Adenovirus E4 Gene Promotes Selective Endothelial Cell Survival and Angiogenesis via Activation of the Vascular Endothelial-Cadherin/Akt Signaling Pathway*

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The early 4 region (E4) of the adenoviral vectors (AdE4+) prolongs human endothelial cell (EC) survival and alters the angiogenic response, although the mechanisms for the EC-specific, AdE4+-mediated effects remain unknown. We hypothesized that AdE4+ modulates EC survival through activation of the vascular endothelial (VE)-cadherin/Akt pathway. Here, we showed that AdE4+, but not AdE4- vectors, selectively stimulated phosphorylation of both Akt at Thr308 and Src kinase in ECs. The phosphatidylinositol 3-kinase (PI3K) inhibitors LY294002 and wortmannin abrogated AdE4+ induction of both phospho-Akt expression and prolonged EC survival. Regulation of phoso-Akt was found to be under the control of various factors, namely VE-cadherin activation, Src kinase, tyrosine kinase, extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK). Downstream targets of Akt signaling resulted in glycolgen synthase kinase-3α/β phosphorylation, β-catenin up-regulation, and caspase-3 suppression, all of which led to AdE4+-mediated EC survival. Furthermore, infection with AdE4+ vectors increased the angiogenic potential of ECs by promoting EC migration and capillary tube formation in Matrigel plugs. This selective AdE4+-mediated enhanced motility of ECs was also blocked by PI3K inhibitors. Taken together, these results suggest that activation of the VE-cadherin/Akt pathway is critical for AdE4+-mediated survival of ECs and angiogenic potential.

Adenoviral (Ad)1 vectors are efficient vectors for gene delivery in vitro and in vivo, especially for local gene delivery to the endothelial cells (EC) within the vascular system. ECs are important target cells for gene therapy because they are readily accessible to Ad vectors via the circulation and play a critical role in the progression of physiological and pathophysiological processes, including wound healing, apoptosis, inflammation, tissue revascularization, and tumor angiogenesis. We and other groups demonstrated previously that infection of ECs with first-generation AdE4+ vectors, but not AdE4- vectors, selectively modulates the angiogenic potential of ECs by altering apoptosis and the inflammatory status of ECs (1–4). Remarkably, infection with AdE4+ vectors resulted in the generation of a unique “suspended animation” state where the cells remain viable without requiring stimulation with serum or growth factors for several months. However, the mechanisms by which AdE4+ promotes long-term survival of ECs have yet to be clarified.

The phosphatidylinositol 3-kinase (PI3K)/Akt signal cascade is involved in the regulation of apoptosis, survival, and proliferation of a wide variety of cell types (5), including ECs (6). PI3K activates the Akt serine/threonine kinase by generating specific inositol phospholipids that recruit Akt to the cell membrane and enable its activation. PI3K/Akt signaling is activated by various growth factors, including VEGF-A, FGF-2, and platelet-derived growth factor (7, 8) as well as the vascular endothelial cadherin (VE-cadherin)/β-catenin complex (9). PI3K is activated by binding to phosphorylated receptors, and the resulting phosphoinositides recruit the kinases Akt and 3-phosphoinositide-dependent protein kinase-1 via pleckstrin homology domains (10).

VE-cadherin is a member of the cadherin superfamily that is specifically expressed at EC junctions (11). Its extracellular adhesive domain interacts via its cytoplasmic tail with the armadillo family proteins β-catenin, plakoglobin, and α-catenin (12), which couple the cadherin-catenin complex to the actin cytoskeleton. This complex is known to be involved in controlling endothelial survival and motility.

We hypothesized that the Akt signaling pathway, through activation of the VE-cadherin, plays an important role in AdE4+ promotion of EC survival and that the VE-cadherin/Akt signaling pathway can regulate the AdE4+ pro-survival effects on EC. Here, we demonstrate that AdE4+ significantly protected ECs against apoptosis through the recruitment of the VE-cadherin/Akt-signaling cascade. Moreover, this AdE4+ effect is associated with alterations of EC migration and vessel-like tube formation, suggesting that AdE4+-mediated activation...
tion of this pathway also regulates the angiogenic potential of ECs.

MATERIALS AND METHODS
Cell Culture—Human umbilical venous endothelial cells (HUVEC) and human umbilical smooth muscle cells were isolated as described (13) and cultured in growth factor-free medium and treated with either AdNull(E4+), AdLuc(E4+), or AdGFP(E4+) at an m.o.i. of 100 and compared with AdLacZ(E4-) and AdE4 ORF 6 at an m.o.i. of 100 or PBS (control). The number of HUVECs was quantified by phase-contrast microscopy for each indicated time point. Cell morphology was evaluated by phase-contrast microscopy. B, HUVEC monolayers were treated with or without 100 m.o.i. of AdNull(E4+) for the indicated time points. Cell morphology was evaluated by phase-contrast microscopy. C, the number of HUVECs infected with Ad vectors were quantified by β-gal staining of HUVECs after infection with 100 m.o.i. of either AdLacZ (E4-), AdLacz (E4+), or PBS (control) for 3 days.

FIG. 1. AdE4+ vectors prolong survival of HUVECs.

A, HUVEC monolayers were cultured in growth factor-free medium and treated with either AdNull(E4+), AdLuc(E4+), or AdGFP(E4+) at an m.o.i. of 100 and compared with AdLacZ(E4-) and AdE4 ORF 6 at an m.o.i. of 100 or PBS (control). The number of HUVECs was quantified by phase-contrast microscopy for each indicated time point. B, HUVEC monolayers were treated with or without 100 m.o.i. of AdNull(E4+) for the indicated time points. Cell morphology was evaluated by phase-contrast microscopy. C, the number of HUVECs infected with Ad vectors were quantified by β-gal staining of HUVECs after infection with 100 m.o.i. of either AdLacZ (E4-), AdLacz (E4+), or PBS (control) for 3 days.

primary human foreskin fibroblasts (HFF) were purchased from the American Type Culture Collection (ATCC, Manassas, VA); human pulmonary microvascular endothelial cells, human liver endothelial cells, and human vein microvascular endothelial cells were obtained from Clonetics. Cell viability was assayed by the trypan blue exclusion method, which indicated that <5% of the cells took up the dye both before and after the infection of Ad vectors.

Construction of Ad Vectors—The Ad vectors used in this study included the following: AdNull (E1-E2-E3-E4; no transgene in the expression cassette) (14); AdLacZ(E4); AdGFP (identical to E1-E2-E3-E4; but with a modified form of the Aequorea victoria green fluorescent protein (GFP) cDNA in place of β-gal); adenoviral luciferase (AdLuc) (E1-E2-E3-E4; cytomegalovirus promoter driving the luciferase gene); AdGFP (identical to AdLacZ, but with a complete deletion of the E4 gene by using the β-gal gene as a spacer in the E4 region); and AdE4 open reading frame (ORF) 6 (E1-E3 expresses only E4 ORF 6 from the E4 promoter, and all other E4 ORFs were deleted). A second generation AdE4 (E1-, E2b-, E3-, and E4+) vector was constructed as described previously (15). Ad vectors were amplified in 293 cells and purified by cesium chloride centrifugation and dialysis as described previously (16). All Ad vectors had a particle/plaque-forming unit ratio of ~100.

Western Blot Analysis—Cells were lysed in radioimmune precipitation buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, and 10 μg/ml aprotinin). Insoluble debris was pelleted,
and the protein concentration of the supernatant was determined with a DC protein assay kit (Bio-Rad). Fifty micrograms of each protein sample were separated on 10% SDS-PAGE gels. The protein samples were then transferred to nitrocellulose membrane. Protein expression was confirmed by immunoblotting with the following antibodies: Ser473-phospho-Akt, Tyr416-phospho-Akt, Akt, phospho-GSK-3β, and cleaved caspase-3 (Cell Signaling); Tyr416-phospho-Src (Upstate Cell Signaling); phospho-Akt, Tyr308-phospho-Akt, and total Akt antibody (Abcam). The cell lysates were then analyzed by immunoblot using the polyclonal anti-phospho-Src antibody (Santa Cruz Biotechnology); and VE-cadherin (Santa Cruz Biotechnology), or PBS (control) for the indicated time points. The cell lysates were then analyzed by immunoblot using the polyclonal anti-phospho-Ser-473 Akt/PKB antibody (pAkt), and total Akt antibody (Akt). C, non-EC cells lines, including the A549 cell line or the HFF cell line, were infected with AdE4+, AdE4−, or PBS (control) for 18 h. The cell lysates were then tested for caspase-3 activity. The data points represent the mean ± S.D. of three separate experiments. **, p < 0.01 compared with control; ##, p < 0.01 compared with AdE4+. C, AdE4+ prevented the A23187-induced apoptosis in HUVECs. Cells were infected with or without the 100 m.o.i. of AdE4+ for 2 h and then treated with or without A23187 (100 mM) or vehicle (control) in EC medium containing serum and EC growth factors. Following treatment for the indicated time points, the number of ECs was quantified by the trypan blue exclusion method.

Determination of the Caspase-3 Activity—The activity of caspase-3 in EC lysates (2 × 106 cells/sample) was determined using the caspase-3 colorimetric assay kit according to the manufacturer’s instructions (R&D Systems). 150 µg of protein were used per reaction and incubated for 2 h at 37 °C. The colorimetric reaction products were determined at 505 nm.

Migration Assays—Migration assays were performed by a modification of the procedure described previously using a Boyden chamber (17). HUVECs were infected with or without Ad vectors overnight in serum-free media (X-vivo medium). Transduced cells were suspended with 1% collagenase and washed with phosphate-buffered saline. Cells were resuspended in serum-free medium and put into the coated fibronectin chamber (3-µm pores size; BD Biosciences). VEGF-A (10 ng/ml) and FGF-2 (10 ng/ml) in 5% fetal bovine serum X-vivo medium were put into the lower chamber. The chamber was incubated for 22 h at 37 °C. The filter was carefully removed, and cells attached on the upper side were wiped off. HUVECs migrating through the filter and appearing on the lower side were fixed by careful immersion of the filter into 70% ethanol.
**Fig. 5. Effects of PI3K inhibitor, Src kinase inhibitor, tyrosine kinase inhibitor, and MAPK inhibitors on AdE4+–induced EC viability and Akt activation.** A, HUVEC monolayers were pre-incubated with 10 μM LY294002 (LY), 500 μM wortmannin, and 10 μM PP2 for 0.5 h and then infected with AdE4+ at 100 m.o.i. in serum- and growth factor-free medium for 48 h. The cell lysates were then analyzed by immunoblot using polyclonal anti-phospho-Ser473 Akt/PKB antibody (pAkt) and total Akt antibody (Akt). B, HUVECs were pre-incubated with 50 μM genistein for 0.5 h and then infected with AdNull(E4−) at 100 m.o.i. in serum- and growth factor-free medium for 48 h. The cell lysates were then analyzed by immunoblot using polyclonal anti-pAkt and total Akt antibody. C, the viability of confluent ECs cultured in growth factor-free medium was measured over the indicated time after infection with AdNull(E4−) at 100 m.o.i. with or without 50 μM genistein or 10 μM PP2 for 0.5 h. The cells were then infected with AdNull(E4−) at 100 m.o.i. in serum- and growth factor-free medium for 48 h, and the cell lysates were analyzed by immunoblot using polyclonal anti-phospho-Ser473 Akt/PKB antibody (pAkt) or polyclonal Akt/PKB (Akt) antibody. Quantitation of pAkt level after normalization to total Akt from Western analyses using a densitometer is also presented.

**RESULTS**

**AdE4+ Vectors Selectively Prolong Survival of ECs by Preventing Apoptosis**—Infection of HUVEC with E4+, but not E4−, adenoviral vectors supports survival of these cells in serum- and growth factor-free culture conditions. Remarkably, HUVEC monolayers infected with AdE4+ vectors show no decrease in cell number for at least 10 days (Fig. 1, A and B). In contrast, HUVEC monolayers infected with either control or E4-deficient Ad (AdE4−) vectors, including AdE4− LacZ and AdE4 ORF 6, do not survive in serum- or growth factor-free conditions by day 10. The infection of HUVEC with adenovirus vectors is highly efficient, and virtually all of the HUVEC express β-gal after infection with AdE4+ LacZ or AdE4 LacZ (Fig. 1C).

Second generation Ad vectors containing E4 with deletions of E1, E3, and, additionally, E2b genes are associated with decreased risk of adenovirus-derived gene expression and, thus, are less likely to induce immune responses or to be cleared via cell-mediated immune responses (15, 18). HUVECs infected with a second generation AdE4− vector survive at rates similar to those treated with first generation AdE4− (Fig. 2A).

Microvascular ECs from each organ have unique physiological attributes. Therefore, we investigated the effects of AdE4+ infection on survival in various organ-specific ECs, namely human pulmonary endothelial cells, human liver endothelial cells, and human vein endothelial cells. AdE4+ infection of these various ECs also resulted in survival effects similar to those observed in HUVECs under serum- and growth factor-free conditions (Fig. 2B).

To assess whether AdE4+ pro-survival effect is restricted to human ECs, we evaluated the effect of AdE4+ in non-ECs, including A549 (lung carcinoma), HL60 (leukemic cell lines), HeLa (cervical carcinoma), HFF cells, and human umbilical smooth muscle cells. Remarkably, in contrast to ECs, there were no alterations in the apoptotic or survival state of these other non-EC cell types after infection with AdE4− vectors (data not shown) as assessed by TUNEL assay or cell number. These findings indicate that the pro-survival effects of AdE4+ are primarily specific for EC type.

To examine the effect of AdE4+ on the apoptosis of ECs, both TUNEL assay and caspase-3 activity were employed to evaluate the extent to which the AdE4+ gene suppressed EC apoptosis. After 48 h of serum and growth factor starvation, HUVEC monolayers began to apoptose, as evidenced by the TUNEL assay (Fig. 3A). In contrast, AdE4+–treated HUVECs remained robust and showed no indication of apoptosis. Caspase-3 activation was also measured as an indicator of apoptosis induction, because different upstream pathways that lead to apoptosis depend on caspase-3 induction for final apoptotic execution. Fig. 3B shows that AdE4+, but not AdE4−, markedly suppressed caspase-3 activity in ECs, even under serum- and growth factor-free conditions. In addition, EC monolayers cultured in the presence of serum and growth factors were also treated with calcium ionophore A23187, which directly increases the concentration of intracellular Ca2+, and induces cell apoptosis. Remarkably, AdE4+ effectively blocked...
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**Fig. 6.** VE-cadherin and β-catenin play an important role in AdE4+-induced EC survival. A, AdE4+ increased VE-cadherin and β-catenin protein expression in HUVECs. HUVECs were infected with AdE4+, AdE4-+, or PBS (control) for 48 h, and cell extracts were analyzed by Western blotting with antibodies against VE-cadherin, β-catenin, and β-actin. B, effect of neutralizing monoclonal antibody to VE-cadherin on AdE4+-induced EC survival. Phase-contrast micrographs of representative monolayers of HUVECs that were infected with AdE4+ with or without VE-cadherin antibody (BV9 10 μg/ml) for 48 h are shown. C, the effect of neutralizing monoclonal antibodies to VE-cadherin on AdE4+-induced, phosphorylated Akt (pAkt). HUVECs were infected with AdE4+ with or without VE-cadherin Ab (BV9) for 48 h, and cell extracts were analyzed by Western blotting with antibodies against pAkt and Akt.

The onset of apoptosis induced by A23187 (Fig. 3C). These data suggest that AdE4+ infection can also protect ECs from Ca2+-influx-induced cell apoptosis.

**AdE4+ Inhibition of EC Apoptosis Is Mediated via Activation of the PI3K/Akt Pathway**—Because the protein kinase Akt is an important survival factor that suppresses EC apoptosis (7), we examined whether AdE4+ regulates the PI3K/Akt signaling cascade. Treatment of EC cultures with two selective and structurally unrelated PI3K inhibitors, LY294002 (10 μM) or wortmannin (500 nM), abolished the ability of AdE4+ to rescue EC from apoptosis following growth factor withdrawal (Fig. 4A). LY294002 and wortmannin also reverted the inhibition of caspase-3 activation by AdE4+ (data not shown). Because Akt activation is a critical downstream effector of PI3K, we initially tested whether AdE4+ infection of EC is associated with Akt phosphorylation. Akt phosphorylation was assessed by immunoblot analysis in EC using a phospho-specific anti-Akt antibody. Immunoblot analysis of EC infected with AdE4+ vector in serum- and growth factor-free conditions revealed significant up-regulation of the expression of Akt phosphorylated at Ser473 at a maximum of 48 h by AdE4+, but not AdE4- infection, and remained constant until at least 72 h (Fig. 4B). Total Akt levels remained unchanged, and Akt phosphorylated at Thr308 expression was not detectable (data not shown). In addition, AdE4+ induced phosphorylation of Src kinase, which is an upstream activator of PI3K (Fig. 4B). However, AdE4+ did not significantly alter the expression of phospho-Akt in non-EC lines, including the A549 cell line or primary HFF cells (Fig. 4C). These data suggest that AdE4+ promotes survival specifically in EC via the Akt phosphorylation pathway.

**AdE4+ Directly Induces Akt Phosphorylation via Tyrosine Kinase, ERK, and JNK Pathways**—To determine whether AdE4+ activates signals leading to phosphorylation of Akt or inhibits the dephosphorylation of Akt, HUVECs were infected with AdE4+ in the presence of PI3K inhibitors LY294002, wortmannin, or Src kinase inhibitor PP2. As shown in Fig. 5A, AdE4+-mediated induction of phospho-Akt expression was blocked by LY294002 or wortmannin. The Src kinase inhibitor PP2, but not the inactive tyrosine kinase inhibitor P3 (data not shown), also partly suppressed AdE4+-mediated induction of phospho-Akt expression (Fig. 5A); however, PP2 did not significantly alter the AdE4+-mediated survival of EC (Fig. 5C). These data suggest that the AdE4+ effect is mediated by phosphorylation of Akt in ECs.

Both receptor tyrosine kinase and nonreceptor tyrosine kinase are involved upstream of PI3K/Akt (19, 20). To dissect the pathway mediating phospho-Akt activation, we examined the effects of genistein, a widely used inhibitor of tyrosine kinase activity, on AdE4+-promoted EC survival. Fig. 5C shows that survival of EC by AdE4+ was significantly inhibited by treatment with 50 μM genistein. Also, pretreatment with genistein abolished AdE4+-induced phospho-Akt expression (Fig. 5B).

As MAPKs have been implicated in the regulation of phosphorylation of Akt, we also assessed the role of MAPKs in the regulation of phospho-Akt in EC after AdE4+ infection. As shown in Fig. 5D, AdE4+-induced activation of phospho-Akt was inhibited by pretreatment with PD98059 (a selective inhibitor of ERK) or SP600125 (a selective inhibitor of JNK), but not by SB203580 (a selective inhibitor of p38-MAPK). These data indicate that ERK and JNK are the upstream activators of PI3K in the signaling pathways activated by AdE4+.

**Selective Activation of EC-specific VE-cadherin by AdE4+ Vectors**—VE-cadherin and the intracellular β-catenin binding region of VE-cadherin have been shown to play an important role in Akt activation and EC survival (9). To assess the effect of AdE4+ on VE-cadherin and β-catenin levels, EC lysates from control and AdE4+ and AdE4- cell cultures were analyzed by Western blot using specific anti-VE-cadherin and anti-β-catenin antibodies. As shown in Fig. 6A, VE-cadherin and β-catenin protein levels were increased after infection with AdE4+. Because the anti-VE-cadherin antibody can directly inhibit VE-cadherin function in ECs (21), we then used neutralizing monoclonal antibody (BV9) to VE-cadherin in cell cultures infected with AdE4+. BV9 significantly suppressed AdE4+-mediated survival of ECs (Fig. 6B) and blocked AdE4+-induced phospho-Akt in ECs (Fig. 6C). These data suggest that VE-cadherin and β-catenin participate as activators of the PI3K signaling pathway in AdE4+-mediated survival of ECs. As VE-cadherin is only expressed on the ECs but not on the other cell types, this may explain why AdE4+-mediated induction of EC survival is restricted to ECs. It is conceivable that AdE4+ gene products specifically recruit VE-cadherin.
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**AdE4** Increased GSK3α/β Phosphorylation and Decreased Caspase-3 Activation—GSK3α/β and caspase-3 are downstream targets of Akt signaling; both phosphorylation of GSK3α/β and suppression of activation of caspase-3 block the pathways leading to EC apoptosis. As shown by Western blot analysis in Fig. 7, AdE4*, but not AdE4*, stimulated GSK3α/β phosphorylation in a time-dependent manner with maximal expression occurring within 48 h and sustained phosphorylation lasting for at least 4 days. At the same time, activation of caspase-3 was suppressed by AdE4*, suggesting that phosphorylation of GSK3α/β and reduction of caspase-3 activity are involved in the AdE4* survival effect.

**Fig. 7.** Time course of AdE4* and AdE4* effects on GSK3α/β phosphorylation and caspase-3 in EC. ECs were infected with AdE4*, AdE4*, or PBS (control) for indicated times (d, day). The cell lysates were then analyzed by immunoblot using anti-phospho-GSK-3α/β, anti-caspase-3, and anti-β-actin.

**AdE4** Promotes EC Migration and Tube Formation in ECs—Migration and tube formation are important angiogenic functions of ECs essential for the assembly of functional neo-vessels. To examine whether AdE4* also affects the angiogenic potential of EC, the migration of AdE4*-infected ECs was assessed in a modified Boyden chamber. AdE4*, but not AdE4*, increased EC migration in response to VEGF-A by 3-fold, whereas AdE4* infection resulted in a statistically insignificant rate of migration (Fig. 8). This response was blocked by prior administration of the PI3K inhibitor LY294002 (10 μM).

A Matrigel tube formation assay was also employed to test the effect of AdE4* on the angiogenic potential of ECs. Infection of AdE4* vectors, but not AdE4* vectors, induced the assembly of typical sprouting and tube-like structures that are reminiscent of vessels typically formed in vivo (Fig. 8B). This angiogenic effect is similar to that of VEGF-A treatment, which induces EC tube formation in Matrigel. These results indicate that AdE4*-infected ECs maintain their angiogenic potential in the absence of growth factors. Therefore, the overall effect of AdE4* gene products is not only to increase survival but also to maintain the pro-angiogenic properties of ECs.

**DISCUSSION**

Dissecting the mechanism by which E4 adenoviral vectors modulate angiogenesis is important to diminish the toxicities associated with adenoviral gene therapy. The importance of VE-cadherin/Akt signaling in the selective modulation of EC survival (9) offers a new perspective for discerning the mechanism whereby E4 gene products specifically support EC survival. In the present study, our results provide further evidence that AdE4* vectors selectively protect ECs from apoptosis and maintain their angiogenic potential through recruitment of the VE-cadherin/Akt signaling pathway.

Our data show that protein tyrosine kinases, such as Src family kinases, are involved in AdE4* -mediated PI3K/Akt activation and EC survival. AdE4* activation of Src or other receptor tyrosine kinase ligands is the key mediator of activation of Akt. Src kinase has been implicated in the control of cell division, the production of autocrine growth factors, and the cell’s survival response (22). The inhibitory effect of the Src inhibitor PP2 and tyrosine kinase inhibitor genistein suggests that the Src family or some other tyrosine kinase is a crucial upstream activator of the PI3K/Akt pathway that is activated by the AdE4* vectors.

AdE4* -induced Akt phosphorylation promotes survival of ECs by modulating downstream targets of this kinase. These include caspase-3, GSK3α/β, Bad, and Bcl2. GSK3α/β is a serine-threonine kinase that is involved in multiple cellular signaling pathways. It is inhibited upon Ser9 phosphorylation by growth factor receptor-induced activation of Akt and has been shown to participate in apoptosis in several cell types and induce caspase-3 activation (23). Increased phosphorylation of GSK3α/β inhibits activity of GSK3α/β, whereas lithium chloride, a GSK3α/β inhibitor, induced GSK3α/β N-terminal phosphorylation through direct inhibition of GSK3α/β (24). AdE4* -induced GSK3α/β phosphorylation may thus regulate β-catenin accumulation and protect against EC apoptosis (25). Moreover, it has been demonstrated that angiogenesis is inhib-
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Ad vectors contain at least six ORFs that confer a variety of regulatory functions. The precise identity of E4 ORF proteins that induce activation of Akt/VE-cadherin is not known and is the subject of ongoing studies.

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