Endothelial p70 S6 Kinase 1 in Regulating Tumor Angiogenesis

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Abstract

The p70 S6 kinase 1 (p70S6K1) exerts its function in regulating protein synthesis, cell proliferation, cell cycle progression, and cell survival in response to growth factors and other cellular signals. But the direct effect of p70S6K1 in regulating tumor growth and angiogenesis remains to be elucidated. Here, we investigated the effect of p70S6K1 expressed in human dermal microvascular endothelial cells (HDMEC) in regulating cancer cell–inducing tumor growth and angiogenesis and found that HDMECs enhance cancer cell–induced tumor growth and angiogenesis. Constitutive activation of p70S6K1 in HDMECs is sufficient to enhance tumor growth and angiogenesis. Inhibition of p70S6K1 by its dominant-negative mutant in HDMECs interferes with tumor growth and angiogenesis, indicating that p70S6K1 activity in endothelial cells is required for regulating tumor angiogenesis. We found that p70S6K1 regulates hypoxia-inducible factor-1α (HIF-1α) expression in the human endothelial cells. Knockdown of HIF-1α in the endothelial cells decreases tumor growth and angiogenesis. These results show that p70S6K1 and HIF-1 play an important role in regulating the endothelial functions for inducing tumor growth and angiogenesis. This study helps to understand the role and molecular mechanism of p70S6K1 in regulating angiogenesis and tumor growth, and the role of endothelial p70S6K1/HIF-1 signaling in the regulation of tumor microenvironment and angiogenesis. [Cancer Res 2008; 68(19):8183–8]

Introduction

The mammalian target of rapamycin (mTOR) plays an important role in regulating cell proliferation and survival (1–3). Ribosomal p70 S6 kinase 1 (p70S6K1) is a major downstream target of mTOR. In response to mitogen stimulation, activated p70S6K mediates the effects of mTOR on protein translation through its phosphorylation of the 40S ribosomal protein S6, leading to the enhancement of translation of mRNAs with a 5′-terminal oligopyrimidine (1–3). Growing evidence indicates that mTOR/p70S6K1 pathway is involved in carcinogenesis, metastasis, and chemotherapeutic drug resistance (1, 4–9). mTOR/p70S6K1 is activated by cytokines and growth factors through the activation of its upstream pathways, such as phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase/ERK pathways, and the somatic mutations or deletions of tumor suppressor PTEN (1, 10). Recent reports indicate that p70S6K1 regulates cell proliferation, apoptosis, and protein synthesis in human endothelial cells in response to growth factors (11–13). Inhibition of mTOR/p70S6K1 in human lymphatic endothelial cells impedes lymphangiogenesis, suggesting that p70S6K1 activation plays an important role in controlling the function of endothelial cells (14). However, the direct role of p70S6K1 in regulating tumor angiogenesis remains to be elucidated.

Tumor angiogenesis is required for tumor development and growth and is regulated by the microenvironments composed of tumor cells, vascular endothelial cells, and stromal cells (15–17). A huge body of information has been accumulated on signaling molecules expressed in cancer cells in regulating angiogenesis. However, little information is known about signaling molecules in human endothelial cells in mediating tumor growth and angiogenesis *in vivo* because of the limitation of the model system. In this study, we established a new chimeric tumor model *in vivo* using human prostate cancer cells and human dermal microvascular endothelial cells (HDMEC) by biodegradable sponges and studied (*a*) the effect of human endothelial cells on tumor growth and angiogenesis, (*b*) whether p70S6K1 expressed in human endothelial cells was required for tumor growth and angiogenesis, (*c*) whether p70S6K1 activation in human endothelial cells was sufficient for inducing tumor angiogenesis, and (*d*) the potential downstream targets of p70S6K1 for mediating the tumor growth and angiogenesis *in vivo*. This study established a new tumor model to study the endothelial signaling pathways for regulating tumor growth and angiogenesis and showed the role of p70S6K1 signaling pathway specifically expressed in human endothelial cells for mediating tumor angiogenesis.

Materials and Methods

Cells. HDMECs were purchased from Clonetics and cultured in microvascular endothelial cell growth medium (Clonetics). Human prostate cancer cells PC-3 and DU145 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin, 5% CO_2 at 37°C.

Adenovirus. The construction of constitutively active p70S6K1 (p70CA), dominant-negative p70S6K1 (p70KD), and green fluorescent protein (GFP) has been described previously (18). Adenovirus carrying small interfering RNA (siRNA) against hypoxia-inducible factor-1α (HIF-1α) was a gift from Dr. Lily Yang (Emory University School of Medicine, Atlanta, GA). Recombinant adenoviruses were made using the AdEasy system (19). Viral titers were determined using the BD Adeno-X Rapid Titer kit (BD Biosciences Clontech) according to the manufacturer's manual.

Biodegradable polymer matrix. The degradable sponges were prepared similarly as previously described (20, 21). Briefly, poly-L-lactide glycolide acid (PLGA) was purchased from Medisorb and was dissolved in chloroform to yield 7% polymer solution. The solution was loaded into a Teflon dish packed with 3 g of sodium chloride particles with 250 to 500 μm. After the evaporation of the solvent, the sponges were immersed in distilled water to leach the salt and coated with 10 mg/mL of polyvinyl alcohol (Aldrich Chemical Co.) in PBS for 16 h. The sponges were removed from the solution, dried, and cut into pieces with 6 × 6 × 1 mm. The day before the

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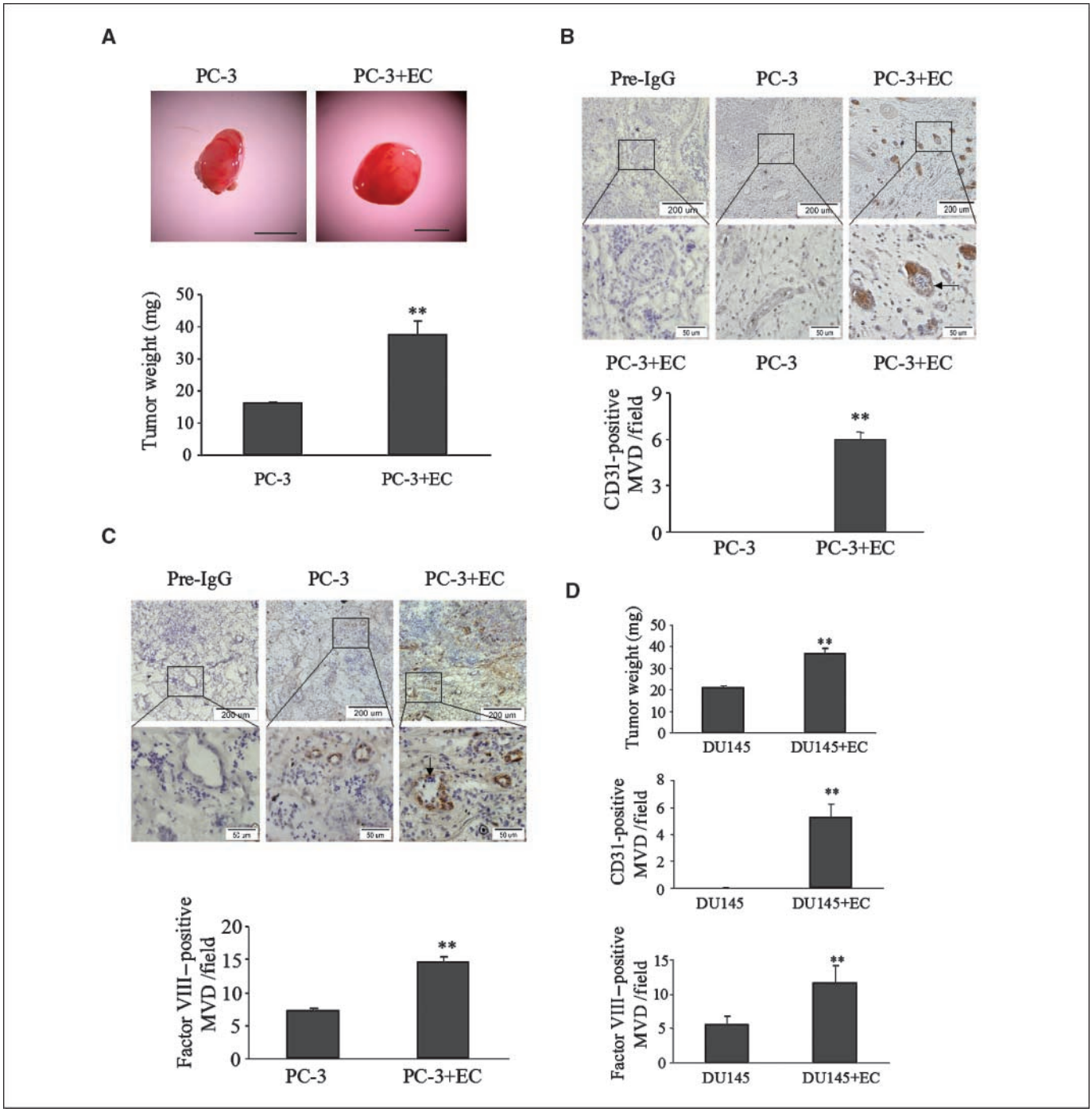

Figure 1. HDMECs enhance prostate cancer cell–induced angiogenesis and tumor growth. *A,* PC-3 cells and HDMECs were trypsinized, resuspended in serum-free medium, and mixed in 1:9 ratio, carried by the PLGA sponges, and implanted onto the chicken CAM of 8-d-old chicken embryos. PC-3 cells alone were used as a control. The tumors were harvested 12 d after the implantation. Representative tumors generated from PC-3 cells alone or PC-3 cells mixed with the human endothelial cells (*EC*). *Bar,* 3 mm (*top*). Tumors were harvested, trimmed of adjacent tissues, and weighed. *Columns,* mean of the net tumor weight that is the total tumor weight minus the weight of implanted sponge from eight replicate tumors; *bars,* SE. **, significant difference when the tumor weight of HDMEC presence was compared with that of the control ($P < 0.01$). *B, top,* representative sections of CD31 staining. Tumor sections were cut at 5 μm and processed for immunohistochemical staining using monoclonal antibody against human CD31. The immunostained sections were developed using the SABC method with diaminobenzidine as the chromogen. *Top,* sections incubated with preimmune IgG instead of the primary antibodies were used as the negative control. Magnification, ×100 (*top*) and ×400 (*bottom*). *Bars,* 200 and 50 μm, respectively. CD31-positive microvessels were counted from five replicate sections, and the microvessels per field were counted with ×400 magnification. **, significant difference when the number of CD31-positive microvessels was compared with that of the control ($P < 0.01$; *bottom*). *MVD,* microvessel density. *C, top,* tumor sections were analyzed by immunohistochemical staining using polyclonal antibodies against factor VIII. Magnification, ×100 (*top*) and ×400 (*bottom*). *Bottom,* quantitative analysis of factor VIII–positive microvessels as described above. **, significant difference when the number of factor VIII–positive microvessels was compared with that of the control ($P < 0.01$). *D,* DU145 cells without or with HDMECs were implanted onto the CAM. Tumor growth and angiogenesis were analyzed as above. *Top,* the weight of tumors from coimplantation of DU145 cell and HDMEC group was 45% more than that from implantation of DU145 cell alone group. The CD31-positive microvessels were not detected in DU145 alone group (*middle*), and number of factor VIII–positive microvessels (*bottom*) significantly increased when compared with the control group. **, significant difference when compared with that of the control group ($P < 0.01$).

transplantation, the sponges were soaked in 100% ethanol for 2 h, washed twice in 1× PBS buffer, and then soaked overnight in fresh 1× PBS buffer.

Transplantation of human endothelial cells and cancer cells to grow chimeric tumor. Fertilized chicken eggs were purchased from SPAFAS and incubated at 37°C with 70% humidity for 7 d. An artificial air sac was created over a region containing small blood vessels in the chicken chorioallantoic membrane (CAM), and a small window was cut in the shell over the artificial air sac as we described (18). HDMECs were infected with adenovirus carrying GFP, p70CA, p70KD, or HIF-1α siRNA at 20 multiplicities of infection (MOI) for 24 h. The HDMECs or the cells reconstituted with one of these signaling molecules (0.9×10^6) were mixed with 0.1×10^6 PC-3 cells and then mixed with 1:1 (v/v) growth factor–reduced Matrigel (BD Labware). The PC-3 cells alone (0.1×10^6) mixed with Matrigel were used as a control. HDMECs infected by adenovirus carrying a specific signaling molecule were compared with HDMECs infected by Ad-GFP. The cell mixture was seeded into the PLGA sponges as was described (22). The sponges were incubated at 37°C for 30 min and transplanted onto the freshly prepared CAM. The eggshell window was resealed after the transplantation. The chicken embryos were incubated at 37°C for 12 d to grow the tumor. The implants and tumors were retrieved from the CAM, photographed, and weighed. The tumors were fixed overnight with Bouin's solution.

Immunohistochemical analysis. Tumors from the CAM were fixed in Bouin's solution and embedded in paraffin. Tumor sections at 5 μm were cut and deparaffinized, and antigen was retrieved by microwave. After incubation with hydrogen peroxide, the sections were washed with 1× PBS thrice. The sections were blocked for 1 h with 10% goat serum in PBS and incubated with a 1:50 dilution of rabbit anti-factor VIII antibodies (BioGenex Laboratories) or 1:50 dilution of mouse anti-human CD31 antibodies (Dako) in a humid chamber at 4°C for 16 h. After washing thrice, the slides were incubated with goat anti-rabbit IgG or goat anti-rat IgG for 2 h. The antibody signals were detected using streptavidin-biotin-horseradish peroxidase complex (SABC) compound, 0.05% 3,3′-diaminobenzidine tetrahydrochloride, and 0.01% hydrogen peroxide Tris-HCl buffer containing 0.15 mol/L NaCl (0.05 mol/L TBS, pH 7.6). Different sections were prepared from five tumors, and the microvessels were counted in five different fields per section as follows: slides were first scanned under low power (×100) to determine three "hotspots" or areas with the maximum number of microvessels, and then the positive stained blood vessels in the selected areas were analyzed at ×400 magnification. Sections incubated with the preimmune IgG were used as the negative control (18, 23).

Immunoblotting. The total cellular protein extracts were prepared in radioimmunoprecipitation assay buffer and separated by 7% SDS-PAGE. Membranes were blocked with 5% nonfat dry milk for 2 h and incubated with primary antibodies of p70S6K1 (Santa Cruz Biotechnology), HIF-1α and HIF-1β (BD Biosciences), HIF-2α (Novus Biologicals), and β-actin (Sigma). Protein bands were detected by incubation with horseradish peroxidase–conjugated antibodies (Perkin-Elmer Life Sciences) and visualized with enhanced chemiluminescence reagent.

Statistical analysis. All values in this study were reported as mean ± SE. Student's unpaired *t* test was used for statistical analyses. The values were considered significantly different at $P < 0.05$.

Results and Discussion

New tumor model is established to study the effect of human endothelial cells in regulating tumor growth and angiogenesis. The interactions of cancer cells and endothelial cells are important for regulating tumor growth and angiogenesis. In addition to cancer cells, the microvascular endothelial cells recruited by the tumor are important for cancer development. A large amount of information has been accumulated about the role of signaling molecules in cancer cells in angiogenesis (24). However, little information is known about human endothelial cells in regulating tumor growth and angiogenesis due to the lack of appropriate tumor models. To determine the signaling molecules

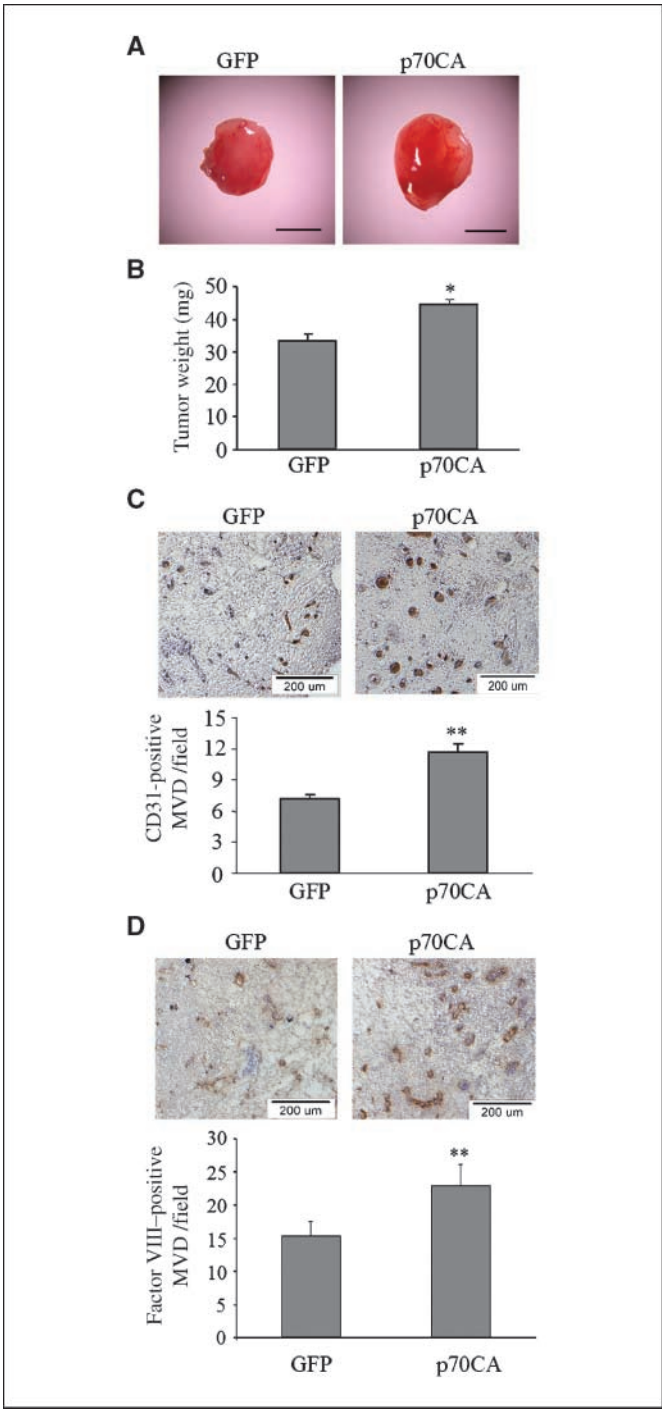

Figure 2. p70S6K1 expressed in HDMECs is sufficient for regulating angiogenesis and tumor growth. *A,* HDMECs were infected with adenovirus carrying GFP or p70CA at 20 MOI for 24 h. Then, cells were trypsinized, mixed with PC-3 cells, and carried by the PLGA sponges for the implantation onto the CAM as previously described. The tumors were harvested 12 d after the implantation, and representative tumors were photographed. *Bar,* 3 mm. *B, columns,* mean of net tumor weight from eight tumors; *bars,* SE. *C, top,* representative tumor sections stained by antibody against human CD31. Magnification, ×100. *Bar,* 200 μm. *Bottom,* CD31-positive microvessels were counted from five replicate sections, and the microvessels per field were counted with ×400 magnification. **, significant increase when the CD31-positive stained microvessel density was compared with the control ($P < 0.01$). *D, top,* representative tumor sections stained by antibody against factor VIII. *Bottom, columns,* mean of factor VIII–positive stained microvessel density from five tumor sections; *bars,* SE. **, significant increase when the factor VIII–positive stained microvessel density was compared with the control ($P < 0.01$).

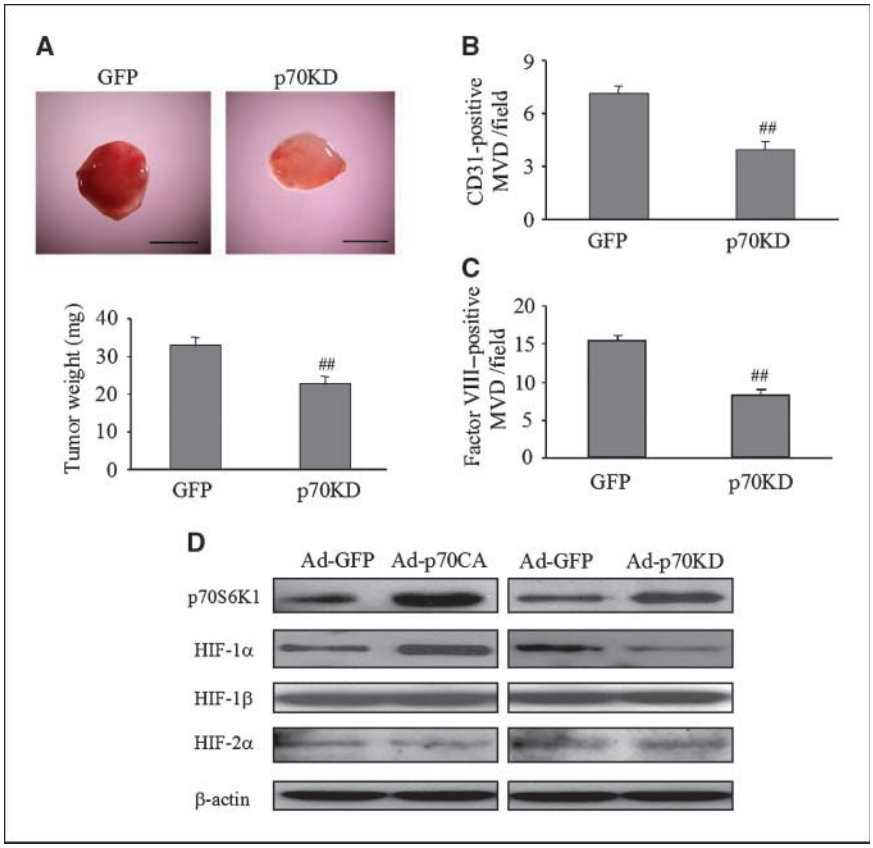

Figure 3. p70S6K1 expressed in HDMECs is required for regulating angiogenesis and tumor growth and HIF-1α is the downstream target of p70S6K1 in the endothelial cells. *A,* HDMECs were infected with adenovirus carrying GFP or p70KD at 20 MOI for 24 h. Then, cells were trypsinized, mixed with PC-3 cells, and carried by the PLGA sponges for the implantation onto the CAM as previously described. *Top,* the tumors were harvested 12 d after the implantation and representative tumors were photographed. *Bar,* 3 mm. *Bottom, columns,* mean of net tumor weight from eight tumors; *bars,* SE. *B,* CD31-positive microvessels were counted from five replicate sections, and the microvessels per field were counted with ×400 magnification. ##, significant decrease when the CD31-positive stained microvessel density of p70KD treatment was compared with that of the control ($P < 0.01$). *C,* factor VIII–positive microvessels were counted from five replicate sections, and the microvessels per field were counted with ×400 magnification. ##, significant decrease when the factor VIII–positive stained microvessel density of p70KD treatment was compared with that of the control ($P < 0.01$). *D,* HDMECs were treated as above for 48 h. The total proteins were extracted and subjected to immunoblotting using antibodies against total p70S6K1, HIF-1α, HIF-1β, HIF-2α, and β-actin.

expressed in human endothelial cells in regulating tumor growth, we established the condition by carrying human prostate cancer cells PC-3 and HDMECs with a highly porous biodegradable sponge and by implanting the cells onto the CAM. During the tumor formation, human cancer cells recruit human endothelial cells to differentiate into functional microvessels that fused with the host chicken microvessels. As shown in Fig. 1*A*, HDMECs increased the tumor size and weight >2-fold when compared with that induced by PC-3 cells alone. To test whether HDMECs were involved in tumor angiogenesis, the tumor sections were stained using monoclonal antibody against human CD31 that specifically recognized and stained human endothelial cells, but not chicken endothelial cells. The immunohistochemical analysis showed that there were a large number of CD31-positive microvessels from the tumor sections prepared from the treatment of PC-3 and HDMECs, indicating that human endothelial cells form the microvessels in the tumors (Fig. 1*B*). These CD31-positive microvessels contained blood cells filled in the lumens as indicated by the arrow, suggesting that the microvessels were functional in supporting the tumor growth. The tumor sections stained by the preimmune IgG and the tumors induced by PC-3 cells alone did not have any CD31-positive microvessels, suggesting that the CD31 antibody specifically recognized human endothelial cells in the experiment. During tumor growth, tumor cells also recruit host microvessels to support tumor development. To analyze the total microvessels in the tumor, tumor sections were stained by antibodies against factor VIII that are a specific marker for both human and chicken endothelial cells. The number of factor VIII–positive microvessels in the tumors induced by PC-3 and HDMECs was >2-fold higher than that in tumors induced by PC-3 cells alone (Fig. 1*C*). Tumor sections stained by the preimmune serum did not detect any positive signal, suggesting the specificity of factor VIII antibodies. Because PTEN is mutated in PC-3 cells, we also use DU145 prostate cancer cells with wild-type PTEN expression in the similar experiment. As shown in Fig. 1*D*, HDMEC cells also significantly induced DU145 cells to grow tumors to induce tumor angiogenesis with significant induction of CD31-positive and factor VIII–positive microvessels in the tumors (Fig. 1*D*). These results show that this new chimeric tumor model system using the CAM is suitable for studying the biological function of human endothelial cells in regulating tumor growth and angiogenesis.

p70S6K1 is an essential target for regulating tumor growth and angiogenesis. mTOR is activated in response to growth factors, which in turn activate p70S6K1 through phosphorylation (1). The activation of p70S6K1 is important to control translation rates and additional metabolic processes for cell growth (25–27). To study the role of p70S6K1 in regulating the function of endothelial cells in PC-3 cell–induced angiogenesis and tumor growth, HDMECs were infected with adenovirus carrying GFP or p70CA at 20 MOI, respectively. The cells were harvested 24 h after the infection and mixed with PC-3 cells to perform tumor assay. After infection by Ad-p70CA for 24 h, the infection rate of the cells was >80% (data not shown). Overexpression of p70CA by adenovirus in HDMECs increased tumor growth by 34% (Fig. 2*A* and *B*), showing that p70S6K1 activation was sufficient to induce tumor growth. To test whether overexpression of p70S6K1 can affect tumor angiogenesis, we showed that the number of CD31-positive microvessels from the tumor sections by the treatment of Ad-p70CA increased at 40% when compared with that of the control group, indicating that p70S6K1 activation enhanced microvessel formation derived from human endothelial cells (Fig. 2*C*). The total number of microvessels in the tumors in

p70CA treatment group increased to 150% of that of the GFP control (Fig. 2*D*), indicating that overexpression of p70CA in the cells is sufficient to induce tumor growth and angiogenesis. mTOR/p70S6K1 pathway in cancer cells also plays a critical role in tumor growth (2, 28). This study also indicates that mTOR/p70S6K1 inhibitors such as rapamycin analogues CCI-779 and RAD001 (29, 30) may be effective for inhibiting tumor angiogenesis.

p70S6K1 in HDMECs is required for regulating PC-3 cell–induced tumor growth and angiogenesis. To further determine whether p70S6K1 activation in HDMECs is required in regulating PC-3 cell–induced angiogenesis and tumor growth, HDMECs were infected with adenovirus carrying GFP or p70KD at 20 MOI, respectively. Inhibition of p70S6K1 by adenovirus carrying p70KD reduced tumor growth by 35% when compared with the Ad-GFP group (Fig. 3*A*), indicating that endothelial p70S6K1 activity is required for PC-3 cell–induced tumor growth. To test the effect of p70S6K1 inhibition on functional microvessel formation from human endothelial cells, we found that the CD31-positive microvessels decreased >44% with the treatment of Ad-p70KD (Fig. 3*B*). Similarly, the total microvessels from both human and chicken endothelial cells represented by factor VIII–positive staining cells reduced by 46%, indicating that p70S6K1 activation in HDMECs specifically reduced the formation of microvessels generated from the human endothelial cells (Fig. 3*C*). These results showed that functional p70S6K1 expression in the endothelial cells was required for tumor angiogenesis. Under normoxia condition, p70S6K1 activation regulated HIF-1α synthesis due to the global rate induction of cap-dependent translation, whereas rapamycin, the inhibitor of mTOR, inhibited HIF-1α synthesis (31). In addition, p70S6K1 regulated human double minute 2, which is important to regulate HIF-1α stability (32, 33). To test the potential downstream target of p70S6K1 in regulating tumor angiogenesis, we found that overexpression of active form of p70S6K1 in HDMECs increased HIF-1α expression, whereas inhibition of p70S6K1 decreased HIF-1α expression (Fig. 3*D*), suggesting that HIF-1α expression is regulated by p70S6K1 in human endothelial cells. The levels of HIF-1β were not affected by p70S6K1 activation in the cells (Fig. 3*D*). The expression of HIF-2α did not change in HDMEC cells with p70S6K1 overexpression or inhibition, indicating that HIF-1α, but not HIF-2α, was the key downstream target of p70S6K1 in HDMECs for regulating tumor angiogenesis.

HIF-1α expression in HDMECs is essential for regulating PC-3 cell–induced tumor growth and angiogenesis. HIF-1 is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits (34, 35). HIF-1α can be induced by hypoxia, growth factors, and oncogenes, whereas HIF-1β protein is constitutively expressed in human cells. It is well known that HIF-1α is one of the key regulators of vascular endothelial growth factor (VEGF). Our previous studies showed that p70S6K1 regulates VEGF expression in cancer cells during tumor growth and angiogenesis (9, 23). To determine whether the expression of HIF-1α in human endothelial cells is required for regulating PC-3 cell–induced tumor growth and angiogenesis, HDMECs were infected with adenovirus carrying GFP or siRNA against HIF-1α (siHIF1α) at 20 MOI, respectively. After 24 h, the infection rate was >80% in HDMECs (data not shown), and the expression of HIF-1α, but not HIF-2α, was strongly abolished in the cells after the infection for 48 h (Fig. 4*A*). Inhibition of HIF-1α expression in HDMECs by Ad-siHIF1α decreased the tumor growth by 42.5% when compared with that of Ad-GFP control (Fig. 4*B*). The number of CD31-positive microvessels from human endothelial cells decreased by >55% with the treatment of Ad-siHIF1α (Fig. 4*C*). The factor VIII–positive microvessels from both human and chicken vascular endothelial

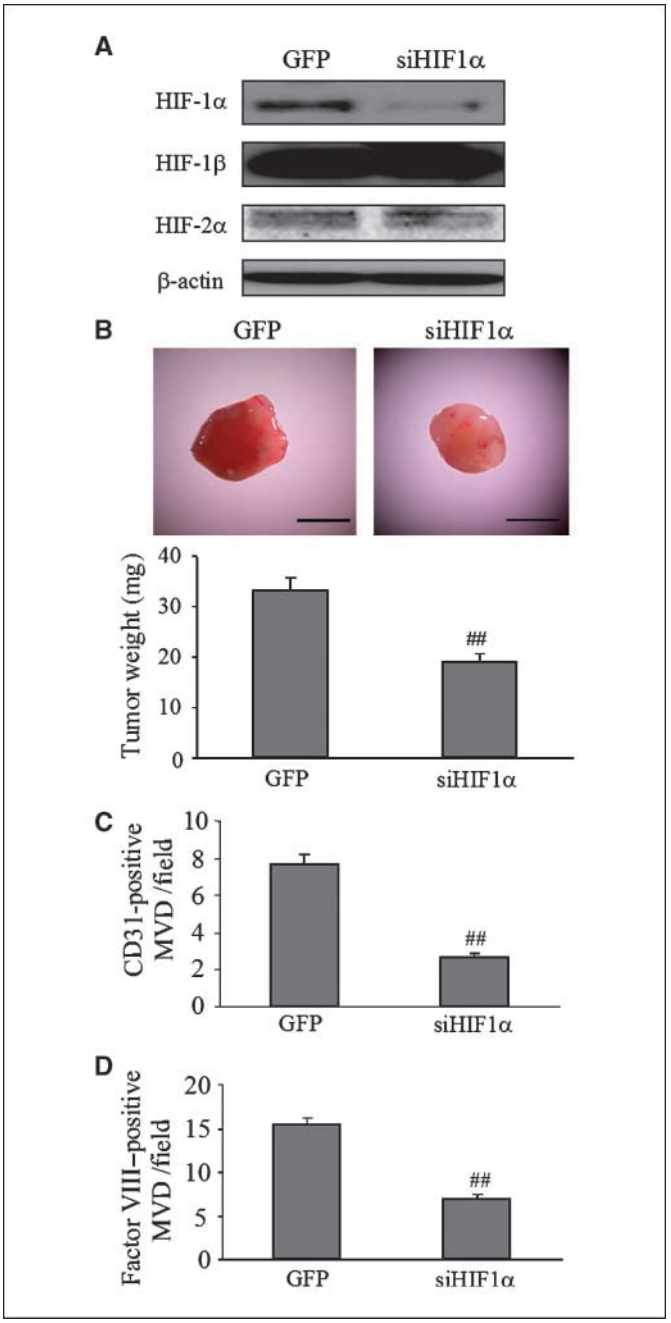

Figure 4. HIF-1α expressed in HDMECs is required for regulating angiogenesis and tumor growth. *A,* HDMECs were infected with adenovirus carrying GFP or siRNA against HIF-1α (siHIF1α) for 48 h. The expression of HIF-1α, HIF-1β, HIF-2α, and β-actin was determined by immunoblotting. *B,* HDMECs were infected with adenovirus carrying GFP or siHIF1α at 20 MOI for 24 h. Then, cells were trypsinized, mixed with PC-3 cells, and carried by the PLGA sponges for the implantation onto the CAM as previously described. *Top,* the tumors were harvested 12 d after the implantation and representative tumors were photographed. *Bar,* 3 mm. *Bottom, columns,* mean of net tumor weight from eight tumors; *bars,* SE. *C,* quantitative analysis of CD31-positive microvessels as described above. *D,* quantitative analysis of factor VIII–positive microvessels as described above. ##, significant difference when the tumor weight of Ad-siHIF1α treatment was compared with that of the control ($P < 0.01$).

cells were decreased by 50% with the treatment of Ad-siHIF1α (Fig. 4*D*). These results suggest that p70S6K1 regulates HIF-1α expression in endothelial cells, and the endothelial expression of HIF-1α is required for the cancer cell–induced tumor growth and angiogenesis.

In summary, we have developed a novel chimeric tumor model system using the CAM to determine the effects of p70S6K1 and its downstream signaling molecule HIF-1α in endothelial cells in regulating cancer cell–induced angiogenesis *in vivo*. p70S6K1 activation in endothelial cells is sufficient and required for PC-3 cells in inducing tumor growth and angiogenesis. HIF-1 is the essential downstream target of p70S6K1 for regulating tumor angiogenesis. This study shows a novel role of endothelial p70S6K1/HIF-1 signaling in tumor growth and angiogenesis and suggests that the interruption of p70S6K1/HIF-1 pathway in endothelial cells may be an effective antiangiogenic therapy for prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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