenylindole; ESCC, esophageal squamous cell carcinoma; MOI, multiplicity of infection; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine.

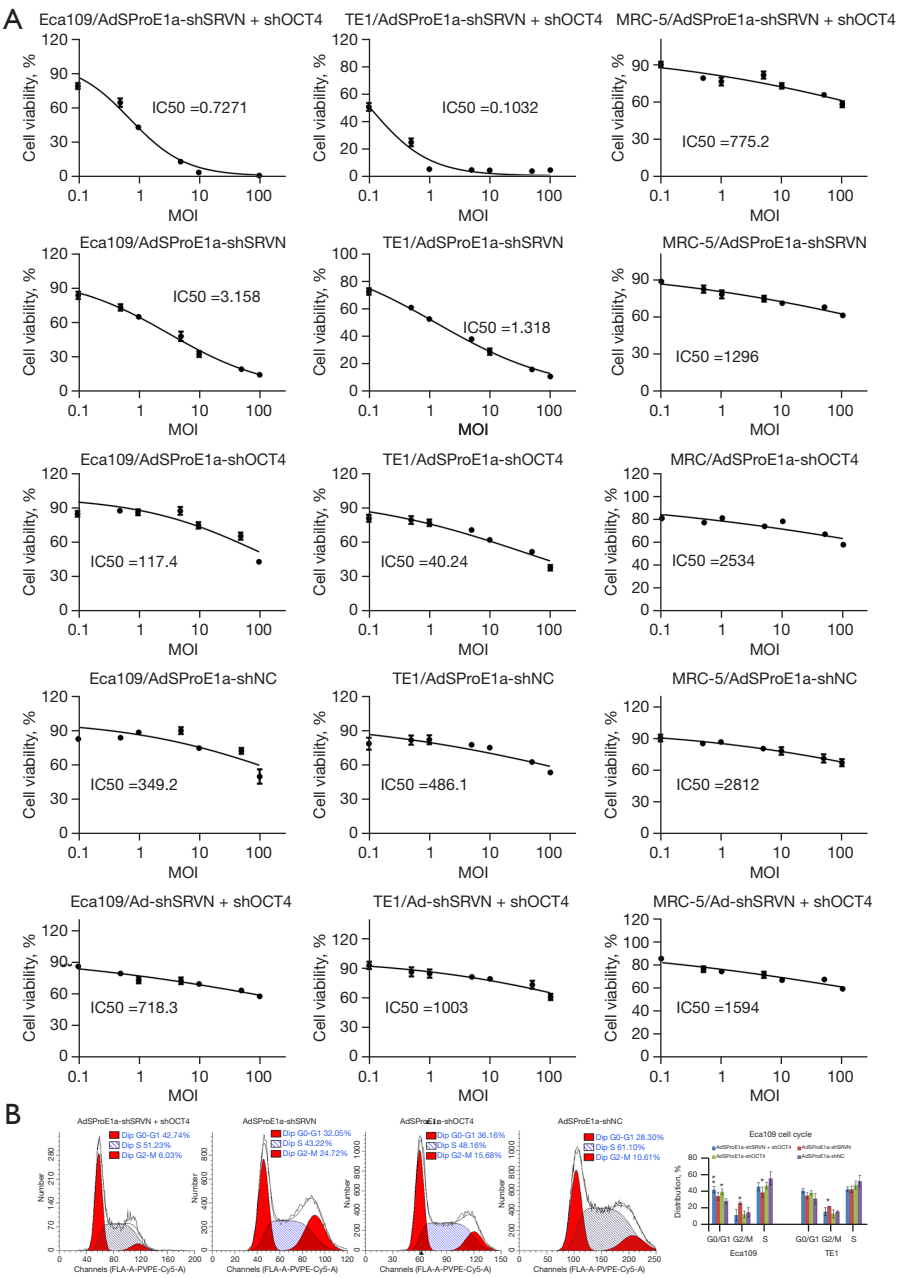

Figure 4 Inhibition of ESCC cell proliferation by oncolytic adenovirus. (A) Effect of oncolytic adenovirus carrying different target genes on cell survival. Cells were seeded in 96-well plates at 1×10^4 cells/well and infected with viruses AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, AdSProE1a-shOCT4, AdSProE1a-shOCT4, AdSProE1a-shNC, and Ad-shSRVN + shOCT4 at a concentration gradient of MOI =0, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 pfu/cell, corresponding to 8 duplicate wells at each MOI value, and cultured for another 7 days. The CCK-8 kit was used to detect the effect of the virus on cell survival and to calculate IC50 values. (B) Effect of oncolytic adenovirus carrying different target genes on cell cycle. Cel ls were seeded in 6-well plates at 1×10^5 cells/well and infected with AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, and AdSProE1a-shOCTNC at a concentration of MOI =5 pfu/cell. The culture was continued for 72 h. The cells were collected, fixed in 75% ethanol, stained with 5 μL PI, and the cell cycle was detected by flow cytometry. Compared with the shNC virus group, *P<0.05 and **P<0.01. shSRVN, survivin shRNA; shOCT4, OCT4 shRNA; IC50, the half maximal inhibitory concentrations of oncolytic adenovirus; MOI, multiplicity of infection; shNC, control shRNA; Dip, diploid; ESCC, esophageal squamous cell carcinoma; CCK-8, Cell Counting Kit-8; PI, propidium iodide.

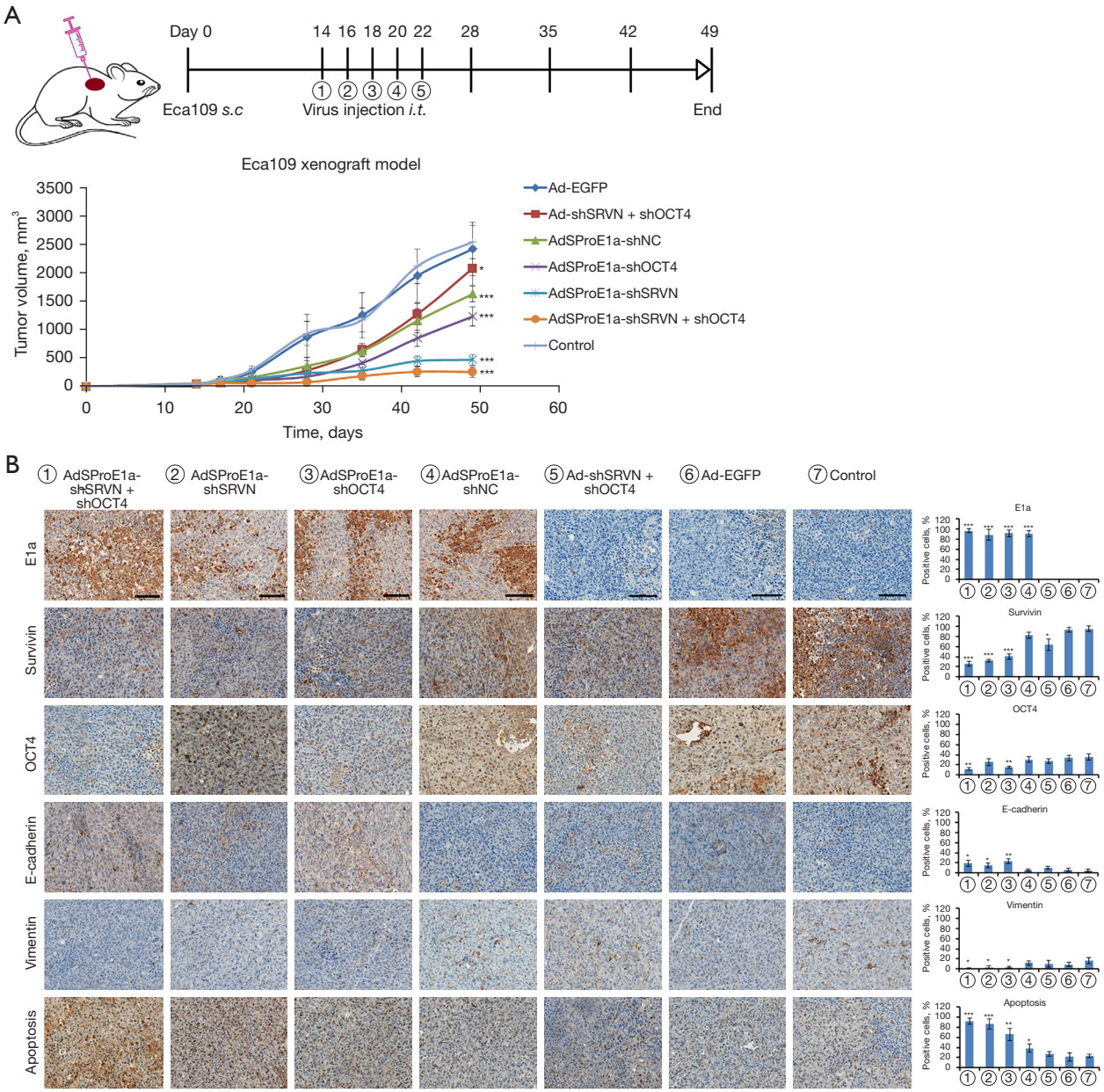

Figure 5 Animal experiments in which oncolytic adenoviruses inhibited Eca109 xenografts. (A) Effect of oncolytic adenovirus carrying different target genes on xenografts in animal model. BALB/c (nu/nu) were subcutaneously implanted with Eca109 cells in the right axilla, 5×10^6 cells/mouse, and after 14 days of tumor formation, they were randomly divided into 7 groups (AdSProE1a-shSRVN + shOCT4, AdSProE1a-shSRVN, AdSProE1a-shOCT4, AdSProE1a-shNC, Ad-shSRVN + shOCT4, Ad-EGFP, and blank control groups), with 6 animals in each group, and treatment was started. The virus-treated group received an intra-tumoral injection of the corresponding virus at 2×10^8 pfu per animal every other day for 5 consecutive times (1–5 represented the order of virus injection). Tumor size was measured regularly by a vernier caliper to calculate the tumor volume, and the observation was ended on day 49 after tumor cell inoculation. Compared with the control group, *P<0.05, and ***P<0.001. (B) Immunohistochemical detection of tumor samples. Tumor samples were taken to make pathological sections, the expression of E1a, survivin, OCT4, E-cadherin, and vimentin was detected by immunohistochemistry, and the apoptosis rate was labeled using TUNEL assay. Compared with the control group, *P<0.05, **P<0.01, and ***P<0.001. Bar: 100 μm (all photographs had the same magnification). EGFP, enhanced green fluorescent protein; shSRVN, survivin shRNA; shOCT4, OCT4 shRNA; shNC, control shRNA; OCT4, octamer-binding transcription factor 4; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

cycle arrest, and inhibit the proliferation of cancer cells (13,19). We further explored the underlying mechanism and found that survivin was positively regulated by OCT4, and patients with a high expression of OCT4 and survivin showed a worse prognosis (13).

Oncolytic virus therapy has become an efficient therapy for malignant tumors (20). Our previous study confirmed that the surviving promoter-containing oncolytic adenovirus could achieve an effective anti-tumor effect. Replicability of oncolytic viruses eliminates the disadvantages of low expression of transfected genes and tumor-targeting inability, which is completely different from traditional chemotherapy, radiotherapy, and other adjuvant therapies (21). For EC, due to the high malignancy rate and the rapid proliferation of cancer cells, especially for tumor stem cells, single-gene intervention therapy was not sufficient to inhibit tumor growth and metastasis, which requires us to further optimize the treatment strategy. Therefore, we considered a gene therapy method combining oncolytic virus therapy and double-gene intervention, hoping to achieve a synergistic effect. Based on the high expression of survivin in EC and that it has been shown to be regulated by OCT4, we synthesized a specific replicative oncolytic adenovirus containing shRNA of survivin and OCT4 to realize dual knockdown, with the intention to establish an effective targeted therapy strategy. In detail, the expression of survivin increased with the proliferation of cancer cells, which initiated replication of the oncolytic virus and shRNAs targeting survivin and OCT4, resulting in cancer cell death. Furthermore, once the cancer cells were eliminated by the oncolytic virus and the expression of survivin was diminished, the virus stopped replicating and functioning. The entire feedback mechanism endowed the therapeutic system with higher specificity and safety, and the presence of the virus appeared to prevent the recurrence and metastasis of cancer cells and sustain an anti-tumor effect.

By detecting the expression of survivin and OCT4 in ESCC cells and the replication ability of the oncolytic adenovirus in cancer cells, we found that survivin was significantly upregulated in cancer cells, which was positively correlated with the replication ability of the oncolytic adenovirus. The massive replication of the oncolytic adenovirus enabled an efficient increase in the expression of EGFP and shRNA while having little effect on normal cells, such as MRC-5. Therefore, the oncolytic adenovirus regulated by the survivin-promoter could achieve higher specificity and safety in treatment used for ESCC.

In our study, it was found that the oncolytic adenovirus-mediated knockdown of survivin or OCT4 could inhibit the proliferative ability of cancer cells and induce cell cycle arrest. Compared to other groups, the inhibitory effect was more pronounced in the dual knockdown group. Target therapy mediated by the replicative oncolytic adenovirus was significantly more effective than that mediated by the replication-deficient adenovirus. It was also found in the experiment that the knockdown of OCT4 could down-regulate the expression of survivin, while the knockdown of survivin had no effect on OCT4, suggesting that OCT4 is an upstream regulator of survivin. The knockdown of survivin and OCT4 also influenced the cell cycle; OCT4 knockdown mainly caused G0/G1 phase arrest, and survivin knockdown mainly caused G2/M phase arrest.

In order to further explore the synergistic effect of the oncolytic adenovirus-mediated dual knockdown of survivin and OCT4, we constructed a xenograft model by subcutaneous injection of Eca109 cells. After intratumoral injection of virus treatment, we found that the curative effect of dual knockdown therapy was significantly better than single-gene intervention. The therapeutic effect mediated by the replication-type oncolytic adenovirus was significantly stronger than the replication-deficient adenovirus vector, confirming that the survivin and OCT4 dual knockdown oncolytic adenovirus therapeutic strategy could produce multiple effects and significantly improve the anti-tumor effect. Since EMT is a common molecular event in EC and is closely related to its recurrence and metastasis, we detected the expression levels of EMT-related molecules in ESCC cell lines and nude mice xenografts. Oncolytic adenovirus-mediated dual knockdown of OCT4 and survivin upregulated E-cadherin and downregulated vimentin in cancer cells and tumor tissues, suggesting the reversal of EMT in ESCC after treatment, which was considered one of the important mechanisms to achieve a therapeutic effect. It was recognized that EC cells highly expressed OCT4 and survivin, which endowed the cells with high proliferative and metastatic abilities by inducing EMT. The process of EMT was reversed after simultaneous interference by OCT4 and survivin led to the inhibition of tumor progression. However, there are some limitations in our study. First of all, the replication ability of oncolytic adenovirus *in vivo* and the corresponding virus titer displaying anti-tumor effect were not discussed. Besides, the consistency and stability of anti-tumor effect of the oncolytic adenovirus need to be explored in the following

study. At last, researches about the side-effects of oncolytic adenovirus-mediated therapy *in vivo* and *in vitro* were not presented.

Conclusions

In conclusion, our study established an oncolytic adenovirus-mediated target therapy against EC. The replicative oncolytic adenovirus-mediated dual knockdown strategy, which was characterized by the survivin promoter as the core regulatory sequence, exerted an inhibitory effect on EC (Figure S1). Our study demonstrated that the dual target design strategy ensured the efficacy and safety of the treatment system and provided a novel and effective adjuvant target therapy for EC. In our future study, we will continue to explore the consistency and stability of anti-tumor effect of the oncolytic adenovirus. Besides, the underlying mechanism, especially the relevant signaling pathways, will be discussed and the side-effect of oncolytic adenovirus-mediated dual knockdown of survivin and OCT4 *in vitro* and *in vivo* will be explored.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-4499/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were approved by the Ethics Committee of Shanghai Chest Hospital, in compliance with guidelines for the care and use of animals of Shanghai Jiao Tong University School of Medicine.

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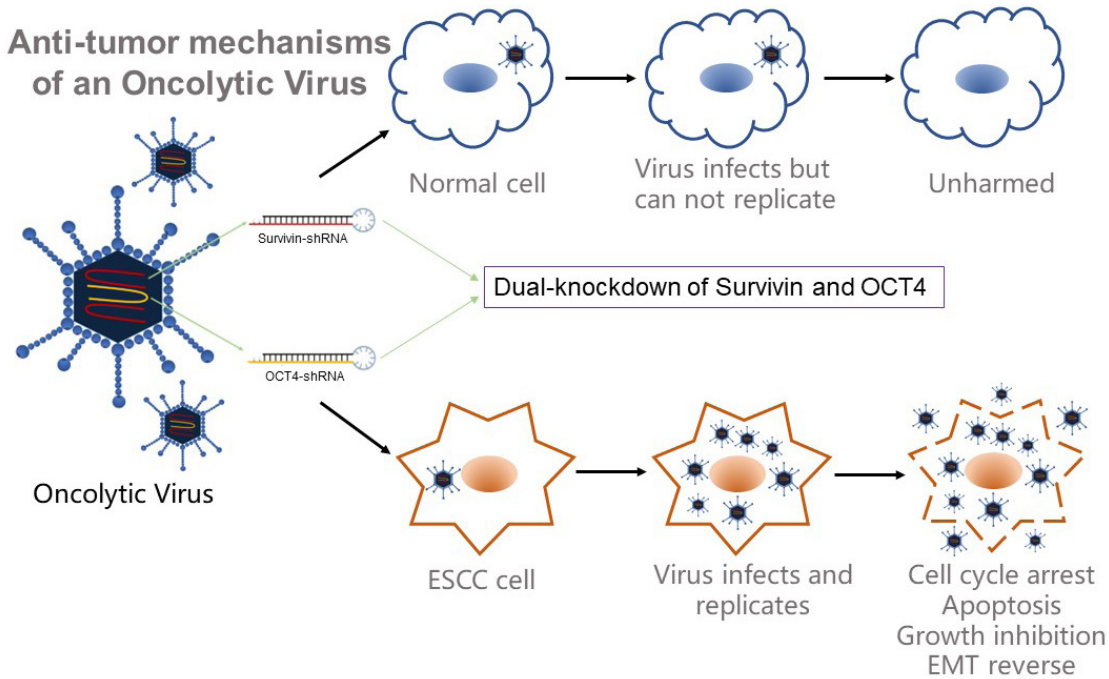

Figure S1 A diagrammatic illustration for the inhibition mechanism of knockdown of survivin and OCT4.

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