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ORIGINAL RESEARCH

Simultaneous overexpression of miR-126 and miR-34a induces a superior antitumor efficacy in pancreatic adenocarcinoma

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Background: Pancreatic adenocarcinoma (PAC) is one of the most fatal cancers due to its high degree of malignancy, increasing incidence, high mortality, and unsatisfactory treatment efficacy. Evidence has suggested that numerous microRNAs (miRNAs), including miR-126 and miR-34a, have potent tumor-suppressive effects on PAC, indicating a possible application of miRNA in tumor therapy. However, the therapeutic effect of a single miRNA on pancreatic cancer is limited.

Methods: We simultaneously delivered miR-126 and miR-34a into PAC cells by a carcinoembryonic antigen promoter-driven oncolytic adenovirus (AdCEAp-miR126/34a), and examined the antitumor efficacy of the therapeutic system in in vitro and in vivo experiments.

Results: In vitro cytological experiment found that the expression levels of miR-126 and miR-34a were significantly increased in the AdCEAp-miR126/34a-infected PAC cells, and the antitumor efficacy was enhanced in aspects of cancer cell viability, migration, invasion, and apoptosis by synergistically combining the antitumor effects of overexpressed miR-126 and miR-34a and the oncolytic effect of viral replication specifically in PAC cells. The expression levels of miR-126 target genes (*vascular endothelial growth factor-A* and *SOX2*) and miR-34a target genes (*cyclin D1*, *E2F1*, and *Bcl-2*) were markedly decreased in the PAC cells after being infected with AdCEAp-miR126/34a. Notable suppression of the therapeutic system on tumor growth was also proven in established PAC xenograft tumor models in nude mice, which demonstrated that the combination of miR-126 and miR-34a exerts more effective antitumor outcomes than a single miRNA.

Conclusion: The therapeutic system co-expressing miR-126 and miR-34a mediated by oncolytic adenovirus is a promising system for PAC target therapy.

Keywords: pancreatic cancer, miR-126, miR-34a, oncolytic adenovirus, target therapy

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Introduction

Pancreatic adenocarcinoma (PAC) is one of the most lethal cancers worldwide. Although great progresses in clinical diagnosis and treatments for PAC have been achieved, its 5-year survival rate is still <5%.[1] MicroRNAs (miRNAs), a class of endogenous small noncoding RNAs that function likely as proto-oncogenes and oncosuppressor genes, are essential for the regulation of target gene expression and posttranscriptional modification, and finally affect the occurrence and development of cancers.[2,3] Different cancers have different miRNA expression profiling. PAC is a kind of polygenic complex disease; the expression profiling of miRNAs in PAC is significantly altered and involved in the control of many corresponding target genes; miRNA abnormalities are widely related to abnormal proliferation, differentiation,

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metastasis, metabolism, chemoresistance, and apoptosis of cancer cells.[4–8] Consequently, miRNA-based gene therapy is becoming a new target strategy in cancer and has demonstrated antitumor effects in vivo and in vitro, which laid the foundation for its safe and effective application in tumor patients.

Studies have reported that the expression of miR-34a and miR-126 is downregulated in PAC tissues and blood. The miR-34 family, including miR-34a, miR-34b, and miR-34c, exhibits an abnormal low expression in a variety of cancers and plays different functions.[9,10] The transcription of miR-34a was adjusted by p53.[11–13] miR-34a was found to inhibit malignant growth of cancer by repressing genes involved in various oncogenic signaling pathways, such as targeting the silent mating type information regulation 2 homologue 1 (*SIRT1*) gene to enhance p53-mediated cell apoptosis,[14–16] repressing CD44 to inhibit the self-renewal of cancer stem cells.[17] Low expression of miR-34 endows cancer cells with high capabilities of proliferation, metastasis, stemness maintenance, apoptosis inhibition, and chemoresistance.[18] The broad anti-oncogenic activity of miR-34a enables it to become an effective target against human cancers. Therefore, the restoring of miR-34a expression or delivery of miR-34a mimics may be a promising therapeutic strategy for PAC treatment. miR-126 is also a tumor-suppressor miRNA which is downregulated in many tumors including PAC. It is known to target a disintegrin and metalloproteinase 9 (*ADAM9*) to control cell migration and invasion in PAC, as well as to induce a reversal of epithelial-to-mesenchymal transition (EMT).[20] Re-expression of miR-126 and siRNA-based knockdown of ADAM9 in PAC cells resulted in reduced cellular migration, invasion, and increased expression of epithelial marker E-cadherin.[21] Therefore, re-expression of miR-126 in PAC patients may be a novel strategy for preventing progression and metastasis.

The potential of miRNAs as treatment targets in cancer has been explored in many studies. Nowadays, vectors of plasmids and viruses are frequently used to mediate miRNAs (or miRNA mimics and inhibitors) into target cells so as to intervene miRNA expression and functions in cancer cells and achieve the effectiveness of tumor treatment.[22] Nanoparticles and corn cystatin 9 peptides were successfully used to carry miR-34a for the treatment of PAC and inhibit the growth of subcutaneous transplanted pancreatic tumors.[23] The viral vector had excellent gene transfer efficiency in cancer cells.[24] For example, miR-122 was efficiently delivered into diverse types of cancer cells and demonstrated strong antitumor efficacy.[25,26] In another study, Kota et al found that adenovirus could mediate miRNA transfer and lead to promotion of cell apoptosis, inhibition of cell proliferation, and blocking of tumor progress without toxicity to normal cells.[27] However, the specificity of those vectors was poor, and the efficiency and functional delivery of miRNA into cancer cells remain a great challenge.[28,29] Oncolytic adenovirus is a type of tumor-selective replicating vector and can mediate the specific expression of transgene with high efficiency in cancer cells and not in normal cells, and simultaneously present oncolytic effect with viral replication. Therefore, oncolytic adenovirus is a suitable vector to deliver miRNA genes for the treatment of cancer.

With the completion of a comprehensive overview about miRNA-based cancer gene therapy, we realized that the therapeutic effect of a single miRNA on pancreatic cancer was limited. That is because many miRNAs are involved in PAC oncogenesis and development by regulating the extensive target genes or many signaling pathways, and cancer cells can acquire tolerance to miRNA intervention for a certain time and easily regain their operative activity through alternative pathways. For this reason, interventions that target more than two miRNAs with complementary functions might inhibit multiple signaling pathways and exert enhanced efficacy for cancer treatment. In this study, we expressed miR-126 and miR-34a by an oncolytic adenovirus vector driven by the carcinoembryonic antigen (CEA) promoter. We explored if the therapeutic system carcinoembryonic antigen promoter-driven oncolytic adenovirus (AdCEAp-miR126/34a) could efficiently co-express miR-126 and miR-34a in PAC cells, and if it could achieve enhanced or synergistic antitumor effects in PAC treatment.

Materials and methods

Cell lines and cell culture

Human PAC cell lines (Panc-1, SW1990) were provided by the Department of Gastroenterology, Changhai Hospital, Second Military Medical University (Shanghai, China). The PAC cell line (Capa-2, BxPC3) and the fibroblast cell line (BJ) were purchased from the American Type Culture Collection (Manassas, VA, USA). The human embryonic kidney cell line HEK293 was obtained from Microbix Biosystems Inc (Mississauga, ON, Canada). All cell lines were analyzed for their short tandem repeat profile originally before experiments, and cultured in Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific) at 37°C under 5% CO_2 condition. An electrochemiluminescence assay was used to measure CEA content in the culture supernatants according to the

reference.[30] The cytological experiments were approved by the Biological Medical Experiment Ethics Committee of Second Military Medical University.

Construction of adenoviral vectors

The 19-bp expression sequence of miR-126 and miR-34a was used to design the small hairpin RNA (shRNA). The synthesis structure of the encoding shRNA was "XbaI + U6 + EcoRI + sense DNA + loop (ttc aag acg) + antisense DNA + TTTTTT + NheI" (miR-126) and "SpeI + U6 + BamHI + sense DNA + loop (ttc aag acg) + antisense DNA + TTTTTT + SalI" (miR-34a), respectively. The two shRNA encoding sequences were cloned into plasmids pDC315, separately or simultaneously, and pDC315-miR126, pDC315-miR34a, and pDC315-miR126/34a were obtained. The shRNA expression sequences in these three plasmids were digested and inserted into the CEA promoter-regulated oncolytic adenovirus AdCEAp-enhanced green fluorescent protein (EGFP), which was constructed in our previous study,[30] to displace the EGFP cassette, and then to generate a set of novel oncolytic adenoviruses, AdCEAp-miR126, AdCEAp-miR34a, AdCEAp-miR126/34a. The non-oncolytic adenovirus Ad5-miR126/34a and the control adenovirus Ad5-EGFP were constructed simultaneously. The viral titer of recombinant adenoviruses was determined with the tissue culture infectious dose 50 (TCID50) assay. The virus replication fold at 48 hours was normalized with that at the beginning of infection.

Identification of viral-specific replication and target expression of transgenes mediated by oncolytic adenoviruses

Cell lines were infected with the recombined adenoviruses at a multiplicity of infection (MOI) of 1 pfu/cell. After 48 hours postinfection, the harvested cells infected with the miR-126 or/and miR-34a expressed adenoviruses were measured as the virus titers by the TCID50 method. The percentages of EGFP-positive cells in the AdCEAp-EGFP- and Ad5-EGFP-infected cells were counted under a fluorescence microscope. The expression of miR-126 and miR-34a was measured by quantitative reverse transcription polymerase chain reaction. Specific primers for miRNAs were purchased from Shanghai Genechem Co, Ltd (Shanghai, China).

Cell proliferation assay

All cell lines were plated in 96-well plates at 1×10^4 per well and treated with adenoviruses at MOIs of 0, 1, 10 pfu/cell. After 48 hours postinfection, cells were added to 20 µL methyl thiazolyl tetrazolium (MTT) solution and incubated at 37°C for 4 hours, followed by removal of MTT solution and addition of 150 µL dimethyl sulfoxide solution. After mixing thoroughly in an oscillator for 10 minutes, the absorbance value at 540 nm was read on a microplate reader and the cell viability in every group was represented in percentages. The cell viability was calculated from the formula, cell viability = (A_{540} of tested group − A_{540} of blank group)/(A_{540} of control group − A_{540} of blank group) ×100%.

Transwell assay for invasion and migration tests

Cells were plated in a transwell chamber (Corning Life Sciences, Tewksbury, MA, USA) of 24-well plates at 5×10^4 per well and treated with adenoviruses at an MOI of 1 pfu/cell. Polycarbonate membrane was plated in the transwell chamber in the invasion test and not in the migration test. After 48 hours postinfection, cells were fixed with 4% formaldehyde for 10 minutes and stained by crystal violet hydrate solution for 20 minutes. Transwell chamber was washed by phosphate-buffered saline (PBS) and photographed within three random fields (200×) under microscope.

Apoptosis assay

In order to study the possible proapoptotic effects of our adenovirus therapy system, the PAC cells were seeded in 6-well plates at 5×10^5 per well and transfected with adenoviruses at an MOI of 1 pfu/cell. After 48 hours postinfection, cells were digested with trypsin-free EDTA solution and harvested, stained with Annexin V/propidium iodide (PI; MultiSciences Biotech, Shanghai, China), and analyzed by flow cytometry.

Western blotting

Cells were cultured in 24-well plates at 1×10^6 cells/well and infected with adenoviruses at an MOI of 1 pfu/cell. After 48 hours, cells were harvested, and total proteins were extracted. Western blotting method was used to detect the expression of the indicated proteins. The primary antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA; CDK4, cyclin D1, E2F1, Bcl-2), Abcam (Cambridge, UK; vascular endothelial growth factor [VEGF]-A, SOX2), and Cell Signaling Technology (Danvers, MA, USA; E1a, GAPDH).

Tumor xenografts in nude mice

The animal study was approved by the Animal Ethics Committee of the Second Military Medical University. All animal

study procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals of the US Department of Health and Human Services.

Forty healthy male purebred BALB/c nude mice, 5 weeks of age, were purchased from Shanghai SLAC Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China) and maintained in a specific pathogen-free grade animal laboratory in the Second Military Medical University. Mice were inoculated subcutaneously in the right axilla with Panc-1 cells, 1×10^6 cells in 100 µL solution for every mouse. Three weeks later after inoculation, three mice with maximal tumors and two mice with minimal tumors were excluded and the remaining 35 mice were randomly divided into seven groups (AdCEAp-miR126/34a, AdCEAp-miR126, AdCEAp-miR34a, Ad5-miR126/34a, AdCEAp-EGFP, Ad5-EGFP, and blank control). Intratumoral multipoint injection of every adenovirus (2×10^8 pfu/dose) in 100 µL of PBS was administrated, once every other day for five times totally. The blank control group was injected with the same volume of PBS synchronously. After treatments, the xenograft tumor development was termly observed and the tumor volume was calculated.

Animals were killed in an anesthetization box and tumors were obtained and weighed. The paraffin-embedded sections were prepared for examining the expression of SOX2 and cyclin D1 by immunohistochemistry, and apoptosis was examined by terminal-deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The percentage of positive cells were counted within five high-power fields.

Statistical analysis

Data from independent cytological experiments performed three times and from five mice in vivo experiments were presented as mean ± standard deviation. Student's *t*-test and one-way analysis of variance were used to calculate differences among the experimental groups according to the type of data. Statistical significance was confirmed when *P*-value was <0.05.

Results

Tumor-selective replication of oncolytic adenoviruses

Molecular structure diagrams of the constructed adenoviruses, including AdCEAp-miR126/34a, AdCEAp-miR126, AdCEAp-miR34a, Ad5-miR126/34a, AdCEAp-EGFP, and Ad5-EGFP, are shown in Figure 1A. The cultured cells showed CEA secretion with high levels in Panc-1, Capa-2, and BxPC3, and negative results in SW1990 and HEK293 cells (Figure 1B). Correspondingly, the expression of E1a was positive in Panc-1, Capa-2, and BxPC3 cells after infection of the oncolytic adenovirus AdCEAp-miR126/34a, in which the E1a gene was under the control of the CEA promoter. On the contrary, the expression of E1a was nearly negative in SW1990 infected with AdCEAp-miR126/34a and completely negative in all PAC cells infected with Ad5-miR126/34a (Figure 1C). We further detected the replication capability of adenoviruses by TCID50 method, and the results showed that the oncolytic adenoviruses AdCEAp-miR126/34a, AdCEAp-miR126, AdCEAp-miR34a, and AdCEAp-EGFP could replicate in Panc-1, Capa-2, and BxPC3 and not in SW1990 cells; the highest replication capacity of AdCEAp-miR126/34a was 6,385.22±1,101.64 fold increase in BxPC3 cells. The non-oncolytic adenoviruses Ad5-miR126/34a and Ad5-EGFP did not replicate in all PAC cell lines. Both the oncolytic adenoviruses and non-oncolytic adenoviruses could replicate in HEK293 cells (Figure 1D). The results suggested that the CEA promoter-driven E1a expression is consistent with the CEA secretion levels in PAC cells.

Target expression of transgenes mediated by oncolytic adenoviruses

After being infected with the oncolytic adenovirus carrying the EGFP gene (AdCEAp-EGFP), the PAC cell lines Panc-1, Capa-2, and BxPC3 showed more cells with EGFP expression, whereas, a few EGFP-positive cells were observed in SW1990 cells. The non-oncolytic adenovirus Ad5-EGFP had no replication activity in all tested cells (Figure 2A). Along with viral replication, the adenovirus AdCEAp-miR126/34a mediated high levels of miR-126 and miR-34a expression in Panc-1, Capa-2, and BxPC3 cell lines. The expression level of miR-126 and miR-34a was low in the above three PAC cell lines infected with Ad5-miR126/34a and SW1990 cells infected with AdCEAp-miR126/34a (Figure 2B). The results suggested that the CEA promoter-controlled oncolytic adenoviruses can specifically and efficiently mediate high levels of transgene expression in CEA-positive PAC cells.

Infection of adenoviruses affects the behavior of PAC cells

As described in the Introduction section, miR-126 and miR-34a are tumor-suppressor miRNAs which are mainly involved in controlling cell proliferation and mobility in cancer; so, we detected cell viability and mobility in PAC cells after infection of adenoviruses. The MTT assay showed that the infection of AdCEAp-miR126/34a, AdCEAp-miR126, and AdCEAp-miR34a resulted in an obvious decreased viability in Panc-1, Capa-2, and BxPC3 cell lines, especially in the

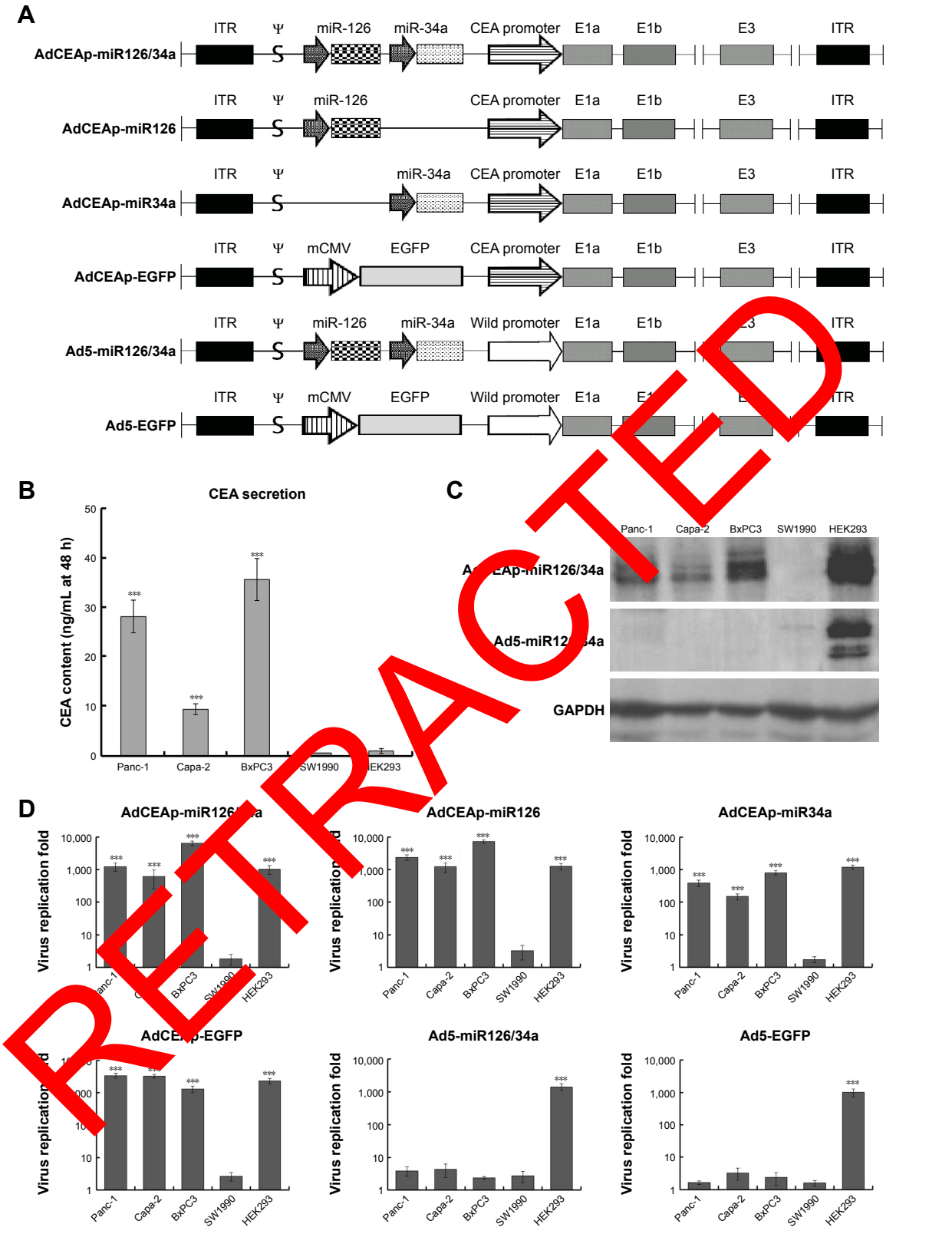


Figure 1 Specific replication of the CEA promoter-regulated oncolytic adenovirus in PAC cells.

Notes: (**A**) Molecular structure diagrams of the constructed adenoviruses. ψ: adenovirus type 5 packaging signal. (**B**–**D**) Cell lines were planted in 6-well plates at 1×10^6 per well and infected with the recombined adenoviruses at an MOI of 1 pfu/cell. (**B**) After 48 hours postinfection, cell culture supernatants were collected, and an electrochemiluminescence assay was used to detect CEA levels. ***$P<0.001$ versus the SW1990 group. (**C**) After 48 hours postinfection, cells were collected and used to detect E1a expression by Western blotting, with GAPDH as the loading control. (**D**) After 48 hours postinfection, cells were collected and the viral titers were quantified using the TCID50 assay. ***$P<0.001$ versus the SW1990 group.

Abbreviations: AdCEAp-miR126/34a, carcinoembryonic antigen promoter-driven oncolytic adenovirus; ITR, inverted terminal repeats; PAC, pancreatic adenocarcinoma; CEA, carcinoembryonic antigen; MOI, multiplicity of infection; TCID, tissue culture infectious dose 50; EGFP, enhanced green fluorescent protein.

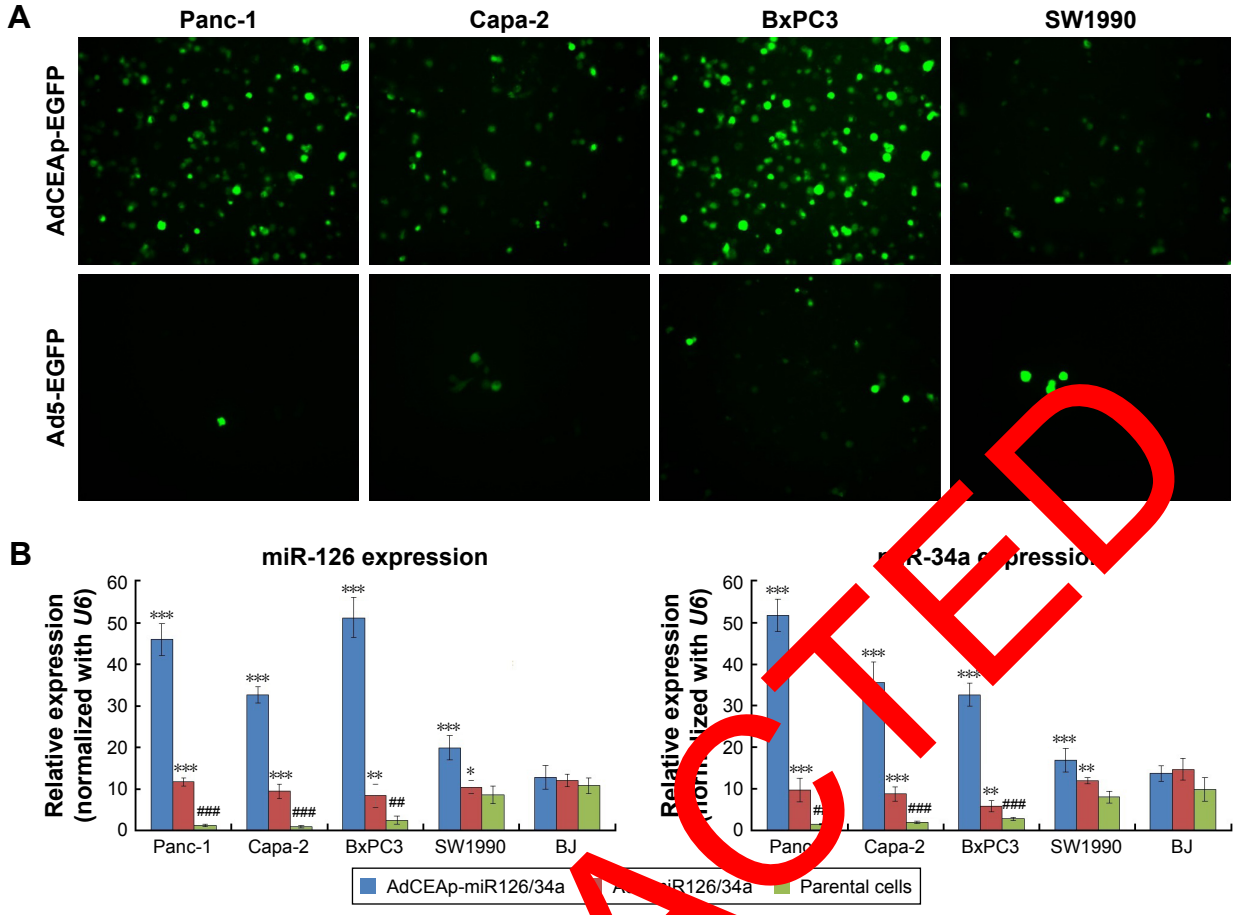


Figure 2 Specific expression of transgenes mediated by oncolytic adenoviruses in PAC cells.

Notes: (**A**) Cell lines were planted in 24-well plates at 1×10^5 per well and infected with the recombined adenoviruses, AdCEAp-EGFP and Ad5-EGFP, at an MOI of 1 pfu/cell. After 48 hours postinfection, EGFP expression was observed under a fluorescent microscope. Magnification: 100×. (**B**) Cell lines were planted in 6-well plates at 1×10^6 per well and infected with the recombined adenoviruses, AdCEAp-miR126/34a and Ad5-miR126/34a, at an MOI of 1 pfu/cell. After 48 hours postinfection, cells were collected and used to detect the expression of miR-126 and miR-34a by RT-PCR. $^{**}P<0.01$, and $^{***}P<0.001$ versus the parental cells within the same cell line; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ versus the SW1990 parental cells.

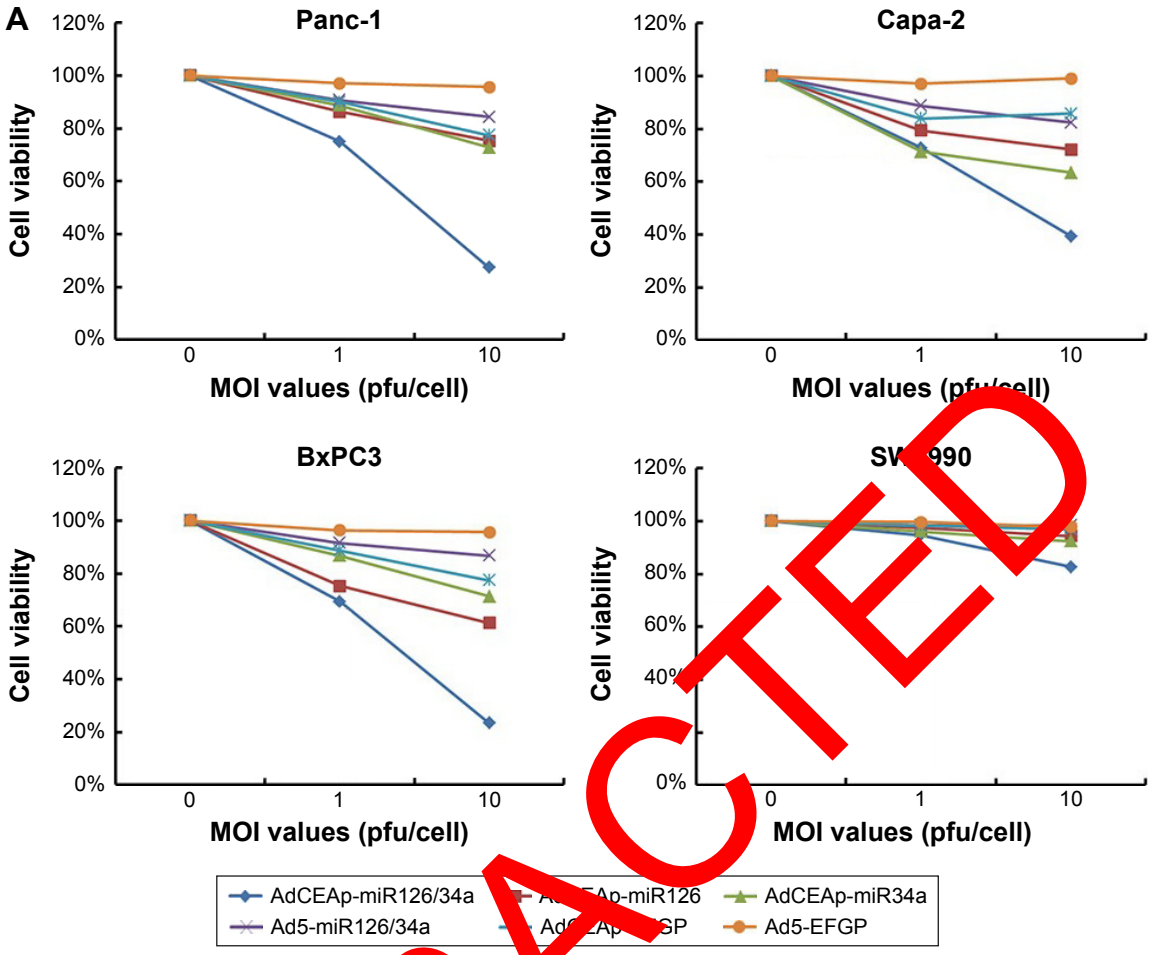
Abbreviations: AdCEAp-miR126/34a, carcinoembryonic antigen promoter-driven oncolytic adenovirus; PAC, pancreatic adenocarcinoma; CEA, carcinoembryonic antigen; MOI, multiplicity of infection; EGFP, enhanced green fluorescent protein; RT-PCR, quantitative reverse transcription polymerase chain reaction.

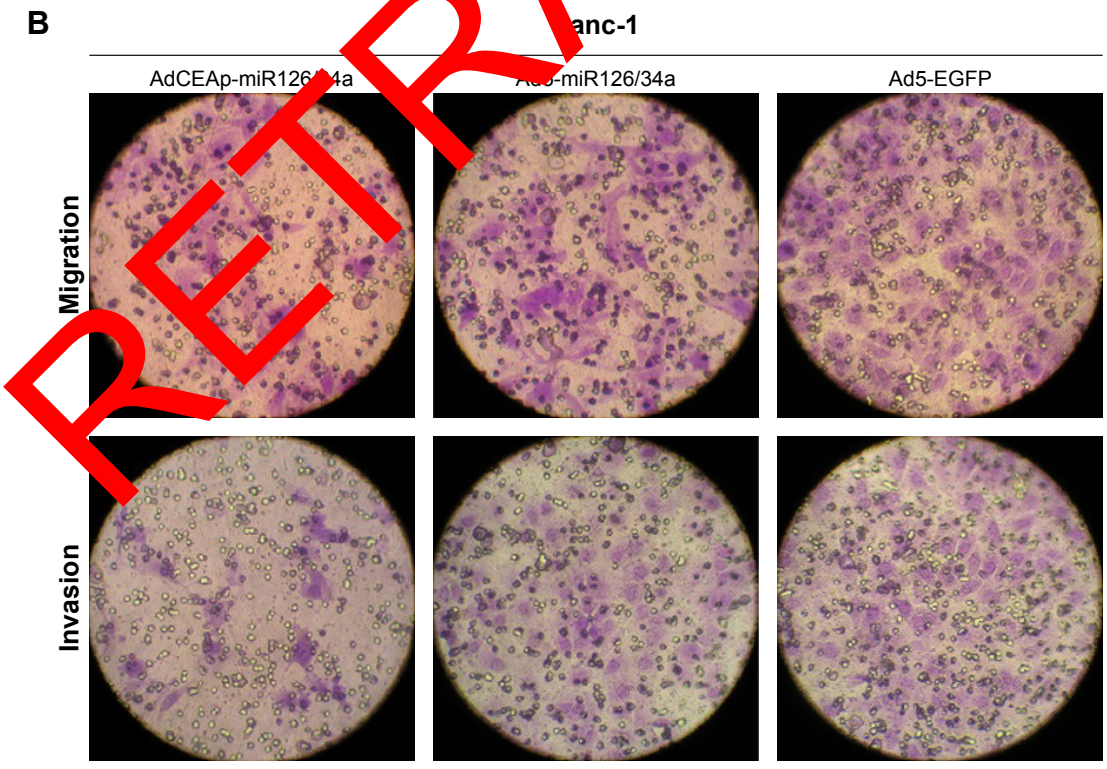
AdCEAp-miR126/34a-infected cells, compared with the parental cells. However, the infection of Ad5-miR126/34a, as well as AdCEAp-EGFP, caused a slightly decreased viability in the above three PAC cells. Ad5-EGFP did not show any effect on PAC cell viability; all adenoviruses did not show any effect on SW1990 cells except AdCEAp-miR126/34a at an MOI of 10 pfu/cell (Figure 3A). The ability of cell migration and invasion was markedly reduced by the infection of AdCEAp-miR126/34a, AdCEAp-miR126, and AdCEAp-miR34a, and slightly reduced by Ad5-miR126/34a and AdCEAp-EGFP in Panc-1, Capa-2, and BxPC3 cells, but nearly did not change in SW1990 cells (Figure 3B).

The expression of miR-34a was regulated by p53 and involved in the induction of p53-mediated cell apoptosis. We therefore detected apoptosis in PAC cells by Annexin V/PI-based flow cytometry. The results showed that the apoptosis rates were markedly increased in Panc-1, Capa-2, and BxPC3 cells after being infected with AdCEAp-miR126/34a, AdCEAp-miR126, and AdCEAp-miR34a, especially in the AdCEAp-miR126/34a- and AdCEAp-miR34a-infected cells, and slightly increased in the Ad5-miR126/34a- and AdCEAp-EGFP-infected PAC cells, compared with the Ad5-EGFP-infected cells (Figure 3C). Only AdCEAp-miR126/34a resulted in a slight increase of apoptosis in SW1990 cells.

Changes of miRNA target gene expression in PAC cells

Studies have demonstrated that *VEGF-A* and sex-determining region of Y chromosome (SRY)-related high-mobility group (HMG) box-2 (*SOX2*) are representative target genes of miR-126, and *cyclin D1*, *E2F1*, and *Bcl-2* are the representative target genes of miR-34a. By Western blotting, we found





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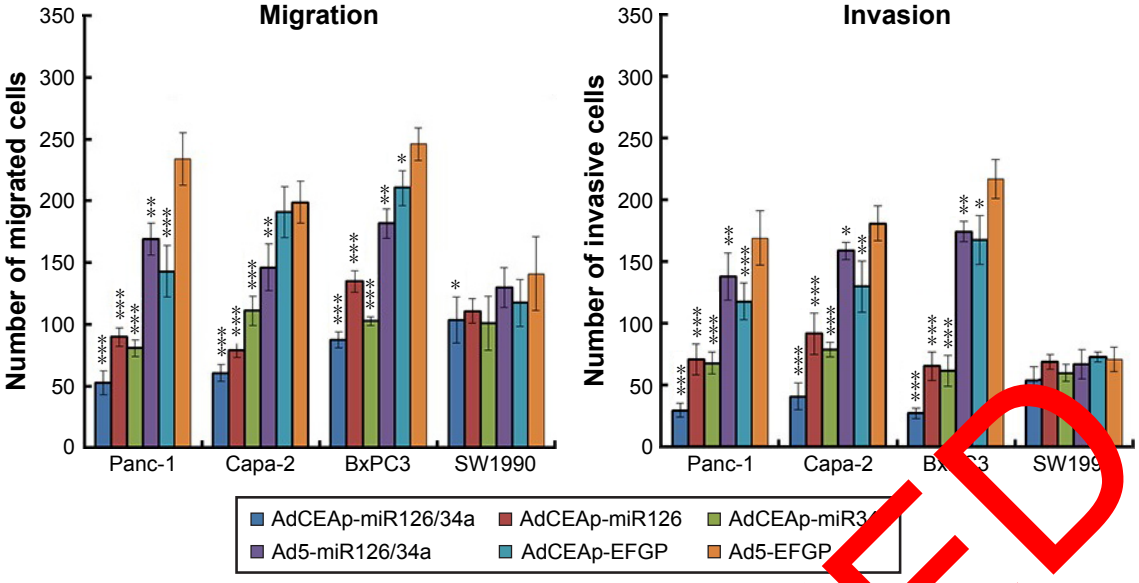

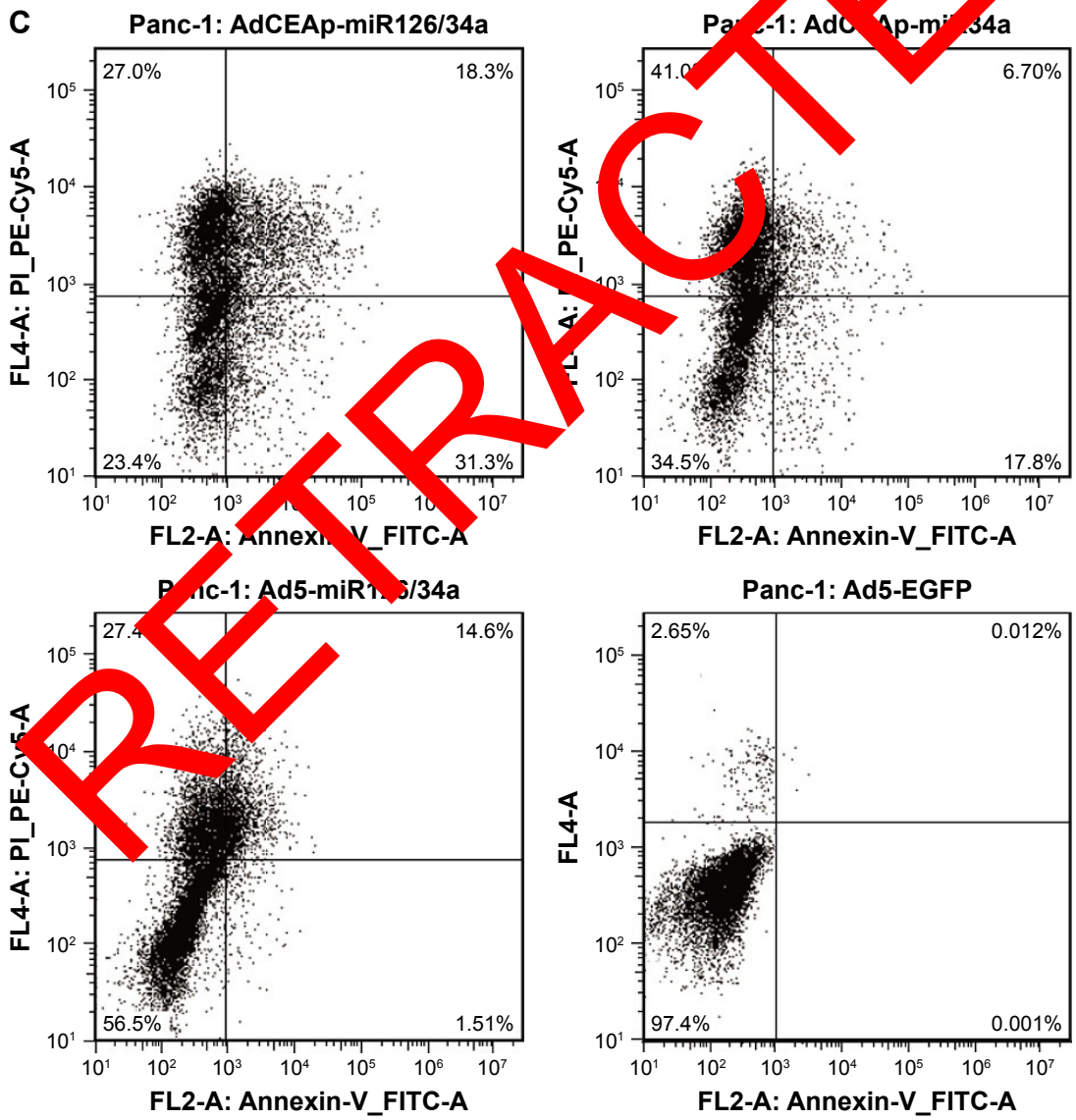

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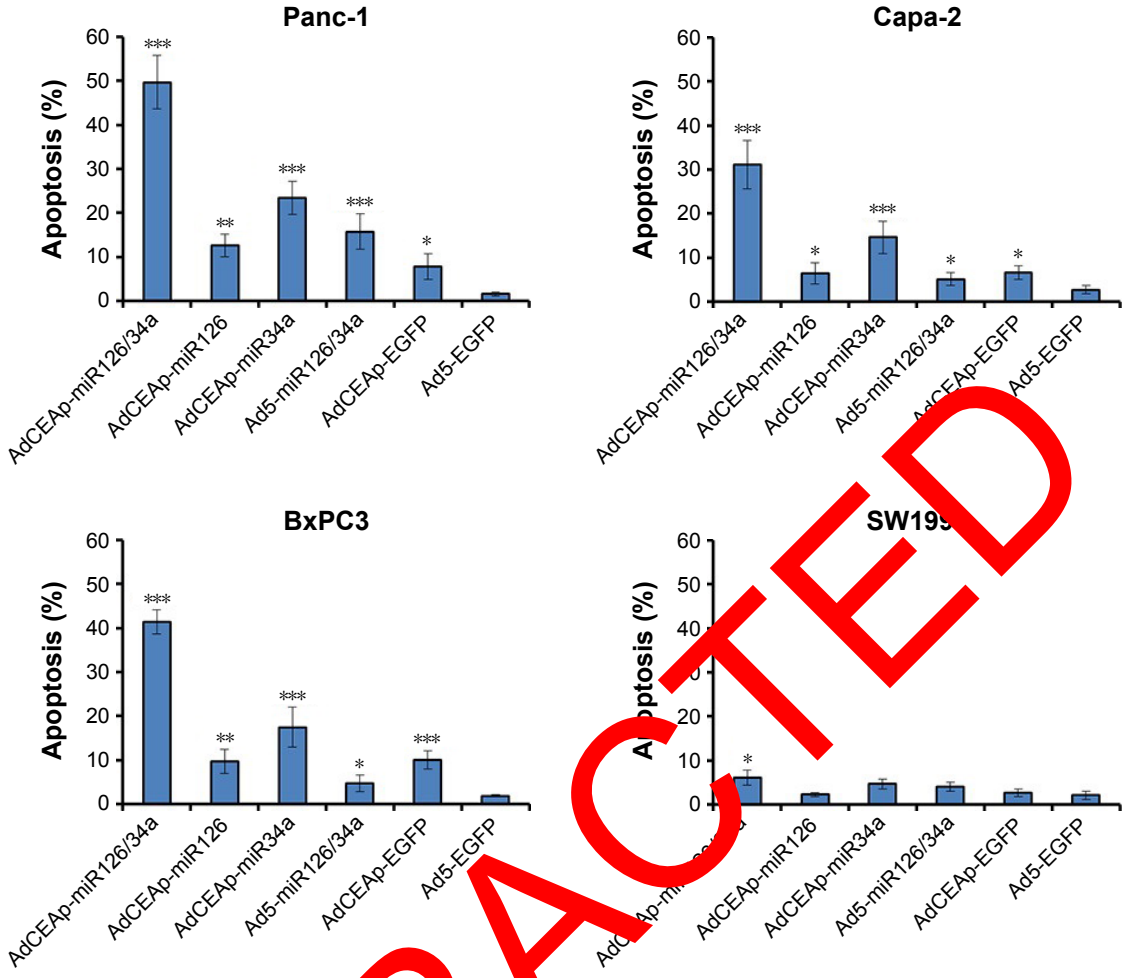

Figure 3 Specific cytotoxicity of oncolytic adenovirus i AC cells.
Notes: (**A**) Cells were seeded in 96-well plates at 1× cells/wel ted with viruses at MOIs of 0, 1, and 10 pfu/cell. After 48 hours postinfection, cell viability was examined using the MTT assay. (**B**) Cells were pl ed in chamber of 24-well plates at 5×10^4 per well and treated with adenoviruses at an MOI of 1 pfu/cell. After 48 hours postinfection, the capacity of cell inv n and migra was assessed. The penetrated cells were counted within five high-power fields. Original magnification ×200; *$P<0.05$, **$P<0.01$, and ***$P<0.001$ versu Ad5-EGFP-infec cells within the same cell line. (**C**) Cell lines were planted in 6-well plates at 5×10^5 per well and infected with adenoviruses at an MOI of 1 pfu/ . Aft 48 hours postin ion, cells were collected and stained with Annexin V/PI, and then analyzed by flow cytometry. The percentages of apoptotic cells included the early optotic cells (FITC-positive and PE-Cy5-negative) and the late apoptotic cells (FITC-positive and PE-Cy5-positive). *$P<0.05$, **$P<0.01$, and ***$P<0.0$ versus the Ad5-E -infected cells within the same cell line.
Abbreviations: AdCEAp-mi 6/34a, carcinoembryoni tigen promoter-driven oncolytic adenovirus; PAC, pancreatic adenocarcinoma; CEA, carcinoembryonic antigen; MOI, multiplicity of infecti EGFP, enh ed green fluorescent protein; PI, propidium iodide; FITC, fluorescein isothiocyanate; MTT, methyl thiazolyl tetrazolium.

that the exp ssi levels f thos arget proteins had obvious change ter inf ion of ad noviruses. The expression levels of VE F-A and SO were markedly downregulated in the AdCEA iR126/34a- and AdCEAp-miR126-infected BxPC3, Panc-1, nd Capa-2 cells, and slightly downregulated in all the Ad5-miR126/34a-infected PAC cells and the AdCEAp-miR126/34a- or AdCEAp-miR126-infected SW1990 cells. The expression levels of cyclin D1, E2F1, and Bcl-2 were significantly downregulated in the AdCEAp-miR126/34a- and AdCEAp-miR34a-infected BxPC3, Panc-1, and Capa-2 cells, and slightly downregulated in all the Ad5-miR126/34a-infected PAC cells and the AdCEAp-miR126/34a- or AdCEAp-miR126-infected SW1990 cells (Figure 4).

Antitumor effect of miRNA-expressed adenoviruses on PAC xenografts in nude mice

Panc-1 cells were used to establish the xenografted tumors in nude mice. After treatments by intratumoral injections of miRNA-expressed adenoviruses, we found that AdCEAp-miR126/34a exhibited the best antitumor effect on the growth of tumors, followed by AdCEAp-miR34a and AdCEAp-miR126. AdCEAp-EGFP and Ad5-miR126/34a exhibited weak antitumor effects, but Ad5-EGFP did not show any antitumor effect. On day 28, the tumor volumes in the Ad5-EGFP-treated group and the blank control group exceeded the upper limit of 3,000 mm^3 permitted by the Ethics Committee of Animal

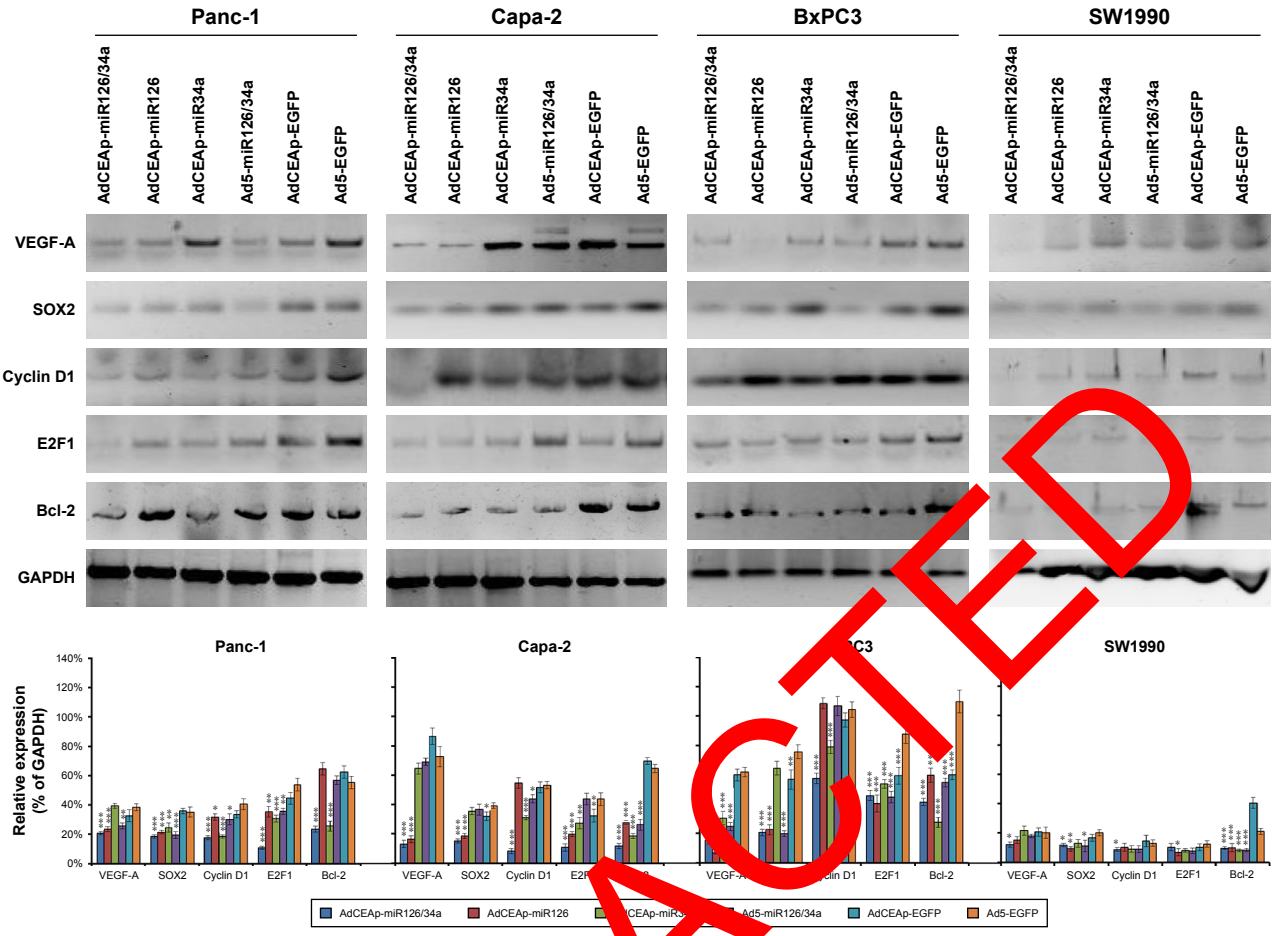

Figure 4 Effect of miR-126 and miR-34a on their target genes.
Notes: Cells were planted in 24-well plates at 1×10^6 cells/well a[nd] infected [w]ith adeno[vi]ruses at an MOI of 1 pfu/cell. After 48 hours, the harvested cells were examined for the expression of indicated proteins by Western blotting. The [de]nsitometr[ic a]nalysis of e[ach] band was normalized with GAPDH density. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ versus the Ad5-EGFP-infected cells.
Abbreviations: AdCEAp-miR126/34a, carcinoembry[onic] antig[en] [pro]moter-driven oncolytic adenovirus; CEA, carcinoembryonic antigen; MOI, multiplicity of infection; EGFP, enhanced green fluorescent protein; VEGF, [vas]cular endothe[lial] growth factor.

Studies, and the experiment was [ter]mina[ted]. The mice were killed and the tumors were re[mo]ved and weigh[ed]. The weight of tumors was least in the Ad[CEA]p-miR126/34a-treated group and the results were consiste[nt] with t[he] tumor volume (Figure 5A).

The tumors w[ere] subj[ected] to i[mmu]nohistochemical staining to an[aly]ze th[e] expres[sion] of miRNA target genes, and the res[ults] reveal[ed] that there was a significant decreased expression le[vel] of SOX2 or cyclin D1 in the Ad5-miR126/34a-treat[ed] group, a certain extent of decrease in the AdCEAp-miR34a- and AdCEAp-miR126-treated groups, compared with the blank control group (Figure 5B). TUNEL labeling indicated a significantly higher apoptosis rate in the AdCEAp-miR126/34a group, followed by the AdCEAp-miR34a and AdCEAp-miR126 groups, compared with the blank control group (Figure 5C).

Discussion

By identifying and applying the variation of miRNA expression profiles between cancer cells and their corresponding normal cells, as well as the specificity of miRNA expression in different tumors, more optimal modalities of cancer therapy can be discovered or designed. The miRNAs that are expressed at low levels in cancer cells often have functions of tumor-suppressor genes; it is feasible to treat cancer by exogenously introducing these miRNAs into cancer cells via vectors.

Studies showed that miR-34a was downregulated in a majority of human cancers, including gastric cancer, breast cancer, colon cancer, hepatocellular carcinoma, non-small cell lung carcinoma, prostate cancer, and PAC.[31–36] A study evaluated two independent cohorts of 268 colorectal cancer (CRC) patients and validated that miR-34a was downregulated in CRC tumor tissues, and its expression level was positively correlated with disease-free survival (cohort I: n=205, $P<0.001$; cohort II: n=63, $P=0.006$). Moreover, the expression of miR-34a was an independent prognostic factor for CRC recurrence by multivariate analysis ($P<0.001$ for cohort I, $P=0.007$ for cohort II). Overexpression of

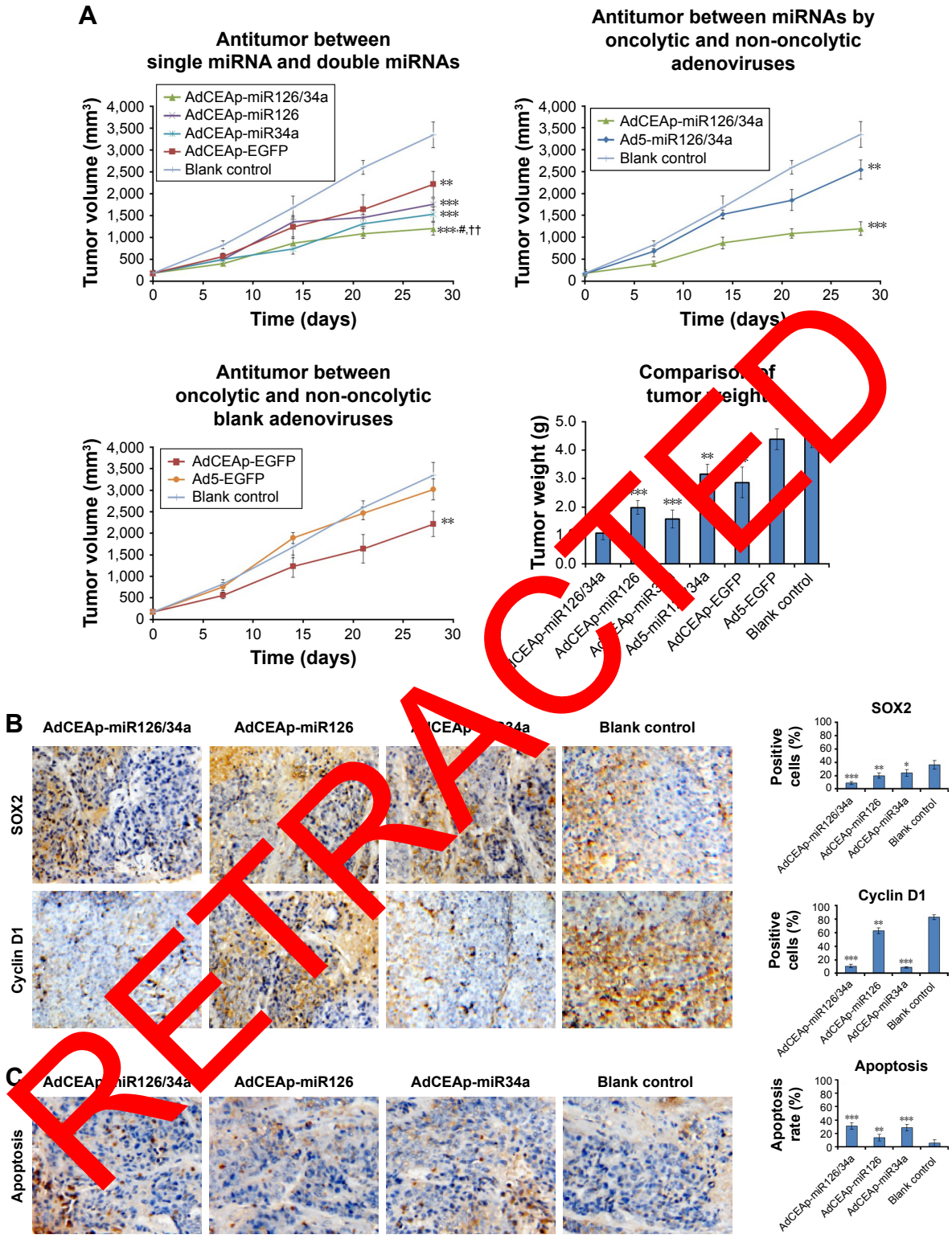

Figure 5 Antitumor efficacy of miR-126 and miR-34a expressed by oncolytic adenoviruses in PAC xenograft models.

Notes: (**A**) Nude mice were implanted with 1×10^6 Panc-1 cells to establish xenograft models, and randomly divided into seven groups (AdCEAp-miR126/34a, AdCEAp-miR126, AdCEAp-miR34a, Ad5-miR126/34a, AdCEAp-EGFP, Ad5-EGFP, and blank control), n=5 for each group. The mice in each group were given intratumoral injections of the corresponding adenovirus at a total dose of 10^9 pfu. The blank control group was injected with the same volume of PBS synchronously. The tumor diameters were measured weekly and the tumor volumes were calculated. At day 28, the xenografted tumors were removed and weighed. $^{**}P<0.01$ and $^{***}P<0.001$ compared with the blank control group, $^{\#}P<0.05$ compared with the AdCEAp-miR34a group, $^{\dagger\dagger}P<0.01$ compared with the AdCEAp-miR126 group. (**B**) The paraffin-embedded sections of the xenografted tumors were prepared for examining the expression of SOX2 and cyclin D1 by immunohistochemistry. The percentages of positive cells were counted within 5 high-power fields. Magnification: 200×. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus the blank control group. (**C**) Apoptosis was examined by the TUNEL assay. The percentages of positive cells were counted within five high-power fields. Magnification: 200×. $^{**}P<0.01$, and $^{***}P<0.001$ versus the blank control group.

Abbreviations: AdCEAp-miR126/34a, carcinoembryonic antigen promoter-driven oncolytic adenovirus; PAC, pancreatic adenocarcinoma; CEA, carcinoembryonic antigen; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; TUNEL, terminal-deoxynucleotidyl transferase-mediated dUTP nick end labeling; miRNA, microRNA.

miR-34a in p53 wild-type colon cancer cell HCT116 significantly inhibited cell growth, migration, invasion, and metastasis, induced cell apoptosis, cell cycle arrest at G1 phase, and p53 transcription activity, and significantly suppressed HCT116 xenograft growth in vivo, but not in the HCT116/p53 knockout cells. The results indicated that miR-34a inhibits CRC in a p53-dependent manner.[37] Transactivation of miR-34a by p53 could promote apoptosis of cancer cells.[13] miR-34a could suppress angiogenesis and metastasis of cancer cells by regulating the expression of target gene *c-MET* and *SIRT1*,[34,38] and could induce apoptosis through repression of *Bcl-2* and *SIRT1*.[16,39] For PAC treatment, miR-34a was also demonstrated to inhibit PAC progression through reversing Snail1-mediated EMT and inactivating the Notch signaling pathway. The *Snail1* and *Notch1* genes were direct targets of miR-34a.[40] miR-126 is also a tumor-suppressor miRNA. A study found that miR-126 has significantly lower expression in esophageal cancer, which could inhibit esophageal cancer cell proliferation. In vivo study showed that tumor growth was significantly suppressed by miR-126 overexpression through targeting *VEGF-A*.[41] The low expression of miR-126 has been identified in the blood of hepatocellular carcinoma patients, and restored miR-126 expression could inhibit cell proliferation, arrest cell cycle progression, and induce cell apoptosis in cancer cells through at least partially targeting *SOX2*.[42] In clinical specimens, miR-126 was strongly downregulated in PAC tissues; a further study showed that miR-126 was found to directly target *KRAS*, and re-expression of miR-126 has potential as a therapeutic strategy against PAC and other KRAS-driven cancers.[43]

However, the intervention of single miRNA expression will produce limited effect in tumor inhibition. PAC is associated with a series of abnormal genes, as well as different clinical behaviors, treatment responses, and prognoses; all of these characteristics involve an extensive and complex miRNA regulation processes, allowing cancer cells to easily regain proliferative activity through alternate bypass pathways. Therefore, we hypothesized that the combined intervention of multiple miRNAs might have the potential to be significantly more effective for PAC treatment. To enhance the miRNA expression efficiency specifically within cancer cells, oncolytic adenovirus could be used as a superior vehicle for delivery of miRNAs to treat cancer.[44,45] The CEA promoter-controlled oncolytic adenoviruses were designed to replicate specifically in the CEA-positive cancer cells and mediate high copies of miRNAs to synergistically exert better antitumor effect and oncolytic effect.[30] In this study, we constructed a therapeutic system AdCEAp-miR126/34a with oncolytic adenovirus vector which could effectively deliver miR-126 and miR-34a simultaneously for the treatment of PAC.

Our cytological experiments found that the CEA promoter-controlled oncolytic adenovirus AdCEAp has the capability to specifically replicate in the CEA-positive cancer cells BxPC3, Panc-1, and Capa-2, but not in the CEA-negative cancer cells SW1990. The high efficiency of viral replication mediated a high effective expression of transgenes in the CEA-positive PAC cells, such as AdCEAp-miR126/34a, AdCEAp-miR126, or AdCEAp-miR34a expressed high levels of miR-126 and/or miR-34a compared with Ad5-miR126/34a, and AdCEAp-EGFP expressed high levels of EGFP compared with Ad5-EGFP. The results suggested that our oncolytic adenovirus is an excellent target vector for PAC gene therapy. Accordingly, the therapeutic system AdCEAp-miR126/34a induced an obvious cytotoxic effect on PAC cells and decreased cell viability in BxPC3, Panc-1, and Capa-2 cell lines, as well as induced cancer cell apoptosis. Meanwhile, we found that AdCEAp-miR126/34a dramatically inhibited the ability of cell migration and invasion of pancreatic cancer cells. To investigate the molecular mechanisms of miRNA function, the expression levels of some representative target genes of miR-126 and miR-34a were examined. The results found that the expression of VEGF-A and SOX2 was markedly downregulated along with restoring miR-126 expression, and the expression of cyclins D1, E2F1, and Bcl-2 was also significantly downregulated along with restoring miR-34a expression. After successfully establishing a pancreatic cancer xenograft model in nude mice, we found that the simultaneous expression of miR-126 and miR-34a mediated by oncolytic adenovirus could suppress the growth of pancreatic xenograft tumors by intratumoral injections of this therapeutic system. The tumor sections were examined by immunohistochemistry, and the results revealed a significant decreased level of SOX2 or cyclin D1 and a significant increased level of cell apoptosis together with miR-126 or miR-34a overexpression. Our data suggested that the simultaneous expression of miR-126 and miR-34a may cause superior antitumor activity in PAC treatment.

The biological safety of oncolytic adenovirus as a gene therapy vector is a considerable issue. We previously studied the side effect of oncolytic adenovirus in rodents, felids, and nonhuman primates and demonstrated that the oncolytic adenovirus is a relatively safe vector in gene therapy, especially for cancer gene therapy, because of its non-integration

and low toxicity.[46] Therefore, the application of the oncolytic adenovirus vector in advanced pancreatic cancer at late stage can completely ignore the problem of viral safety.

Conclusion

This study provides a potent strategy for PAC therapy by simultaneously restoring two different antitumor miRNAs with oncolytic adenovirus. More importantly, the combined expression of two kinds of tumor-suppressor miRNAs, miR-126 and miR-34a, enabled a superior outcome for cancer therapy.

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Disclosure

The authors report no conflicts of interest in this work.

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