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PI3K/PTEN/AKT Signaling Regulates Prostate Tumor

Angiogenesis

Jing Fang^{a,*}, Min Ding^b, Lily Yang^c, Ling-Zhi Liu^a, and Bing-Hua Jiang^{a,#}

^a Mary Babb Randolph Cancer Center, Department of Microbiology, Immunology and Cell Biology, West Virginia University, Morgantown, WV 26506-9300

^b Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV 26505

^c Department of Surgery and Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA 30322.

Abstract

PI3K pathway exerts its function through its downstream molecule AKT in regulating various cell functions including cell proliferation, cell transformation, cell apoptosis, tumor growth and angiogenesis. PTEN is an inhibitor of PI3K, and its loss or mutation is common in human prostate cancer. But the direct role and mechanism of PI3K/PTEN signaling in regulating angiogenesis and tumor growth *in vivo* remain to be elucidated. In this study, by using chicken chorioallantoic membrane (CAM) and in nude mice models, we demonstrated that inhibition of PI3K activity by LY294002 decreased PC-3 cells-induced angiogenesis. Reconstitution of PTEN, the molecular inhibitor of PI3K in PC-3 cells inhibited angiogenesis and tumor growth. Immunohistochemical staining indicated that PTEN expression suppressed HIF-1 α , VEGF and PCNA expression in the tumor xenographs. Similarly, expression of AKT dominant negative mutant also inhibited angiogenesis and tumor growth, and decreased the expression of HIF-1 α and VEGF in the tumor xenographs. These results suggest that inhibition of PI3K signaling pathway by PTEN inhibits tumor angiogenesis and tumor growth. In addition, we found that AKT is the downstream target of PI3K in controlling angiogenesis and tumor growth, and PTEN could inhibit angiogenesis by regulating the expression of HIF-1 and VEGF expression through AKT activation in PC-3 cells.

Keywords

PTEN; AKT; Hypoxia inducible factor 1; Vascular endothelial growth factor

1. Introduction

The phosphatidylinositol 3-kinase (PI3K) is a heterodimeric enzyme composed of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit [1]. PI3K catalyzes the production of PtdIns-3,4-P2 and PtdIns-3,4,5-P3 helping to recruit AKT (protein kinase B) and PDK1 to the membrane [1-5]. When activated, AKT transmits survival signals from growth factors and inactivates the apoptotic pathways [6,7].

#Corresponding author: E-mail address: bhjiang@hsc.wvu.edu (B.H. Jiang)

*Present Address: The Institute for Nutritional Sciences, SIBS, Chinese Academy of Sciences, Shanghai 200031, China.

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PTEN (phosphatase and tensin homolog deleted on chromosome ten)/MMAC (mutated in multiple advanced cancers) is a tumor suppressor gene [3,8]. The PTEN tumor suppressor gene is lost or mutated in a large number of human cancers including prostate cancer [8-10]. Deregulation of PI3K pathway is commonly observed in many human cancers. This deregulation can be caused either by loss of PTEN, or by constitutive activation of PI3K or its target AKT. Activation of PI3K/AKT may potentiate cell survival, cell migration, proliferation, and cytoskeletal rearrangement. In addition, PI3K pathway is implicated in upregulating vascular endothelial growth factor (VEGF). Studies show that overexpression of PI3K or AKT correlates with increased VEGF levels [11-14], and overexpression of PTEN in chicken embryo inhibited embryonic angiogenesis [15].

Angiogenesis is formation of new blood vessels from preexisted ones. It is critical for tumor growth. The tumor cannot grow more than 1-2 mm in diameter without angiogenesis [16]. Tumor angiogenesis is stimulated by a few angiogenic factors, among which VEGF is a fundamental regulator [17]. Hypoxia inducible factor 1 (HIF-1) is a transcription factor that activates the transcription of many genes, including VEGF [18]. HIF-1 is composed of HIF-1 α and β subunits [19,20]. HIF-1 α expression can be stimulated by a low concentration of oxygen or loss of function of VHL and PTEN [21,22]. HIF-1 α expression can be induced by growth factors through activation of PI3K/AKT signaling [23,24]. PI3K/AKT signaling regulates tumor angiogenesis via HIF-1 α /VEGF.

Prostate cancer is one of the most commonly diagnosed male malignancy in the United States, affecting 1 in 9 men over 65 years of age [25]. Several lines of evidence implicate that loss of PTEN is a major factor causing human prostate carcinogenesis. PTEN was found to be mutated or deleted in many prostate cancer cell lines including LNCaP, PC-3, NCI H660 [8,10]. It was shown that PTEN is frequently lost in prostate cancer cells [26], that it undergoes homozygous deletions in ~10% of primary prostate tumors [27], and that alterations are frequently occurred in metastatic prostate cancer [28].

Elevated levels of VEGF are found in the serum and urine of prostate cancer patients, suggesting that VEGF plays an important role in prostate cancer. PI3K/PTEN pathway regulated HIF-1 α and VEGF expression in prostate cancer cell lines [11,14,28], indicating that this pathway may play an important role in regulating prostate tumor angiogenesis. However, there is a lack of direct evidence to show that PI3K/PTEN pathway regulates prostate tumor angiogenesis *in vivo*. We have successfully established tumor cells-induced angiogenesis and tumor growth models on chicken chorioallantoic membrane (CAM) and in nude mice [12,15, 29]. The goal of this study is to investigate: 1) whether PI3K activity is required for angiogenesis of prostate tumor; 2) whether restoration of PTEN expression in prostate cancer cells inhibits tumor angiogenesis and growth on CAM *in vivo*; 3) whether the level and activity of PI3K and PTEN affect HIF-1 and VEGF expression in tumors; and 4) whether AKT is downstream target of PI3K for regulating angiogenesis.

2. Materials and methods

2.1. Reagents and antibodies

The antibodies against phospho-AKT, AKT, and PTEN were purchased from Cell Signaling (Beverly, MA). Antibodies against VEGF and PCNA were products of Santa Cruz (Santa Cruz, CA). The antibodies against β -actin and CD31 were from Sigma (St. Louis, MO) and BD Bioscience (Bedford, MA), respectively. Trizol reagent was from Invitrogen. AMV Reverse Transcriptase and oligo(dT) primer were from Promega (Madison, WI).

2.2. Cell culture

The PC-3 cells were cultured in RPMI 1640 medium, and human embryonic kidney 293 cells were cultured in MEM medium, supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated under 5% CO₂ at 37 °C as described [30].

2.3. Infection of cells with adenovirus

The PTEN, PTEN mutant with cysteine 124 to serine (PTEN-C124S), and AKT dominant negative mutant (AKT-DN) were previously described [15]. These constructs were inserted into adenoviral vector using Ad-Easy System (Stratagene, San Diego, CA). The PC-3 cells were infected with adenovirus carrying GFP (Ad-GFP), PTEN (Ad-PTEN), PTEN-C124S (Ad-PTEN-C124S), or AKT-DN (Ad-AKT-DN) at 20 MOI. The infection rate of the cells was approximately 80%.

2.4. Angiogenesis and tumor growth assay on the CAM

Fertilized White Leghorn chicken eggs were incubated at 37°C under conditions of constant humidity. The developing CAM was separated from the shell by opening a window at the broad end of the egg above the air sac on Day 9. The opening was sealed and the eggs were returned to the incubator. To investigate tumor angiogenesis, the cells were suspended in medium containing 50% Matrigel (BD Biosciences, Bedford, MA). Aliquots (30 µl) of the mixture were then applied onto the CAM of 9-day-old embryos. The area around the implanted Matrigel was photographed 4 days after the implantation, and the blood vessels were counted by two observers in a double-blind manner. Assays for each treatment were carried out using 8–10 chicken embryos. For determining tumor growth, the cells were implanted onto the CAM to grow tumors for 9 days.

2.5. Matrigel plug angiogenesis assay and tumor growth experiments in nude mice

Male BALB/cA-nu nude mice (4 weeks old) were purchased from Shanghai Experimental Animal Center (Chinese Academy of Sciences, China) and maintained in pathogen-free conditions. PC-3 cells were infected with adenovirus carrying GFP, PTEN, PTEN -C124S or AKT-DN. The cells were harvested 24 h after infection, and re-suspended in serum-free medium. The cells (0.2 ml, 4 million cells) were mixed with 0.4 ml of growth factor-reduced Matrigel, and the mixture was immediately injected into the flanks of nude mice. The mice were sacrificed 11 days after the injection, and the Matrigel plugs were taken out of the mice. The hemoglobin content of the Matrigel was determined using Drabkin reagent. Each treatment had 8 mice and the experiment was repeated twice. For tumor growth assay, the infected PC-3 cells were re-suspended in culture medium and 4 million of the cells (in 0.1 ml) were injected into the nude mice at the flank sites. Tumor growth was determined by measuring the size of the tumors, starting at Day 11 and ending at Day 21. Tumor volumes were calculated according to the formula $(width^2 \times length)/2$.

2.6. Immunoblotting and immunohistochemistry

Immunoblotting and immunohistochemistry were performed as described previously [29]. Tumor tissues were fixed in 10% buffered formalin for 24 h and processed by conventional method. For CD31 staining, tumor tissues were fixed in zinc fixative (BD Biosciences) and embedded in paraffin. The relative angiogenesis was calculated as micro-vessel density (MVD) as described [31]. Briefly, slides were first scanned under low power ($\times 40$) in order to determine three 'hot-spots' or areas with the maximum number of micro-vessels, which were then evaluated at magnification of 200. The number of micro-vessels in each field was determined and expressed as MVD per field.

2.7. RT-PCR

Tumor tissues prepared from the cells infected by Ad-GFP and Ad-PTEN groups were ground in liquid nitrogen to extract total RNAs. Total RNAs were used for cDNA synthesis by reverse transcription, and cDNAs of VEGF and GAPDH were amplified by PCR. The primers used for the PCR are as follows: VEGF upstream primer 5'-TCGGCCTCCGAAACCATGA-3', VEGF downstream primer 5'-CCTGGTGAGAGATCTGGTTC-3'; GAPDH upstream primer 5'-CCACCCATGGCAAATCCATGGCA-3', GAPDH downstream primer 5'-CCTGGTGAGAGATCTGGTTC-3'.

2.8. Tunnel assay and Statistical analysis

In Situ Cell Death Detection kit was purchased from Roche Co, Switzerland. Tunnel staining of the tissue sections was performed per the manufacture's instruction. The data represent mean \pm SD from three independent experiments except where indicated. Statistical analysis was performed by student t test at a significant level ($p < 0.05$).

3. Results

3.1. LY294002 inhibited PC-3 xenograft-induced angiogenesis on the CAM

We used the CAM to test whether PI3K in PC-3 cells plays a role in tumor-induced angiogenesis. PC-3 cells greatly induced angiogenesis (Fig. 1A). PI3K inhibitor LY294002 decreased PC-3 xenograft-induced angiogenesis in a concentration dependent manner (Fig. 1). This result showed that PI3K activation in PC-3 cells is required for tumor-induced angiogenesis.

3.2. Over-expression of PTEN inhibited angiogenesis and tumor growth

Since PC-3 cells contain a mutated PTEN gene, we transected a wild type PTEN gene to restore PTEN expression in PC-3 cells. The cells were infected by adenovirus carrying GFP or wild-type PTEN. The PTEN expression in PC-3 cells was confirmed by immunoblotting, which inhibited AKT phosphorylation and activation (Fig. 2A). PTEN expression decreased cell proliferation when compared to the cells expressing GFP and the parental cells (Fig. 2B). To analyze the effect of PTEN restoration in PC-3 cells in affecting angiogenesis, the cells alone or expressing GFP or wild-type PTEN were used for the angiogenesis assay *in vivo*. PC-3 xenografts induced angiogenesis on the CAM, and overexpression of PTEN significantly inhibited tumor-induced angiogenesis while the tumor size was similar 4 days after the implantation (Fig. 2C). This result suggested that PTEN expression initially inhibited angiogenesis before affecting tumor growth. To test the effect of PTEN on tumor growth, the tumor sizes were analyzed 9 days after the implantation of PC-3 cells on the CAM cells. Over-expression of PTEN greatly inhibited the tumor growth when it was analyzed 9 days after the implantation (Fig. 2D). Immunohistochemical staining of the tumor sections showed that PTEN restoration in PC-3 cells decreased the expression of HIF-1 and VEGF in tumor sections (Fig. 2E). The levels of HIF-1 α protein and VEGF mRNA from the tumor tissues infected by adenovirus carrying PTEN showed 70% decrease when compared to those from the Ad-GFP control (Fig. 2F), further confirming the crucial role of PTEN in regulating the expression of HIF-1 and VEGF during the tumor angiogenesis.

To test whether PTEN restoration in PC-3 cells expression has similar effect in regulating angiogenesis and tumor growth in a different animal model, we performed similar experiments using nude mice. Tumor angiogenesis was determined using Matrigel plug assay. PC-3 cells dramatically induced angiogenesis, and PTEN expression decreased the angiogenesis (Fig. 3A). The relative angiogenesis was analyzed by the levels of hemoglobin in the Matrigel plugs. PTEN expression in the cells leads to the hemoglobin level 6-fold less than that of the control

in the Matrigel plugs (Fig. 3A). We also studied tumor growth by the subcutaneous injection of the cells in the nude mice.

The tumor growth assay showed that PTEN expression in PC-3 cells significantly inhibited the tumor growth when compared to the control (Fig. 3B). Micro-vessel density (MVD) of tumor sections was determined as an index of tumor angiogenesis. Expression of PTEN in PC-3 cells significantly decreased the tumor MVD (Fig. 3C). The expression of HIF-1 α and VEGF was decreased in tumor tissues from the cells infected with Ad-PTEN, indicating that PTEN may inhibit angiogenesis through the expression of HIF-1 and VEGF (Fig. 3D). We stained the tumor sections using antibodies against PCNA, and found that more than 80% of the cells were PCNA-positive in the control tumors versus only about 40% of the cells that were PCNA-positive in the PTEN-expressing tumors (Fig. 3E). Tunnel assay indicated that there was no apparent difference of apoptotic cells between the control and PTEN-treated tumors (Fig. 3F). Taken together, these results suggest that the inhibition of tumor growth by PTEN is partially due to the cell proliferation decrease.

3.3 .PTEN lipid phosphatase activity is required for inhibiting tumor-induced angiogenesis and tumor growth

To study whether PTEN lipid phosphatase activity is required for PTEN-inhibiting angiogenesis, we expressed PTEN-C124S, a point mutant of PTEN that abolishes the lipid phosphatase activity. The PC-3 cells were infected with adenovirus carrying GFP or PTEN-C124S. Over-expression of PTEN-C124S was confirmed by immunoblotting, which did not affect the level of phospho-AKT (Fig. 4A) and cell proliferation (Fig. 4B), suggesting that PTEN phosphatase activity is required for inhibiting AKT activation and cell proliferation. The tumor angiogenesis assay was performed using the CAM assay. The forced expression of PTEN-C124S in PC-3 cells did not inhibit tumor-induced angiogenesis using the CAM (Fig. 4C), suggesting that PTEN phosphatase activity is required for inhibiting PC-3 tumor-induced angiogenesis. We used Matrigel plug assay, and also showed that PTEN-C124S expression did not inhibit PC-3 cell-induced angiogenesis in nude mice (Fig. 4D). To determine the effect of PTEN-C124S expression in PC-3 cells in tumor growth, the cells were implanted onto the CAM to grow tumor for 9 days. Expression of PTEN-C124S did not inhibit tumor growth and the levels of HIF-1 α and VEGF in the tumor (Fig. 5B).

3.4 AKT is required for tumor-induced angiogenesis and tumor growth

We next determined the effect of AKT dominant negative mutant (AKT-DN) in PC-3 cells in regulating angiogenesis. PC-3 cells were infected with adenovirus carrying GFP or AKT-DN. The AKT-DN expression in the cells was confirmed by immunoblotting, and its inhibitory effect was showed through decreasing the level of phospho-GSK-3 β , a downstream target of AKT in the cells (Fig. 5A). Forced expression of AKT-DN inhibited tumor growth (Fig. 5B). The immunohistochemical staining of the tumor sections showed that expression of AKT-DN in PC-3 cells inhibited the expression of HIF-1 α and VEGF (Fig. 5C). Expression of AKT-DN in PC-3 cells greatly inhibited PC-3 cell-induced angiogenesis (Fig. 5D), showing that AKT is a PTEN-inhibited downstream target that is required for PC-3 cell-induced angiogenesis *in vivo*.

4. Discussion

The PI3K/AKT signaling has been shown to play an important role in survival and proliferation of human prostate cancer cells [32-35]. The tumor suppressor PTEN is frequently mutated in human prostate cancer. It has been shown that inhibition of PI3K by LY294002 abrogated the expression of HIF-1 α and VEGF in prostate cancer cell lines [11,14]. However, the role and mechanism of PI3K and AKT in prostate tumor angiogenesis *in vivo* remain to be elucidated.

To determine the role and mechanism of PTEN in tumor growth and angiogenesis, in this study we used PC-3 human prostate cancer cell line to perform angiogenesis assay *in vivo* using the CAM and nude mouse models. PC-3 contains PTEN mutation, which is good to test the functions of the reconstituted PTEN construct in the tumor growth without the interference of endogenous PTEN.

The PC-3 xenograft greatly induced angiogenesis on the CAM, and inhibition of PI3K by LY294002 significantly suppressed angiogenesis, suggesting that PI3K is required for prostate tumor angiogenesis. PTEN is a molecular inhibitor of PI3K, and is one of the most commonly mutated tumor suppressor genes, which is ranked the Number 2 after p53 mutations [3]. PTEN mutation is frequently observed in prostate cancer [8,10,26,27]. Inactivation of PTEN leads to the activation of PI3K/AKT signaling. The mutation of PTEN in PC-3 cells leads to the high levels of HIF-1 α and VEGF expression. In order to determine the role and mechanism of PTEN in PI3K-regulated angiogenesis and tumor growth, we found that introduction of PTEN into PC-3 cells inhibited AKT activation and cell proliferation. Over-expression of PTEN inhibited angiogenesis and tumor growth in CAM model. Immunohistochemical staining of the tumor xenografts showed that the expression of PTEN decreased HIF-1 α and VEGF protein levels, suggesting that the inhibition of angiogenesis could be due to the decreased expression of HIF-1 α and VEGF. To further confirm our results obtained by CAM model, we used similar method to test angiogenesis and tumor growth in nude mice model. CD31, which is a specific marker to determine vascular endothelial cells, was used to determine tumor angiogenesis. We found that the number of CD31-positive microvessels and the expression of HIF-1 α and VEGF decreased significantly in tumor xenografts from the Ad-PTEN-treated group, confirming our previous results that over-expression of PTEN inhibited angiogenesis through HIF-1 α and VEGF expression. Previous studies have showed that angiogenesis is required for tumor growth [16,36]. In addition, inhibition of PI3K resulted in inactivation of AKT and decreased cell proliferation. PCNA staining showed that the cell proliferation in PTEN-expressing cells was inhibited while there was no apparent difference of apoptotic cells between the control and PTEN-treated tumors, indicating that the inhibition of tumor growth was also caused by the inhibition of cell proliferation by PTEN. Our results suggest that PI3K is required and plays an important role in prostate tumor growth and angiogenesis.

Next, in order to determine whether the function of PTEN is required in inhibiting angiogenesis and tumor growth, we introduced mutant PTEN-C124S to PC-3 cells. Expression of mutant PTEN had no effects on phospho-AKT and cell proliferation of PC-3 cells. Mutant PTEN expression in PC-3 cells had no inhibitory effect on angiogenesis and tumor growth induced by PC-3 xenografts. Immunohistochemical staining of the tumor xenografts indicated that expression of PTEN mutant could not inhibit HIF-1 and VEGF expression, either. These results suggest that as a tumor suppressor, PTEN plays an important role in regulating angiogenesis and tumor growth.

AKT is one of the important downstream targets of PI3K. Finally, we determined whether AKT activation is necessary during tumor angiogenesis and tumor growth. As shown in Fig. 4A, expression of AKT dominant-negative inhibited AKT activity, and decreased cell proliferation. Inhibition of AKT by Ad-AKT-DN infection significantly suppressed PC-3 cells-induced angiogenesis and tumor growth with decreased expression of HIF-1 α and VEGF by immunohistochemical staining. AKT lies downstream of PTEN, and does not affect PTEN expression in the PC-3 cells. These results suggest that the inhibition of AKT resulted in abrogation of HIF-1 α and VEGF expression, which could lead to the inhibition of angiogenesis and tumor growth, suggesting that AKT plays an important role in angiogenesis. In summary, this study shows that PI3K/PTEN/AKT signaling plays an important role in regulating tumor angiogenesis and growth through HIF-1 α and VEGF expression.

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Abbreviation

CAM, chicken chorioallantoic membrane; PI3K, phosphatidylinositol 3-kinase; MVD, microvessel density; MOI, multiplicity of infection.

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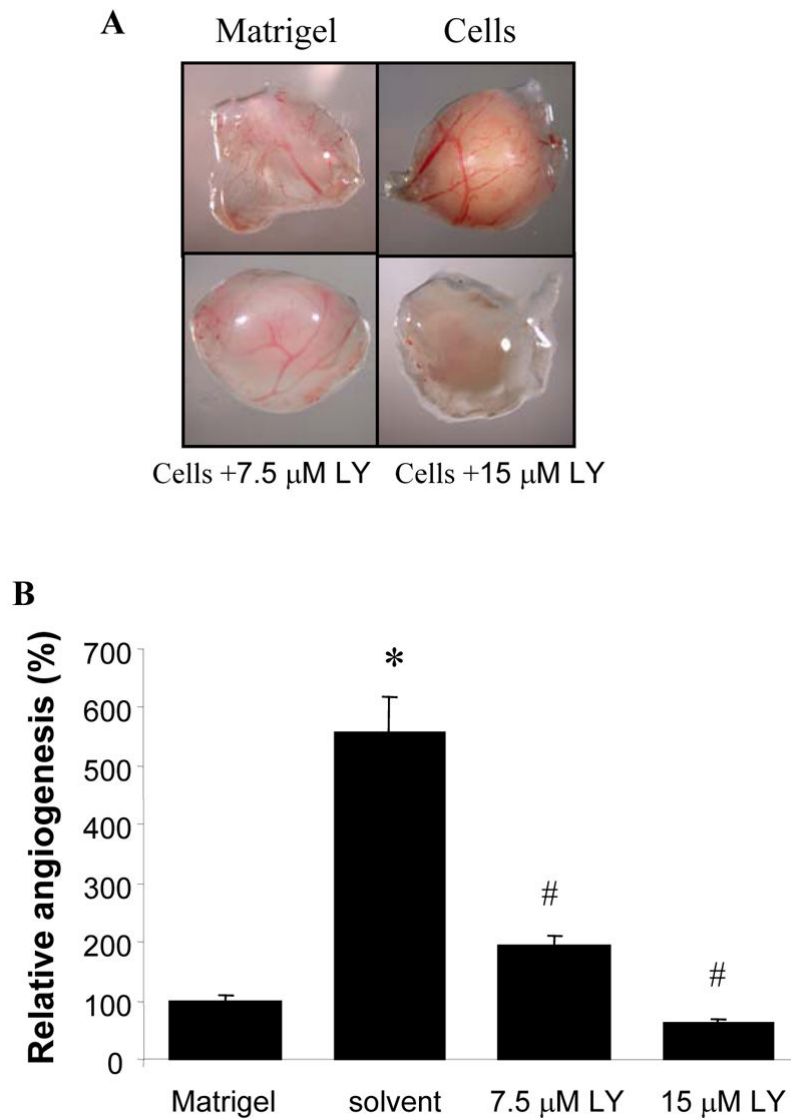
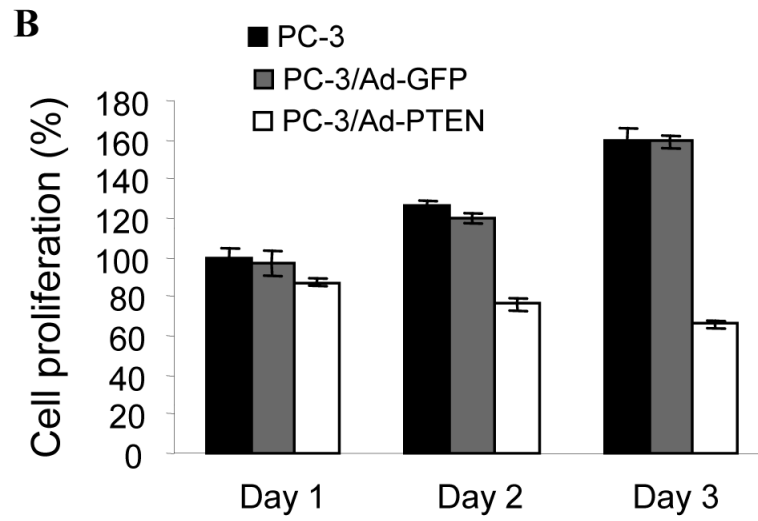
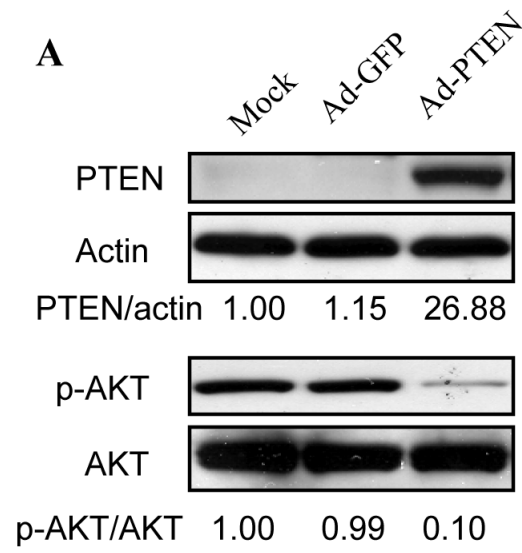
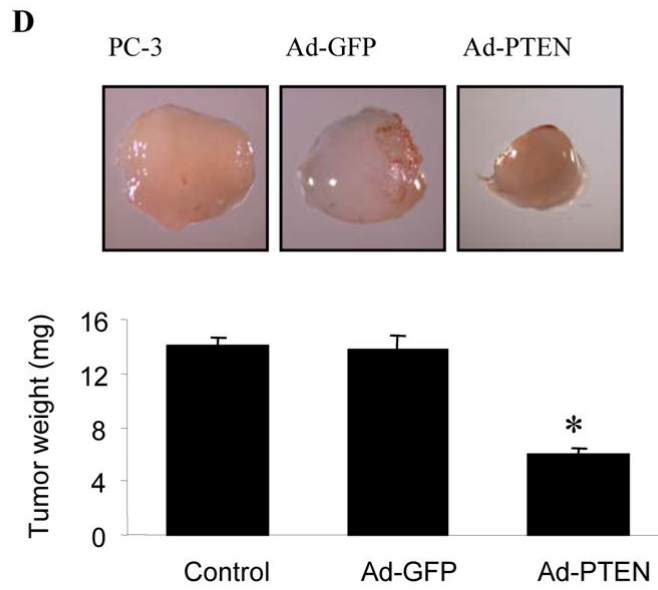
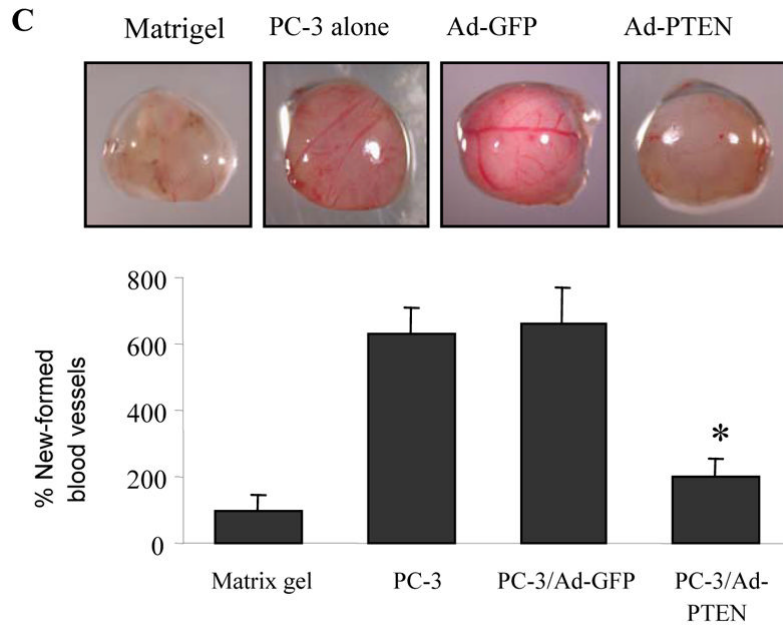


Fig. 1. LY294002 inhibited tumor-induced angiogenesis. PC-3 cells (15 μ l) were mixed with 15 μ l Matrigel in the presence or absence of LY294002. The cell mixture was implanted onto the CAM of a 9-day old chicken embryo. After 4 days of implantation, the CAM was cut off, and the amount of blood vessels on the CAM induced by the PC-3 xenografts was determined from eight different replicated experiments. (A) Representative tumors on the CAM from Matrigel alone, PC-3 cells alone, PC-3 cells with 7.5 μ M LY294002, or 15 μ M LY294002. (B) The relative angiogenesis in the CAM. The number of blood vessels were counted from replicate experiments, and normalized to that of the Matrigel alone as relative angiogenesis. The experiment was performed twice, and the data are mean and SD from 8 replicates. * indicates the significant difference when compared to the Matrigel alone, # indicates the significant difference when compared to the PC-3 cells alone ($p < 0.05$).





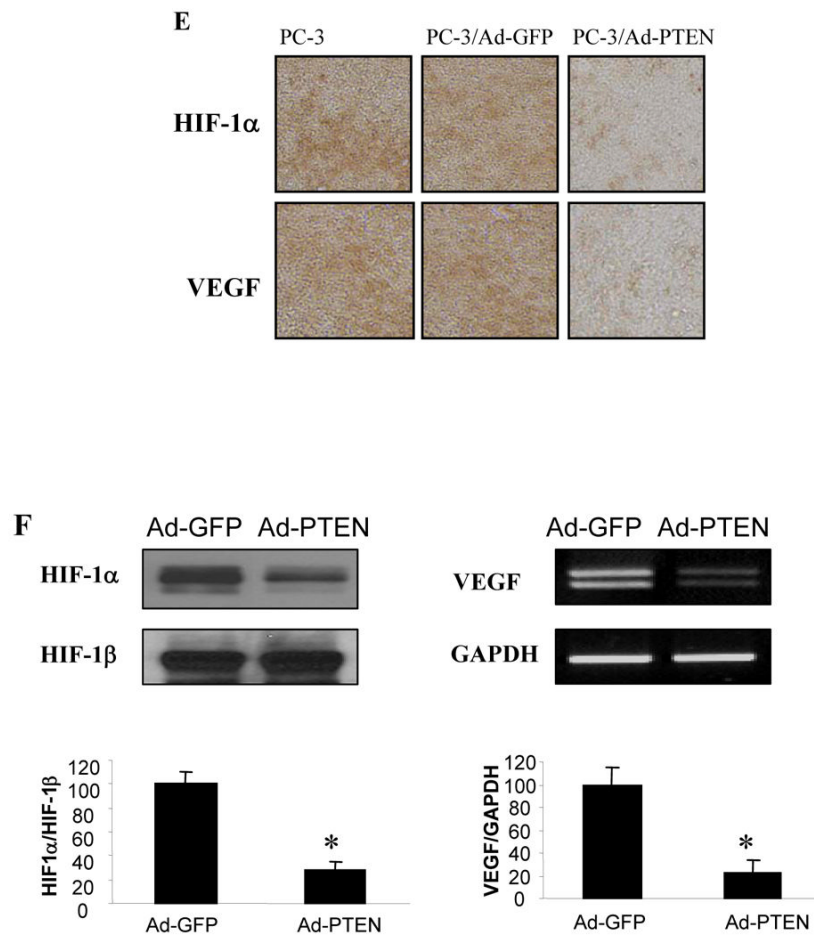
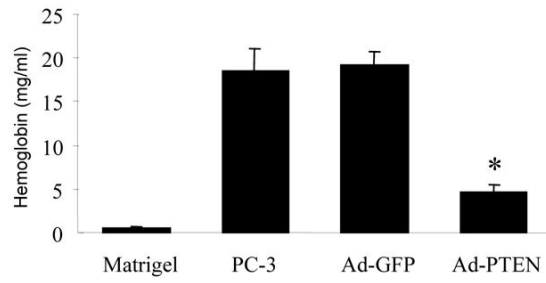
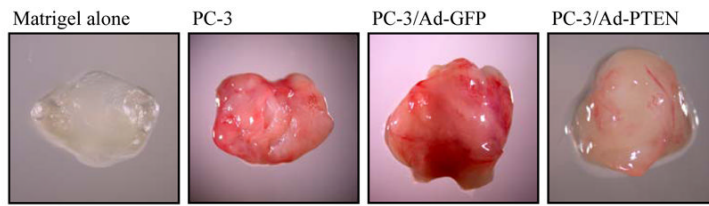


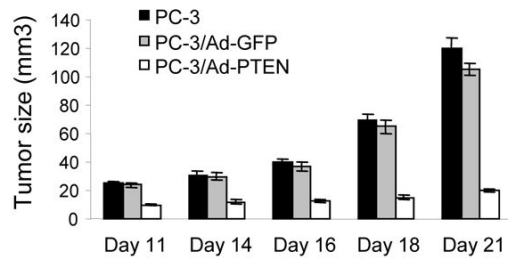
Fig. 2. Over-expression of PTEN inhibited angiogenesis and growth of tumor. The PC-3 cells were infected with adenovirus vector or adenovirus carrying PTEN. Relative density signals were quantified, and normalized to those of actin levels and to those of the mock control. (A) The infected cells were harvested 48 h after the infection, and used for immunoblotting analysis. (B) The infected cells were split into 96-well plates at 1×10^4 cells/well. Cell proliferation was assayed 24 h post-infection (Day 1) using MTT assay [30]. The value of MTT reduction at Day 1 was defined as 100%. (C) PC-3 cells were infected with adenovirus or Ad-PTEN. The cells were harvested 24 h after the infection, and re-suspended in the serum-free medium. The cells were mixed with Matrigel at 1:1 ratio, and 30 μ l of the mixture (3×10^6 cells) was implanted onto the CAM of a 9-day old chicken embryo. The CAM was cut off 4 days after the implantation, and the number of blood vessels on the CAM was counted under microscope. At least five unit areas of each CAM were counted under the tumor. The results are mean and SD from two experiments ($n = 8$). * indicates the significant difference when compared to the GFP control ($p < 0.05$). (D) PC-3 cells were implanted onto the CAM as above. The tumors were harvested and weighed 9 days after the implantation. The data indicate the mean \pm SD ($n = 8$) of three independent experiments. * indicates the significant difference when compared to the GFP control ($p < 0.05$). (E) Immunohistochemical staining of tissues using antibodies against HIF-1 α and VEGF. (F) PC-3 cells were infected with Ad-GFP and Ad-PTEN for 24 h. The cells were mixed and implanted onto the CAM of a 9-day old chicken embryo as above. After 9 days of incubation, tumor tissues were ground by liquid nitrogen. Tissue lysates were analyzed by immunoblotting for HIF-1 α and HIF-1 β expression (left, up panel). Relative

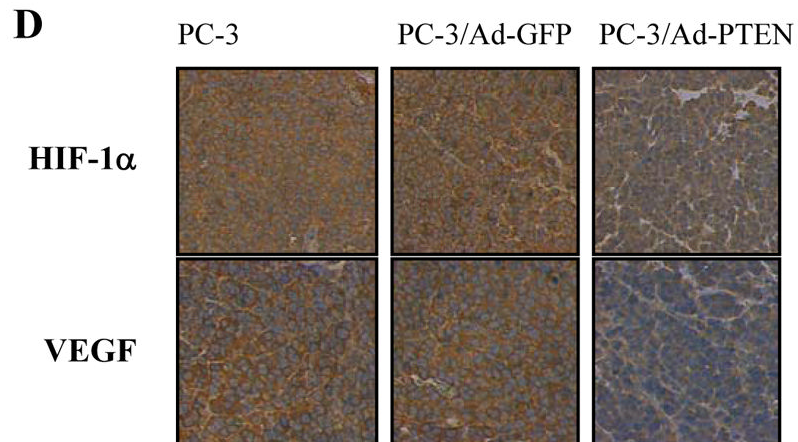
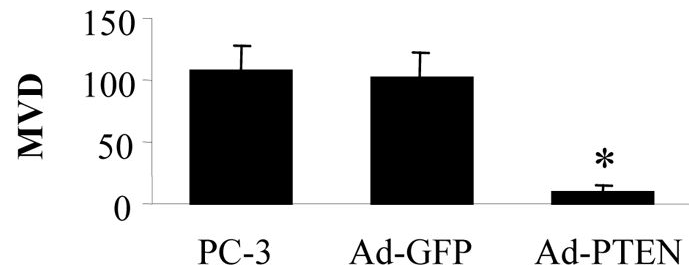
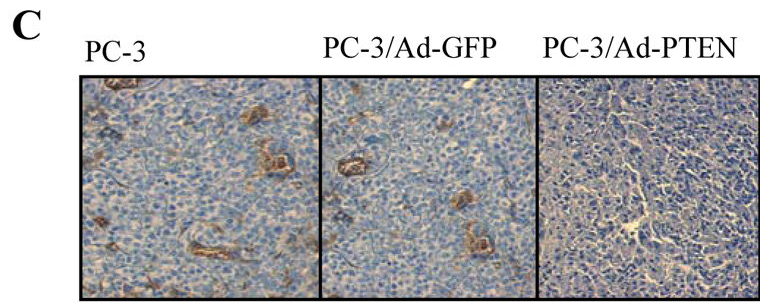
HIF-1 α protein signals were quantified and normalized to those of HIF-1 β and to the control from three replicate experiments. Total RNAs were extracted from the tumor tissues, and VEGF mRNA levels were determined by RT-PCR (right, up panel). Relative VEGF mRNA levels were from three replicate experiments. * indicates the significant difference when compared to the vector control ($p < 0.05$).

A



B





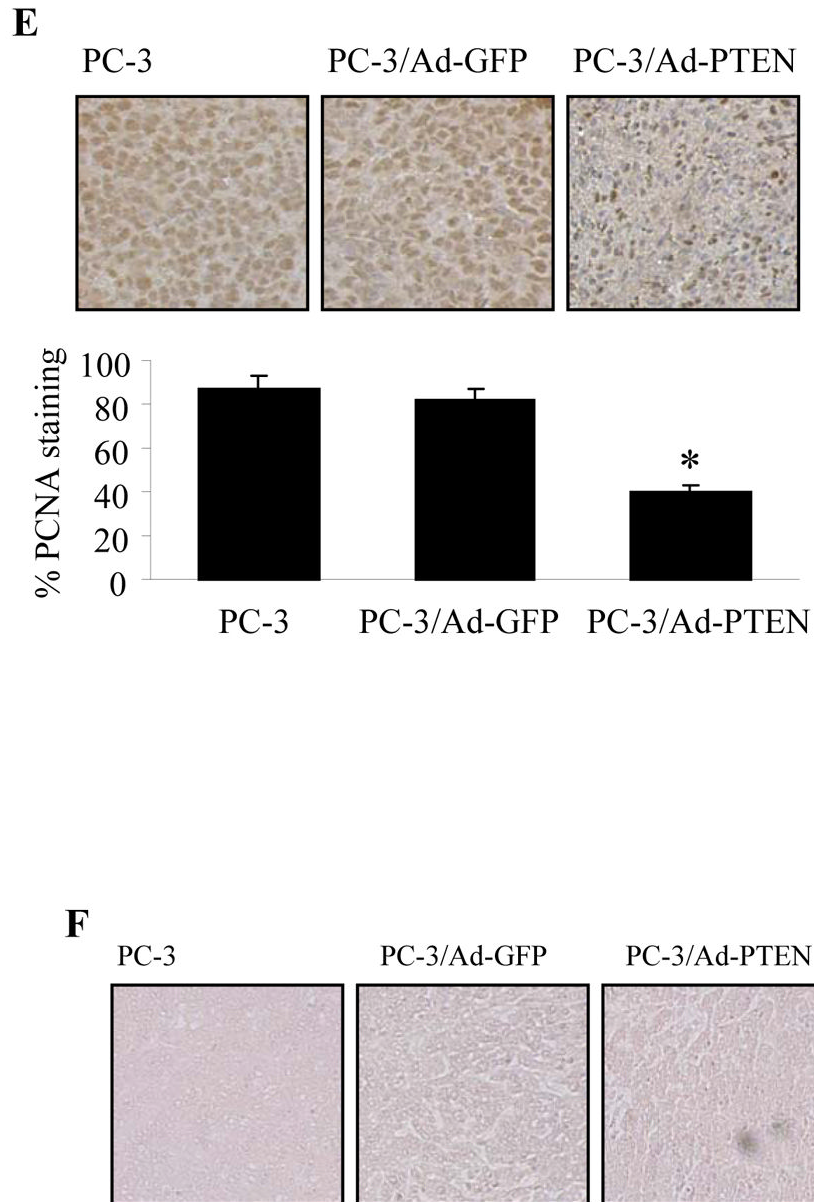
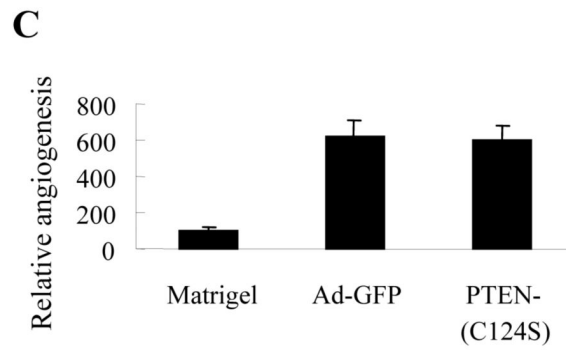
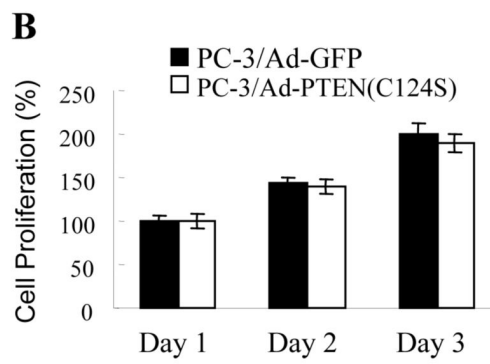
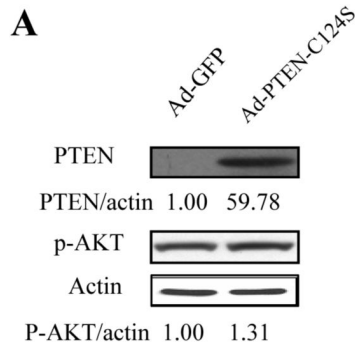


Fig. 3. Over-expression of PTEN inhibited tumor-induced angiogenesis and tumor growth. (A) PC-3 cells were infected as described above. The cells were harvested 24 h after infection, and re-suspended in serum-free medium. The cells (0.2 ml, 4 million cells) were mixed with 0.4 ml of growth factor-reduced Matrigel, and the mixture was immediately injected into the flanks of nude mice. The mice were sacrificed 11 days after the injection, and the Matrigel plugs were taken out of the mice. The hemoglobin content of the Matrigel was determined using Drabkin reagent. Each treatment had 8 mice and the experiment was repeated twice. * indicates the significant difference when compared to the GFP control ($p < 0.05$). (B) The infected PC-3 cells were re-suspended in culture medium and 4 million of the cells (in 0.1 ml) were injected into the nude mice at the flank sites. Tumor growth was determined by measuring the size of the tumors, starting at Day 11 and ending at Day 21. The data indicate mean \pm SD from two experiments ($n = 8$). (C) CD31 immunohistochemical staining. Tumor sections were stained

using antibodies against CD31, and microvessel density of the tumors was analyzed as described under Materials and Methods. * indicates the significant difference when compared to the GFP control ($p < 0.05$). (D) Immunohistochemical staining for HIF-1 α and VEGF expression in tumor sections. (E) The PCNA staining of the tumor sections. Proliferating cells were quantified by counting the PCNA-positive (brown) cells and the total number of cells at 5 arbitrarily selected fields at $\times 200$. The data are mean \pm SD ($n = 5$). * indicates the significant difference when compared to the GFP control ($p < 0.05$). (F) Tunnel assay of the tumor sections.



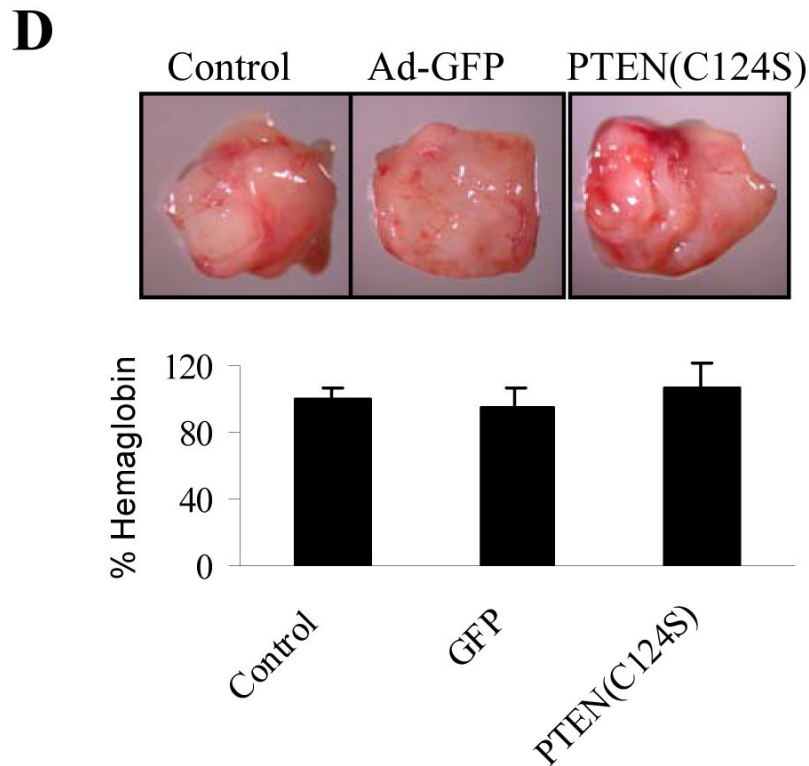
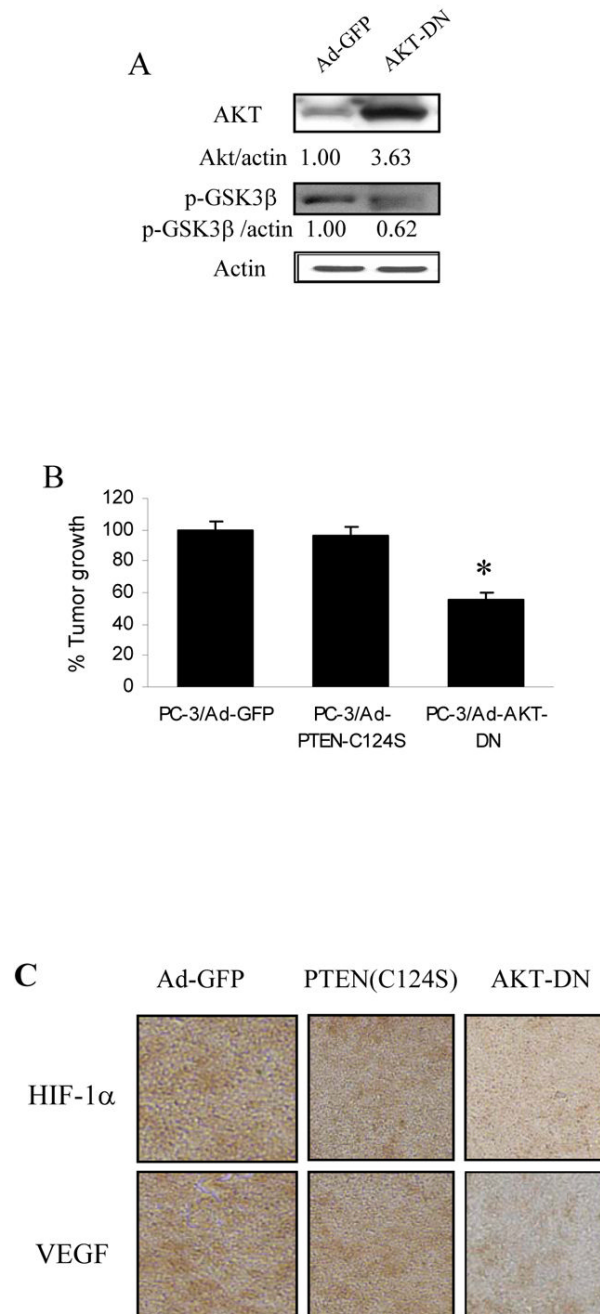


Fig. 4. Over-expression of PTEN-C124S did not inhibit angiogenesis. The PC-3 cells were infected by adenovirus carrying GFP or PTEN-C124S. (A) The infected cells were harvested 48 h after infection, and used for immunoblotting analysis. Relative values of density were quantified as Fig. 1A. (B) The infected cells were split into 96-well plates (1×10^4 cells/well) 24 h post-infection. The cell proliferation was assayed as in Fig. 2B. (C) PC-3 cells were infected with adenovirus carrying GFP or PTEN-C124S. The infected cells were harvested 24 h after infection, mixed with Matrigel, and implanted onto the CAM of 9-day old embryos as described in Fig. 2C. The CAM was excised 4 days after implantation, and blood vessels were counted as described in Fig. 2C. * indicates the significant difference when compared to the GFP control ($p < 0.05$). (D) PC-3 cells were infected with adenovirus carrying GFP or PTEN-C124S. The infected cells were harvested 24 h after infection, mixed with Matrigel, and injected into the nude mice. The mice were sacrificed 11 days after implantation, and the Matrigel plugs were harvested for hemoglobin assay as described above. * indicates the significant difference when compared to the GFP control ($p < 0.05$).



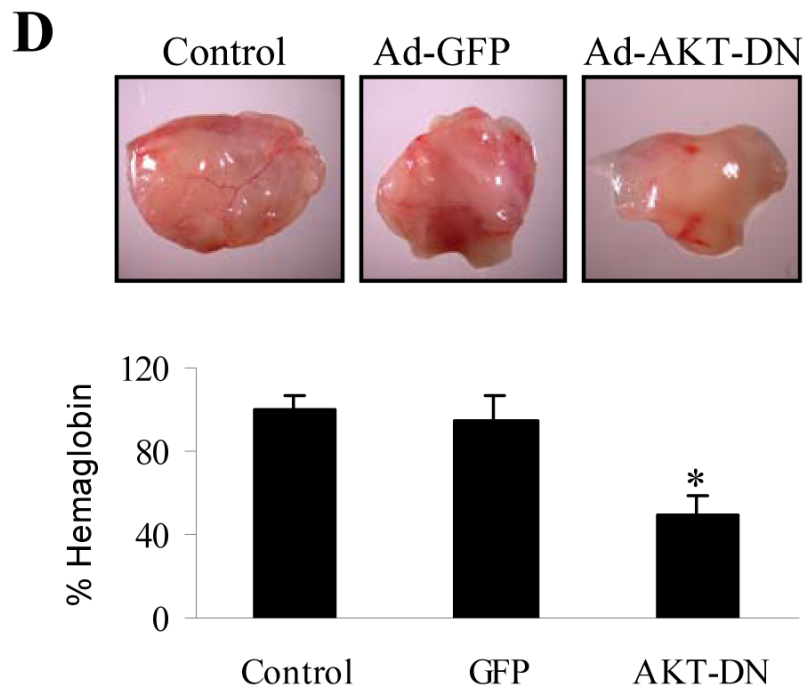


Fig. 5. Expression of AKT-DN inhibited PC-3-induced angiogenesis and tumor growth. PC-3 cells were infected with adenovirus carrying GFP, PTEN-C124S or AKT-DN. The cells were harvested 24 h after infection. (A) The cells were used for immunoblotting for total AKT and phospho-GSK3 levels. Relative values of density were quantified as previously described. (B) The cells were mixed with Matrigel, and implanted onto the CAM of 9-day old embryos as described above. Total cells implanted on each embryo were 3 million. The tumors were harvested and weighed 9 days after implantation. The data are the mean \pm SD of 8 tumor weight. * indicates the significant difference when compared to the GFP control ($p < 0.05$). (C) Immunohistochemical staining for HIF-1 α and VEGF expression in tumor sections from representative tumors from the above experiment. (D) The cells infected with adenovirus carrying GFP, PTEN-C124S or AKT-DN were mixed with Matrigel, and injected into the nude mice. The mice were sacrificed 11 days after implantation, and the Matrigel plugs were harvested for hemoglobin assay as described above. * indicates the significant difference when compared to the GFP control ($p < 0.05$).