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Tumor-Suppressive Effect of Adenovirus-Mediated Inhibitor of Growth 4 Gene Transfer in Breast Carcinoma Cells *In Vitro* and *In Vivo*

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Abstract

The inhibitor of growth (ING) family proteins have been defined as candidate tumor suppressors. ING4 as a novel member of ING family has potential suppressive effect on different tumors via multiple pathways. However, the role of adenovirus-mediated ING4 (Ad-ING4) gene therapy for human breast carcinoma remains unknown. This study investigates the therapeutic effect of Ad-ING4 on human breast cancers *in vitro* and *in vivo* in an athymic nude mouse model, using two human breast carcinoma cell lines MDA-MB-231 (mutant *p53*) and MCF-7 (wild-type *p53*) and elucidated its underlying mechanism. It was found that Ad-ING4 treatment could induce *in vitro* significant growth suppression in both mutant *p53* MDA-MB-231 and wild-type *p53* MCF-7 breast carcinoma cells despite *p53* status. This study further demonstrates that Ad-ING4 gene transfer resulted in G2/M phase arrest and apoptosis, upregulated P21, P27, and Bax, downregulated Bcl-2, IL-8, and Ang-1, promoted cytochrome *c* release from mitochondria into cytosol, and activated caspase-9, caspase-3, and PARP in mutant *p53* MDA-MB-231 breast carcinoma cells. Moreover, intratumoral injections of Ad-ING4 in nude mice bearing mutant *p53* MDA-MB-231 breast tumors remarkably inhibited the human breast xenografted tumor growth and reduced CD34 expression of tumor vessels and microvessel density. This retarded MDA-MB-231 breast carcinoma growth *in vitro* and *in vivo* elicited by Ad-ING4 closely correlated with the upregulation of cell cycle-related molecules P21 and P27, decrease in the ratio of anti- to proapoptotic molecules Bcl-2/Bax, release of cytochrome *c* from mitochondria into cytosol followed by caspase-9 and -3 activation leading to apoptosis via intrinsic apoptotic pathway, and the reduced expression of proangiogenic factors IL-8 and Ang-1 involved in the inhibition of tumor angiogenesis. Thus, the results indicate that Ad-ING4 is a potential candidate for breast cancer gene therapy.

Key words: adenoviral vector, antitumor effect, breast carcinoma, ING4

Introduction

Breast cancer is a common malignancy among women, which is a leading cause of cancer-related mortality ranking second after lung cancer.[1] The incidence of breast cancer has drastically increased over the past several decades. In the United States, the number of new breast cancer cases in 2008 was estimated to be 184,450 and it was predicted to have 40,930 deaths due to breast cancer.[1] Treatment of breast cancer includes surgery, radiation, and drugs (hormone therapy and chemotherapy). A major problem in the treatment of breast cancer is tumor cells' acquisition of resistance to chemotherapeutic agents. Thus, there is increased interest in finding new agents for breast cancer treatment. Adenovirus is one of the most promising vectors for cancer gene therapy. Adenoviral vectors harboring therapeutic genes have been used successfully for gene transfer *in vitro* and *in vivo*. A great deal of data have been accumulated, with nonreplicating adenoviral vectors suggesting a reasonable safety profile in humans.[2] Novel approaches using replication-defective adenoviral vectors encoding tumor suppressor genes are gaining popularity, which may be a novel therapeutic modality for breast cancer.

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Inhibitor of growth (ING) is a candidate tumor suppressor gene family, which is involved in apoptosis, cell cycle regulation, and DNA repair. There are at least five known members including ING1, ING2, ING3, ING4, and ING5. ING4, a novel member of the ING family, was first isolated and characterized by Shiseki et al.[3] ING4 has a conserved plant homeodomain (PHD)-finger motif transcriptional regulator in the COOH-terminal region involved in chromatin remodeling[4,5] and a potential bipartite nuclear localization signal (NLS) in the middle region associated with the binding to p53.[6] ING4 has recently attracted much attention as a strong candidate tumor suppressor. ING4 markedly downregulated glioblastoma,[7] head and neck carcinoma,[8] hepatocellular carcinoma,[9] melanoma,[10,11] and gastric carcinoma,[12] which was closely associated with tumor grade, metastasis, and prognosis.[7,9,10] ING4 can significantly inhibit tumor cell growth, induce cell cycle alteration and apoptosis in different tumor types including colorectal, melanoma, hepatocellular, myeloma, lung and pancreatic carcinomas, and glioblastoma,[3,7,10,11,13–16] and enhance chemosensitivity to doxorubicin and etoposide in HepG2 hepatocarcinoma cells.[13] ING4 can also suppress the activity of NF-κB and HIF-1α, leading to inhibition of tumor angiogenesis.[7,14,17] Further, ING4 can suppress the loss of contact inhibition elicited by MYCN or MYC.[18] More recently, it has been shown that ING4 can exhibit marked inhibitory effect on tumor cell spreading, migration, and invasion.[10,15,19] Therefore, ING4 is a potent tumor suppressor that exerts its tumor-suppressive effect via multiple pathways in a variety of tumor types.

Previous studies have shown that adenovirus-mediated ING4 (Ad-ING4) gene transfer significantly suppressed *in vitro* and *in vivo* lung and pancreatic carcinoma cell growth.[15,16] However, the application of Ad-ING4 expression in breast cancer gene therapy has not been reported. The present study assesses the suppressive effect of Ad-ING4 against MDA-MB-231 (mutant *p53*) and MCF-7 (wild-type *p53*) human breast carcinoma cell lines *in vitro* and mutant *p53* MDA-MB-231 breast xenografted tumors *in vivo* in an athymic nude mouse model and also elucidates its potential mechanism.

Materials and Methods

Adenoviral vectors, cell lines, reagents, and mice

The Ad-ING4 and Ad-green fluorescent protein (GFP) replication-incompetent adenoviral vectors were constructed in the laboratory[15] of the Cell and Molecular Biology Institute, College of Medicine, Soochow University (Suzhou, China). The QBI-293A human embryonic kidney cell line was kindly provided by Prof. Jiang Zhong (Ph.D.) of Fudan University (Shanghai, China). The MDA-MB-231 (mutant *p53*) and MCF-7 (wild-type *p53*) human breast adenocarcinoma cell line was purchased from the American Type Culture Collection (Rockville, MD). The QBI-293A, MDA-MB-231, and MCF-7 cells were cultured separately in RPMI-1640 (Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). The reverse transcriptase–polymerase MuMLV was purchased from MBI (Shanghai, China). The polyclonal goat anti-ING4 antibody was purchased from Abcam (Shanghai, China). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit was purchased from Sigma (Shanghai, China). The annexin V-PE/7-AAD apoptosis detection kit was purchased from BD Biosciences (Shanghai, China). The mammalian cell lysis kit and mitochondria/cytosol isolation kit were purchased from Sigma. The antibodies specific for P21, P27, Bcl-2, Bax, cytochrome *c*, cleaved caspase-9, cleaved caspase-3, cleaved PARP, β-actin, and CD34 were purchased from Santa Cruz (Shanghai, China). The IL-8, VEGF, and Ang-1 enzyme-linked immunosorbnent assay (ELISA) kits were purchased from Jingmei (Shanghai, China). The SuperEnhanced chemiluminescence detection kit was purchased from Applygen Technologies (Beijing, China). The UltraSensitiveTM SP kit was purchased from Maixin (Fuzhou, China). The female athymic nude mice were obtained from Shanghai Experimental Animal Center (Shanghai, China) and maintained in the animal facility at Soochow University according to the university's animal research committee's guidelines.

Analysis of adenoviral infection efficiency and ING4 transgene expression

The recombinant replication-incompetent adenovirus Ad-ING4 expressing ING4 and GFP and its control adenovirus Ad-GFP expressing GFP were prepared in the laboratory as described previously.[15] To assess the optimal multiplicity of infection (MOI) for a maximal infection and transgene expression in MDA-MB-231 and MCF-7 tumor cells, the MDA-MB-231 and MCF-7 human breast carcinoma cells were infected with Ad-GFP and Ad-ING4 at various MOIs (0, 1, 10, 25, 50, 100, and 200), respectively. The adenoviral infection efficiency was examined according to GFP expression by fluorescence microscopy. In addition, the ING4 transgene expression in MDA-MB-231 and MCF-7 tumor cells mediated by adenoviral infection was determined using RT-PCR and western blot analysis, as described previously.[15]

MTT assay

The cytotoxic activity of Ad-ING4 on MDA-MB-231 (mutant *p53*) and MCF-7 (wild-type *p53*) human breast carcinoma cells was determined by MTT assay. Briefly, the MDA-MB-231 and MCF-7 tumor cells were dispensed in a 96-well culture plate at a density of 1×10^4 cells per well and incubated at 37°C. After 24 hours of incubation, they were treated with Ad-ING4 at the optimal MOI of 100 (MDA-MB-231) and 50 (MCF-7) for the indicated time periods (0–4 days), respectively. The medium containing Ad-GFP (100 or 50 MOI) was used as a blank adenovirus control, whereas the medium containing phosphate-buffered saline (PBS) without Ad-ING4 or Ad-GFP was used as a cell control (PBS control). Before treatment and at different time points after treatment, the cells were incubated with 10 μL MTT (5 mg/mL) for 4 hours at 37°C. The formazan crystals in the cells were solubilized with stop solution and the plate was then read at 570 nm using a Microplate Reader Model 550 (Bio-Rad, Shanghai, China).

Clonogenic survival assay

The effect of Ad-ING4 on MDA-MB-231 and MCF-7 human breast carcinoma cell survival was also assessed by colony-forming assay. Briefly, the MDA-MB-231 and MCF-7 tumor cells were dispensed in 60-mm culture plates at a

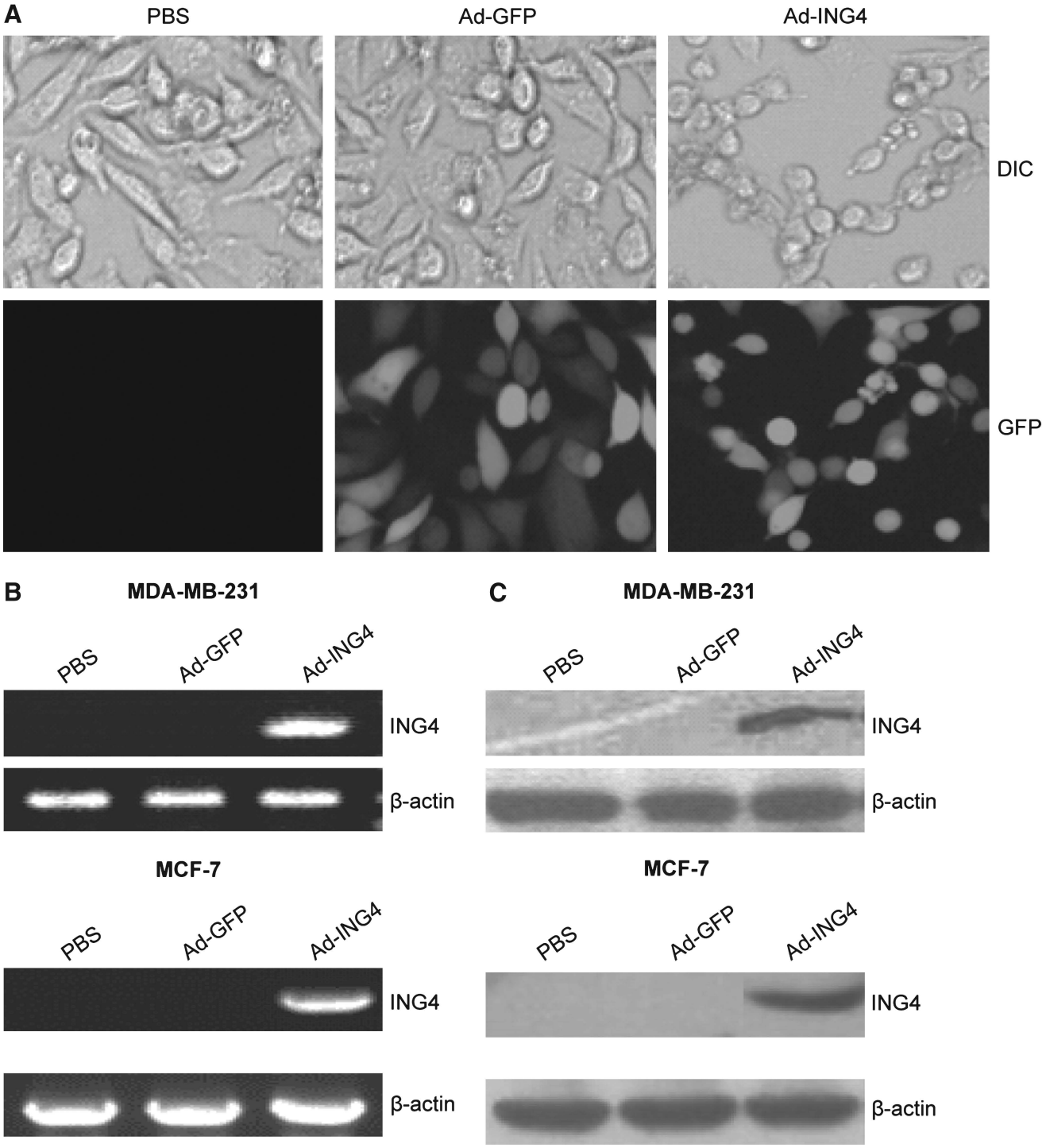


FIG. 1. Transgene ING4 expression in Ad-ING4-infected MDA-MB-231 and MCF-7 breast carcinoma cells. (**A**) GFP expression and infection efficiency examined by fluorescence microscopy. The MDA-MB-231 human breast carcinoma cells were infected with Ad-GFP and Ad-ING4 at various MOIs (0, 1, 10, 25, 50, 100, and 200), respectively, and checked under fluorescence and DIC images by fluorescence microscopy. More than 95% of GFP expression was found in the MDA-MB-231 tumor cells infected with Ad-GFP or Ad-ING4 at an MOI of 100 and above. (**B**) Ad-ING4 transcriptional expression by RT-PCR analysis. The total cellular RNAs were obtained from MDA-MB-231 and MCF-7 tumor cells infected with Ad-ING4 or Ad-GFP, which served as a blank adenovirus control and untreated control cells, respectively. The first-strand cDNA was synthesized from RNA using reverse transcriptase; PCRs were conducted using primer sets specific for *ING4* and the housekeeping gene *β-actin* was used as an internal control. (**C**) Ad-ING4 translational expression by western blot analysis. The total cellular lysates derived from Ad-ING4- or Ad-GFP-infected MDA-MB-231 and MCF-7 tumor cells and untreated control cells were analyzed by immunoblotting with anti-ING4 and anti-*β*-actin (an internal control) antibody, respectively. Data shown are representative of three independent experiments. ING, inhibitor of growth; Ad-ING4, adenovirus-mediated ING4; GFP, green fluorescent protein (shown in black and white); DIC, differential interference contrast.

density of 200 cells per plate, respectively. After 24 hours of incubation, they were treated with or without Ad-ING4 at an MOI of 100 (MDA-MB-231) or 50 (MCF-7), as described earlier. Then the tumor cells were cultured for another 2 weeks. The colonies were fixed with 95% ethanol, stained with Giemsa, and manually counted. The colonies greater than 50 cells were considered to be survivors.

Flow cytometric analysis

Cell cycle conditions of MDA-MB-231 tumor cells with mutant *p53* induced by Ad-ING4 were further determined using propidium iodide (PI) staining by flow cytometric analysis. The MDA-MB-231 human breast carcinoma cells (1×10^6) were cultured with or without Ad-ING4 at an MOI of 100. Three (3) days later, the cells were harvested and washed in cold PBS. The cell pellets were fixed in 70% cold alcohol for more than 24 hours at 4°C, then washed in cold PBS, and stained with PI solution at 4°C in the dark for 30 minutes. Ad-ING4-mediated MDA-MB-231 breast tumor cell apoptosis was assessed using annexin V-PE/7-AAD double staining following manufacturer's instructions by flow cytometric analysis. Briefly, these treated or untreated cells were collected and washed in cold PBS. Then the cells were incubated for 15 minutes at room temperature in the presence of 5 μL annexin V-PE and 5 μL 7-AAD in 100 μL of 1× binding buffer. After incubation, 400 μL of 1× binding buffer was added, and the apoptotic cells were analyzed by flow cytometry.

Western blot analysis

The mutant *p53* MDA-MB-231 human breast carcinoma cells treated with Ad-ING4 (100 MOI), Ad-GFP (100 MOI), or PBS for 48 hours were collected, washed with cold PBS, and lysed in lysis buffer (10^7 cells/1 mL lysis buffer) for preparation of total cell lysates using a mammalian cell lysis kit. In addition, the mitochondrial and cytosolic protein fractions were isolated using a mitochondria/cytosol isolation kit. The protein concentration was determined by BCA protein assay using a spectrophotometer. The total cell lysates and mitochondrial or cytosolic proteins (100 μg/lane) were loaded on 12% acrylamide gels, subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and subsequently transferred onto a polyvinylidene difluoride membrane. The membrane was blocked by incubation with 5% (w/v) nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 hour at 37°C. For western blot analysis, the membrane transferred with total cell lysates was incubated with primary antibodies P21, P27, Bcl-2, Bax, cleaved caspase-9, cleaved caspase-3, cleaved PARP, and β-actin in blocking solution for 1 hour, respectively. Additionally, the membrane transferred with mitochondrial or cytosolic proteins was incubated with cytochrome *c* antibody. The membranes were washed with TBST and incubated with peroxidase-conjugated secondary antibody in blocking solution for 1 hour. The membranes were then washed and developed by use of a SuperEnhanced chemiluminescence detection kit. The protein bands were observed after exposure of the membranes to Kodak X-ray film.

IL-8, VEGF, and Ang-1 expression determined by ELISA

The supernatants of Ad-ING4- or Ad-GFP-infected mutant *p53* MDA-MB-231 human breast carcinoma cells and uninfected MDA-MB-231 control cells were collected after 48 hours of incubation, respectively, and the amounts of IL-8, VEGF, and Ang-1 in the supernatants were assessed by ELISA according to manufacturer's instructions.

Animal studies

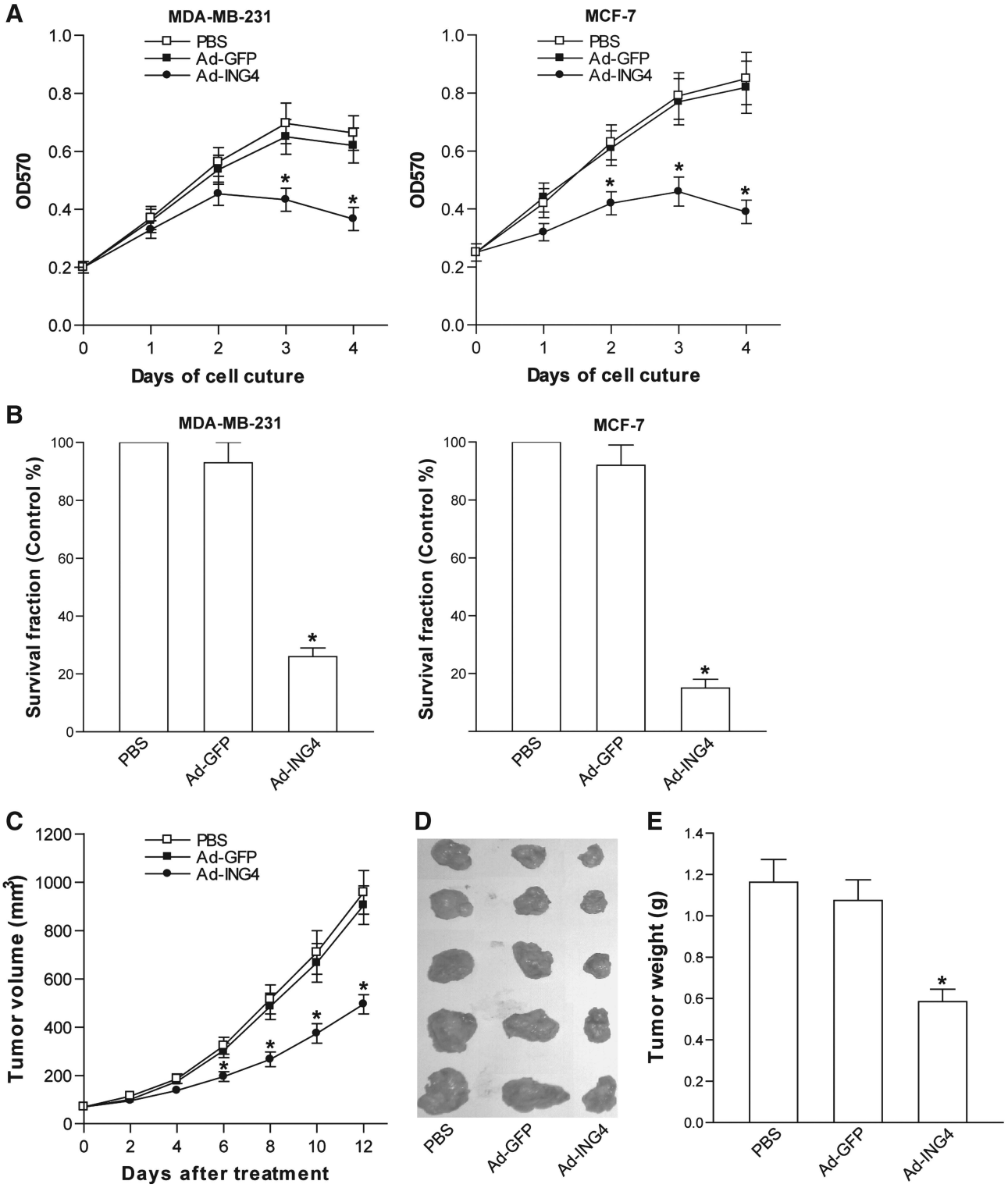
Female athymic nude mice (5 each group) were subcutaneously inoculated on their armpits of right anterior limbs with 2×10^6 mutant *p53* MDA-MB-231 human breast carcinoma cells and then monitored daily for tumor growth. When the tumors grew up to a volume of around 60–80 mm^3, the mice were intratumorally (i.t.) injected with Ad-ING4 (1×10^7 plaque-forming units [pfu]), Ad-GFP (1×10^7 pfu), or PBS every other day for a total of five times, respectively. Tumor progression and regression were monitored daily. Tumor volumes were measured with a caliper before and after treatment and calculated by the following

FIG. 2. Ad-ING4 suppresses *in vitro* and *in vivo* breast carcinoma cell growth. (**A**) The cytotoxic effect of Ad-ING4 on MDA-MB-231 and MCF-7 breast carcinoma cells assessed by MTT assay. The MDA-MB-231 and MCF-7 human breast carcinoma cells were treated with Ad-ING4 at an MOI of 100 or 50 and with Ad-GFP (100 or 50 MOI) or PBS as controls for 4 days, respectively. The survival cells were evaluated at days 0, 1, 2, 3, and 4 after infection by using MTT assay. The *in vitro* growth of MDA-MB-231 and MCF-7 tumor cells treated with Ad-ING4 was significantly inhibited after infection ($^*p < 0.05$, compared with the Ad-GFP and PBS groups at day 3 or 4 [MDA-MB-231] and day 2, 3, or 4 [MCF-7], respectively, whereas $p > 0.05$ when the Ad-GFP group is compared with the PBS group, by two-way repeated measures ANOVA and multiple comparisons; $n = 4$ replicates per condition). (**B**) The cytotoxicity of Ad-ING4 on MDA-MB-231 and MCF-7 tumor cells evaluated by clonogenic survival assay. Ad-ING4 inhibited the *in vitro* proliferation of MDA-MB-231 and MCF-7 tumor cells ($^*p < 0.05$, compared with the Ad-GFP and PBS groups, respectively, whereas $p > 0.05$ when the Ad-GFP group is compared with the PBS group, by one-way repeated measures ANOVA and multiple comparisons; $n = 3$ replicates per condition). (**C–E**) Ad-ING4-mediated *in vivo* growth inhibition of MDA-MB-231 breast xenografted tumor. Athymic nude mice bearing MDA-MB-231 breast tumors (60–80 mm^3) were intratumorally injected with Ad-ING4 (1×10^7 pfu), Ad-GFP (1×10^7 pfu), or PBS every other day for a total of five times, respectively. The tumor volume (**C**) was measured before and after treatment. The MDA-MB-231 breast xenografted tumors were removed (**D**) at 12 days after treatment and tumor weight (**E**) was measured. Ad-ING4 significantly suppressed the MDA-MB-231 breast xenografted tumor growth *in vivo* in an athymic nude mouse model ($^*p < 0.05$, compared with the Ad-GFP and PBS groups, whereas $p > 0.05$ when the Ad-GFP group is compared with the PBS group, by two-way or one-way repeated measures ANOVA and multiple comparisons; $n = 5$ mice per condition). Data shown are representative of three independent experiments. ING, inhibitor of growth; Ad-ING4, adenovirus-mediated ING4; GFP, green fluorescent protein (shown in black and white); PBS, phosphate-buffered saline; ANOVA, analysis of variance; pfu, plaque-forming units.

formula: tumor size $= ab^2/2$, where a is the larger and b is the smaller of the two dimensions. In addition, the tumor-bearing mice were sacrificed at 12 days after injection with Ad-ING4, Ad-GFP, or PBS, and the xenografted tumors were removed, weighted, fixed by 10% neutral formalin, and embedded in paraffin for hematoxylin and eosin staining and immunohistochemistry analysis.

CD34 immunostaining and microvessel density counting

The CD34 expression of vascular endothelial cells in mutant *p53* MDA-MB-231 human breast carcinoma xenografted tumors was tested by immunohistochemistry analysis using UltraSensitiveTM SP kit. The presence of buffy or brown



precipitates is indicative of positive immunoreactivity. The integral optical density (IOD) of CD34 expression was calculated by Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD). The mean IOD value represents average number derived from five visual fields of each case. Microvessel density (MVD), detected by immunostaining for CD34, was determined as described previously by Weidner et al.[20] The areas containing a large number of microvessels or "hot spots" of immunoreactivity against CD34 were counted at a high-power view (400×). Any immunoreactive endothelial cell cluster clearly separated from adjacent microvessels was considered as a single countable vessel. Each MVD value represents the number of vessels counted at high-power field (400×) by microscopy. The mean MVD value represents the average number derived from five high-power fields of each case.

Statistical analysis

All data are presented as the means ± standard deviation (SD). The significance of the difference between groups was evaluated by one-way and two-way repeated measures analysis of variance and multiple comparisons with SPSS 10.0 software (SPSS, Chicago, IL). A p-value of <0.05 was considered statistically significant.

Results

Transgene ING4 expression

To determine the optimal MOI for a maximal infection and transgene expression, the MDA-MB-231 and MCF-7 human breast carcinoma cells were infected with Ad-GFP and Ad-ING4 at various MOIs, respectively, and examined by fluorescence microscopy. As shown in Figure 1A, more than 95% of GFP expression was found in the MDA-MB-231 tumor cells infected with Ad-GFP or Ad-ING4 at an MOI of 100 and above, whereas the GFP expression was not found in the uninfected MDA-MB-231 control cells. In addition, there was also more than 95% of GFP expression in Ad-GFP- or Ad-ING4-infected MCF-7 tumor cells at an MOI of 50 and above (data not shown). Therefore, MOIs of 100 and 50 were selected as the optimal dose for adenoviral infection of MDA-MB-231 and MCF-7 breast tumor cell line in the following experiments, respectively. To further assess Ad-ING4 expression, the total cellular RNAs and lysates extracted from Ad-ING4- or Ad-GFP-infected and uninfected MDA-MB-231 and MCF-7 human breast carcinoma cells were subjected to RT-PCR and western blot analysis. As shown in Figure 1B and C, a significant amount of ING4 expression was found in the Ad-ING4-infected MDA-MB-231 and MCF-7 tumor cells, but not in the Ad-GFP-infected MDA-MB-231 and MCF-7 cells and uninfected MDA-MB-231 and MCF-7 control cells, indicating that transgene ING4 mediated by adenovirus is expressed in Ad-ING4-infected MDA-MB-231 and MCF-7 human breast carcinoma cells at both the transcriptional and translational levels.

ING4 overexpression suppresses in vitro and in vivo breast carcinoma cell growth

To assess its cytotoxic effect on breast cancer cells *in vitro*, the MDA-MB-231 (mutant *p53*) and MCF-7 (wild-type *p53*) human breast carcinoma cells were cultured in the presence or absence of Ad-ING4 at the optimal MOI of 100 and 50, respectively. The tumor cell growth was examined daily for 4 days, using MTT assay. Ad-ING4 treatment significantly inhibited the mutant *p53* MDA-MB-231 and wild-type *p53* MCF-7 tumor cell growth in a time-dependent manner, compared with the Ad-GFP- and PBS-treated control groups ($p < 0.05$; Fig. 2A), indicating that transgene ING4 expression is capable of efficiently inhibiting breast tumor cell growth in a p53-independent fashion. The reasons why the Ad-ING4-treated MDA-MB-231 and MCF-7 tumor cells stopped growing at days 3 and 4 after infection, respectively, may be due to Ad-ING4-induced cytotoxicity, tumor cells' characteristics, and culture conditions such as number of tumor cells seeded and consumption of culture medium's nutrition. The *in vitro* tumor-suppressive effect of Ad-ING4 in MDA-MB-231 and MCF-7 breast carcinoma cells was also evaluated and confirmed using colony-forming assay (Fig. 2B). To further investigate its *in vivo* antitumor effect, the athymic nude mice (5 each group) bearing mutant p53 MDA-MB-231 breast tumors were i.t. injected with Ad-ING4 (1×10^7 pfu), Ad-GFP (1×10^7 pfu), or PBS for a total of five times. The tumor growth *in vivo* was monitored daily and tumor volume and weight were measured. As shown in Figure 2C–E, the tumor growth was remarkably retarded in the Ad-ING4-treated group, compared with the Ad-GFP- and PBS-treated control groups ($p < 0.05$), indicating that Ad-ING4 expression also potentially suppresses *in vivo* MDA-MB-231 breast xenografted tumor growth in an athymic nude mouse model.

ING4 overexpression induces MDA-MB-231 breast carcinoma cell cycle alteration and apoptosis

To explore the potential mechanism by which Ad-ING4 suppresses tumor growth, the cell cycle conditions and apoptosis of mutant *p53* MDA-MB-231 human breast carcinoma cells treated with Ad-ING4 for 72 hours were further analyzed using PI staining, and annexin V-PE (early apoptotic marker) and 7-AAD (late apoptotic marker) double staining by flow cytometry, respectively. As shown in Figure 3A, a significant increase in G2/M phase was observed in MDA-MB-231 breast tumor cells treated with Ad-ING4, but not with Ad-GFP or PBS ($p < 0.05$), indicating that transgene ING4 expression inhibits the proliferation of MDA-MB-231 tumor cells via inducing the G2/M phase arrest. Moreover, Ad-ING4 treatment induced 56.50% of MDA-MB-231 breast tumor cell apoptosis including 21.38% annexin V-PE single-positive cells representing that these tumor cells are in the early stage of apoptosis, and 35.12% annexin V-PE and 7-AAD double-positive cells representing that these tumor cells are in the late stage of apoptosis ($p < 0.05$), whereas there was only ~2%–4% spontaneous tumor cell apoptosis occurring in MDA-MB-231 breast tumor cells grown in the medium with Ad-GFP or PBS (Fig. 3B, C), indicating that transgene ING4 expression efficiently induces MDA-MB-231 breast tumor cell apoptosis that closely correlates with Ad-ING4-mediated significant growth inhibition of MDA-MB-231 tumor cells.

ING4 overexpression alters P21 and P27 expression and induces intrinsic apoptotic pathway

To further address the molecular mechanism involved in Ad-ING4-induced cell cycle alteration and apoptosis in

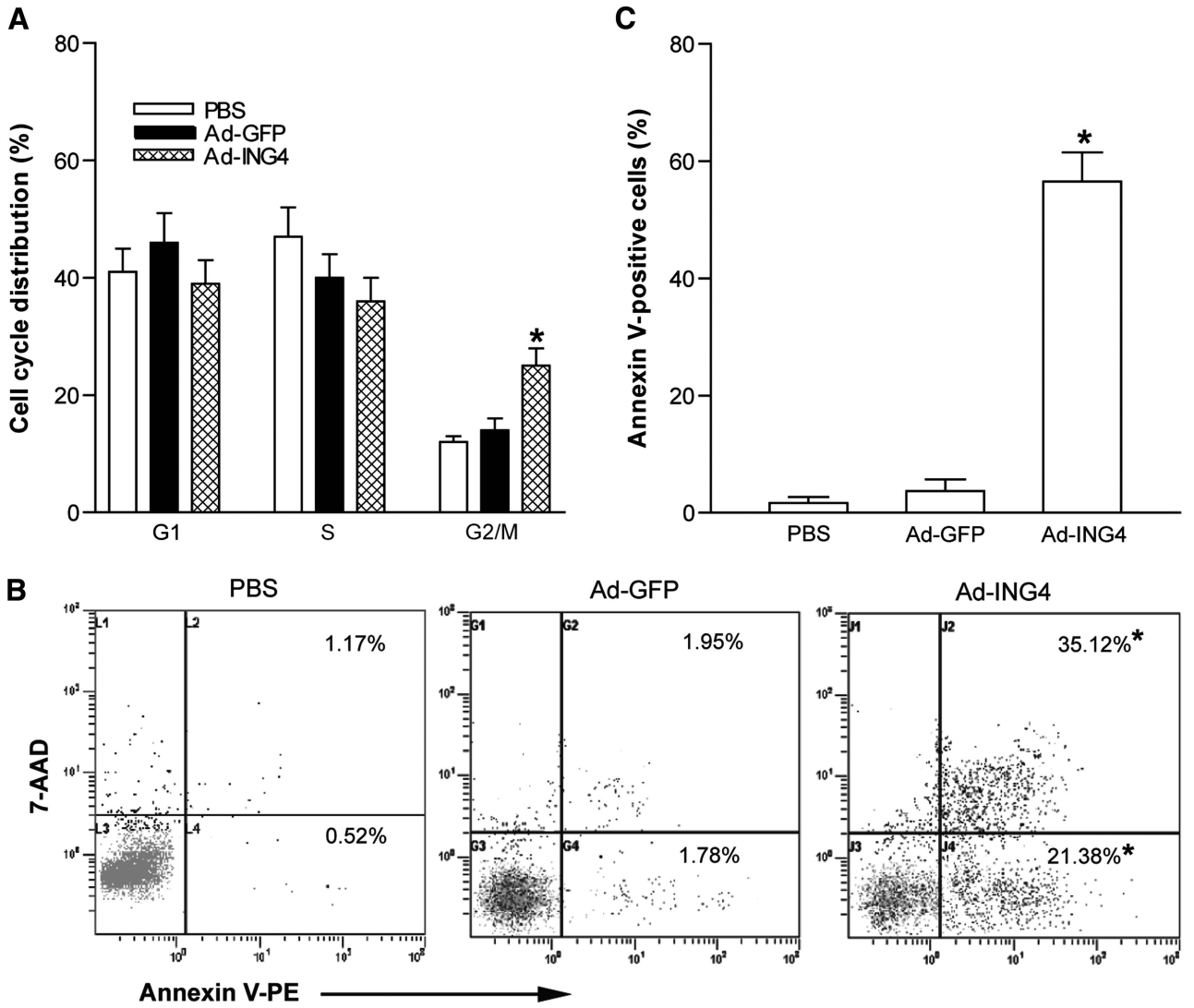


FIG. 3. Ad-ING4 induces MDA-MB-231 breast carcinoma cell cycle alteration and apoptosis. The MDA-MB-231 human breast carcinoma cells were cultured with Ad-ING4 (100 MOI), Ad-GFP (100 MOI), or PBS for 72 hours and then analyzed by flow cytometry. (**A**) Cell cycle analysis using propidium iodide staining. Ad-ING4 significantly induced G2/M phase arrest in MDA-MB-231 tumor cells (*$p < 0.05$, compared with the Ad-GFP and PBS groups, respectively, whereas $p > 0.05$ when the Ad-GFP group is compared with the PBS group, by one-way repeated measures ANOVA and multiple comparisons; $n = 3$ replicates per condition). (**B, C**) Apoptosis analysis using annexin V-PE and 7-AAD double staining. The annexin V-positive cells in the total cell population represented apoptotic cells. The percentage of apoptotic cells was significantly higher in the Ad-ING4 group than that in the Ad-GFP and PBS groups (*$p < 0.05$, compared with the Ad-GFP and PBS groups, respectively, whereas $p > 0.05$ when the Ad-GFP group is compared with the PBS group, by one-way repeated measures ANOVA and multiple comparisons; $n = 3$ replicates per condition). Data shown are representative of three independent experiments. ING, inhibitor of growth; Ad-ING4, adenovirus-mediated ING4; GFP, green fluorescent protein (shown in black and white); PBS, phosphate-buffered saline; ANOVA, analysis of variance.

mutant *p53* MDA-MB-231 tumor cells, the expression of cell cycle- and apoptosis-related proteins P21, P27, Bcl-2, Bax, cytochrome *c*, cleaved caspase-9, cleaved caspase-3, and cleaved PARP in the Ad-ING4-infected MDA-MB-231 human breast carcinoma cells was determined by western blot analysis. As shown in Figure 4, the expression of P21, P27, and Bax in the Ad-ING4-treated group was significantly increased by 5.7-, 2.3-, and 3.1-fold, respectively, whereas the expression of Bcl-2 was decreased by 20%, compared with the Ad-GFP- and PBS-treated control groups. Moreover, cytochrome *c* accumulated in the cytoplasmic protein fraction of Ad-ING4-treated MDA-MB-231 tumor cells (Fig. 4), indicating Ad-ING4-induced downregulation of Bcl-2/Bax contributes to the release of cytochrome *c* from the mitochondria into the cytosol. Accompanied by the cytochrome *c* release, the activation of caspase-9, caspase-3 (cleaved caspase-9 and -3), and cleaved PARP was also found in the Ad-ING4-treated MDA-MB-231 tumor cells but not in the Ad-GFP- or PBS-treated control group (Fig. 4). The results indicated that Ad-ING4 suppresses MDA-MB-231 tumor cell growth and induces apoptosis closely associated with the upregulation of cell cycle-related molecules P21 and P27, and

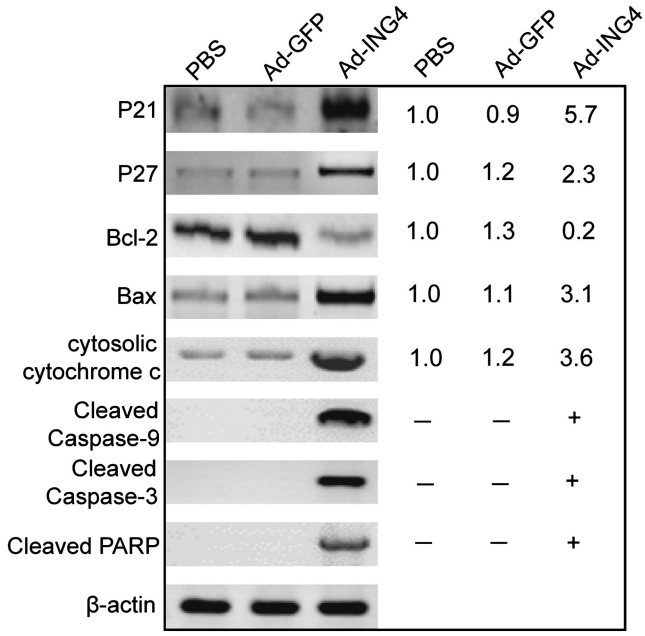


FIG. 4. Ad-ING4 alters expression of cell cycle- and apoptosis-related protein and induces intrinsic apoptotic pathway. The MDA-MB-231 breast carcinoma cells were cultured with Ad-ING4 (100 MOI), Ad-GFP (100 MOI), or PBS for 48 hours, and their cellular lysates and cytosolic proteins were subject to western blot analysis for expression detection of P21, P27, Bcl-2, Bax, cleaved caspase-9, cleaved caspase-3, cleaved PARP, cytochrome *c*, and *β*-actin (used as an internal control). The quantities of protein expression were normalized to the internal control *β*-actin measured in the same samples. The MDA-MB-231 tumor cells treated with Ad-ING4 expressed significant higher levels of P21, P27, and Bax than those in the Ad-GFP and PBS groups, whereas significant lower level of Bcl-2 than that in the Ad-GFP and PBS groups was expressed. In addition, Ad-ING4 induced the release of cytochrome *c* from the mitochondria into the cytosol and subsequent activation of caspase-9 and -3 and cleavage of PARP in MDA-MB-231 tumor cells. Data shown are representative of three independent experiments. ING, inhibitor of growth; Ad-ING4, adenovirus-mediated ING4; GFP, green fluorescent protein (shown in black and white); PBS, phosphate-buffered saline.

the decrease in the ratio of anti- to pro-apoptotic molecules Bcl-2/Bax followed by the release of cytochrome *c* from the mitochondria into the cytosol and the subsequent activation of caspase-9 and -3, leading to apoptosis via intrinsic apoptotic pathway.

ING4 overexpression reduces tumor vessel CD34 expression and MVD

The positive expression of CD34 was mainly presented as brownish yellow or brownish granules in vascular endothelial cells. In all mutant *p53* MDA-MB-231 human breast xenografted tumors collected, the CD34 expression of vascular endothelial cells in the Ad-ING4-treated group was weaker or less, compared with the Ad-GFP- and PBS-treated control groups (Fig. 5A, B), indicating that Ad-ING4 downregulates CD34 expression of MDA-MB-231 human breast xenografted tumor vessels. In addition, the MVD (Fig. 5C) counted in the Ad-ING4-treated group was significantly less than that in the Ad-GFP- or PBS-treated control group ($p < 0.05$), indicating that Ad-ING4 also reduces MVD in the MDA-MB-231 human breast xenografted tumors, which may be involved in the Ad-ING4-mediated *in vivo* growth inhibition of MDA-MB-231 human breast xenografted tumor in an athymic nude mouse model.

ING4 overexpression downregulates IL-8 and Ang-1 expression

To elucidate the potential mechanism responsible for the *in vivo* antiangiogenic effect of Ad-ING4 in an athymic nude mouse model, the effect of Ad-ING4 on the expression of proangiogenic factors such as IL-8, VEGF, and Ang-1 in mutant *p53* MDA-MB-231 breast carcinoma cells was examined using ELISA. As shown in Figure 5D, the amounts of IL-8 and Ang-1 expression in MDA-MB-231 tumor cells treated with Ad-ING4 were less than those in the cells treated with Ad-GFP or PBS ($p < 0.05$), whereas VEGF expression was not changed by Ad-ING4 treatment. These results indicate that transgene ING4 expression suppresses tumor angiogenesis possibly via indirect downregulation of the expression of proangiogenic factors IL-8 and Ang-1.

Discussion

ING4 is a novel tumor suppressor that has been implicated in a variety of processes including oncogenesis, cell cycle control, apoptosis, DNA repair, angiogenesis, migration, and gene transcription regulation. ING4 has a functionally conserved PHD structural domain in the COOH-terminal region involved in the interaction with histone acetyltransferase and histone deacetylase complexes and subsequent gene transcriptional regulation.[21] It also has a potential bipartite NLS domain in the middle region, which is essential for nuclear localization of ING4 and its binding and functional interaction with p53.[6] Recent studies have showed that ING4 can significantly inhibit tumor cell growth, induce apoptosis, suppress tumor angiogenesis[7,14,15] and invasion[10,15,22] in different tumor types, and enhance chemosensitivity to doxorubicin and etoposide in HepG2 hepatocarcinoma cells,[13] suggesting that ING4 is a potent tumor suppressor that negatively modulates tumor growth via multiple pathways.

Cancer gene therapy that transfers tumor suppressor gene into tumor cells by virus or other approaches may represent a rational and potentially successful treatment for breast cancer. The therapeutic potential of Ad-ING4 gene therapy for human breast carcinoma remains to be determined. Therefore, the present study assessed the tumor-suppressive effect of Ad-ING4 on MDA-MB-231 (mutant *p53*) and MCF-7 (wild-type *p53*) human breast carcinoma cells *in vitro* and MDA-MB-231 breast xenografted tumors *in vivo* in an athymic nude mouse model. The present study demonstrated that (1) Ad-ING4 significantly suppressed the MDA-MB-231 and MCF-7 breast carcinoma cell growth *in vitro*, indicating that Ad-ING4 expression is capable of inhibiting breast tumor cell growth in a p53-independent manner; (2) Ad-ING4 induced G2/M phase arrest and apoptosis, upregulated P21, P27, and Bax, downregulated Bcl-2, IL-8, and Ang-1, promoted cytochrome *c* release from mitochondria into cytosol, and activated caspase-9, caspase-3, and PARP in mutant *p53* MDA-MB-231 breast carcinoma cells; and (3) Ad-ING4

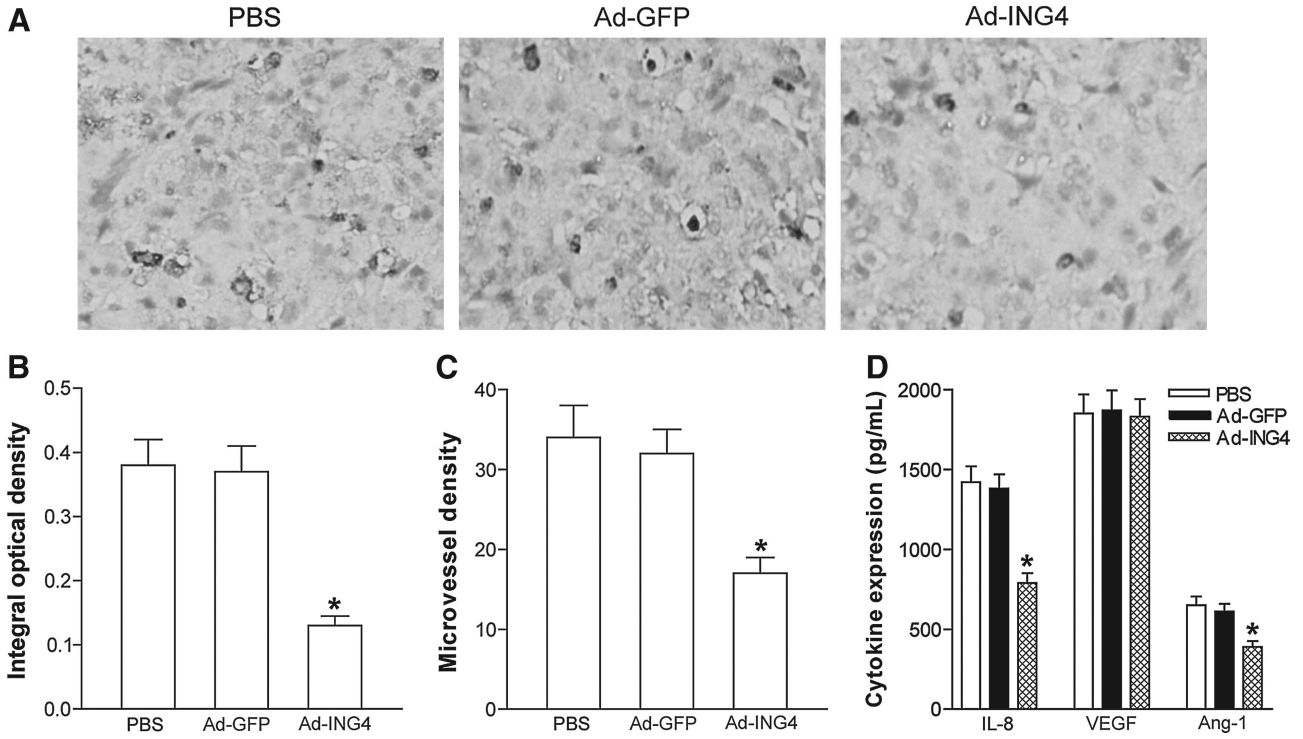


FIG. 5. Ad-ING4 downregulates proangiogenic factors IL-8 and Ang-1 expression involved in the inhibition of tumor angiogenesis. (**A**) Immunohistochemical detection of vascular endothelial cells for CD34 expression. Representative pictures for different treatment groups are shown. The positive expression of CD34 was mainly presented as brownish yellow or brownish granules in vascular endothelial cells (shown in black and white). (**B**) The integral optical density of CD34 expression quantified by Image-Pro Plus 6.0 software. Ad-ING4 downregulates CD34 expression of MDA-MB-231 breast xenografted tumor vessels (*$p < 0.05$, compared with the Ad-GFP and PBS groups, respectively, whereas $p > 0.05$ when the Ad-GFP group is compared with the PBS group, by one-way repeated measures ANOVA and multiple comparisons; $n = 5$ mice per condition, $n = 5$ observations per representative section). (**C**) The tumor MVD in different treatment groups. The MVD in the Ad-ING4-treated group was significantly lower than that in the Ad-GFP- and PBS-treated groups (*$p < 0.05$, compared with the Ad-GFP and PBS groups, respectively, whereas $p > 0.05$ when the Ad-GFP group is compared with the PBS group, by one-way repeated measures ANOVA and multiple comparisons; $n = 5$ mice per condition, $n = 5$ observations per representative section). (**D**) Ad-ING4 downregulates proangiogenic factors IL-8 and Ang-1 expression. The MDA-MB-231 breast carcinoma cells were cultured in medium with Ad-ING4 (100 MOI), Ad-GFP (100 MOI), or PBS. The supernatants were harvested after 48 hours of incubation and the amounts of IL-8, VEGF, and Ang-1 were determined by enzyme-linked immunosorbnent assay. Ad-ING4 significantly downregulated the expression of proangiogenic factors IL-8 and Ang-1 in MDA-MB-231 tumor cells (*$p < 0.05$, compared with the Ad-GFP and PBS groups, respectively, whereas $p > 0.05$ when the Ad-GFP group is compared with the PBS group, by one-way repeated measures ANOVA and multiple comparisons; $n = 3$ replicates per condition, $n = 3$ replicates per sample). Data shown are representative of three independent experiments. ING, inhibitor of growth; Ad-ING4, adenovirus-mediated ING4; GFP, green fluorescent protein (shown in black and white); PBS, phosphate-buffered saline; ANOVA, analysis of variance; MVD, microvessel density.

remarkably inhibited the MDA-MB-231 breast xenografted tumor growth and reduced CD34 expression of tumor vessels and MVD.

The activities of cyclins, cyclin-dependent kinases (cdk), and cdk inhibitors are necessary for cell cycle progression.[23] p21 and p27 as important members of Cip/Kip family can inhibit cyclin E-CDK2, cyclin A-CDK2, cyclin D-CDK4, and cyclin B1/CDC2 complexes, leading to G1 and G2/M arrest.[24–28] Bcl-2 family proteins are known to be key regulators of apoptosis and important determinants of cell fate.[29] The ratio of anti- to pro-apoptotic molecules such as Bcl-2/Bax constitutes a rheostat that sets the threshold of susceptibility to apoptosis for the intrinsic pathway, which utilizes organelles such as the mitochondrion to amplify death signals.[29,30] To explore the potential molecular mechanism, the present study assessed the cell cycle- and apoptosis-related molecules such as P21, P27, Bcl-2, Bax, cytochrome *c*, cleaved caspase-9, cleaved caspase-3, and cleaved PARP in the Ad-ING4-infected mutant *p53* MDA-MB-231 human breast carcinoma cells by western blot analysis. It was found that Ad-ING4 increased the expression of P21 and P27, decreased the ratio of Bcl-2/Bax, promoted cytochrome *c* release from mitochondria into cytosol, and induced the activation of caspase-9 and -3 and cleavage of PARP in MDA-MB-231 breast carcinoma cells, which may closely account for the Ad-ING4-induced growth inhibition, G2/M arrest, and apoptosis in MDA-MB-231 tumor cells. Previous studies showed that IL-8, VEGF, and Ang-1 are important proangiogenic cytokines involved in tumor angiogenesis.[31–34] Further, to elucidate the underlying mechanism of Ad-ING4-mediated antiangiogenic regulation, the effect of Ad-ING4 on the expression of proangiogenic factors including IL-8,

VEGF and Ang-1 in mutant *p53* MDA-MB-231 breast carcinoma cells was examined by ELISA analysis. The present study shows that Ad-ING4 significantly downregulated IL-8 and Ang-1 expression in MDA-MB-231 tumor cells, indicating that Ad-ING4 suppresses tumor angiogenesis possibly via indirectly reducing proangiogenic factors production. In athymic nude mice bearing MDA-MB-231 breast tumors, it was demonstrated that intratumoral injections of Ad-ING4 reduced CD34 expression of tumor vessels and MVD. The inhibition of tumor angiogenesis that correlates with the decreased expression of proangiogenic factors such as IL-8 and Ang-1 may be another important mechanism involved in Ad-ING4-mediated *in vivo* growth inhibition of MDA-MB-231 breast xenografted tumor in an athymic nude mouse model.

Taken together, Ad-ING4 gene transfer significantly induced *in vitro* and *in vivo* growth suppression, G2/M phase arrest, and apoptosis in MDA-MB-231 human breast carcinoma cells in a p53-independent manner. This retarded tumor growth elicited by Ad-ING4 was closely associated with the upregulation of cell cycle-related molecules P21 and P27, decrease in the ratio of anti- to pro-apoptotic molecules Bcl-2/Bax, release of cytochrome *c* from mitochondria into cytosol followed by caspase-9 and -3 activation leading to apoptosis via intrinsic apoptotic pathway, and the reduced expression of proangiogenic factors IL-8 and Ang-1 involved in the inhibition of tumor angiogenesis. Thus, the results indicate that Ad-ING4 is a potential candidate for breast cancer gene therapy.

Disclosure Statement

The authors have no financial conflict of interest.

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