The Akt-regulated Forkhead Transcription Factor FOXO3a Controls Endothelial Cell Viability through Modulation of the Caspase-8 Inhibitor FLIP*

Carsten Skurk‡, Henrike Maatz‡, Hyo-Soo Kim§, Jiang Yang‡, Md Ruhul Abid¶, William C. Aird§, and Kenneth Walsh‡

From the ‡Department of Internal Medicine, Seoul National University Hospital, 28 Yongon-dong, Chongna-gu, Seoul 110-744, Korea, and the §Department of Medicine, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215.

FLICE-inhibitory protein (FLIP) is a homolog of caspase-8 that lacks catalytic activity and has been shown to be important in protecting endothelial cells from apoptosis. The serine/threonine kinase Akt/PI3K was recently reported to promote FLIP expression in endothelial and tumor cells. Here we examined the role of the forkhead transcription factor FOXO3a, a downstream target of Akt, in controlling FLIP regulation in endothelial cells. FOXO3a nuclear translocation was regulated by Akt in human umbilical vein endothelial cells. Transduction of a nonphosphorylatable, constitutively active mutant of FOXO3a (TM-FOXO3a) led to the down-regulation of FLIP levels. Transduction with TM-FOXO3a also increased caspase-8 activity and promoted apoptosis in endothelial cells. Conversely, transduction of a dominant-negative mutant of FOXO3a up-regulated FLIP levels and protected endothelial cells from apoptosis under serum deprivation conditions. Restoration of intracellular FLIP blocked caspase-8 activation and inhibited apoptosis in TM-FOXO3a-transduced cells. These data suggest that FOXO3a is a downstream target of Akt in endothelial cells that can promote apoptosis via FLIP down-regulation and activation of the extrinsic apoptotic pathway.

Apoptotic cell death of vascular cells is a prominent feature of blood vessel remodeling in normal development and fibroproliferative disorders (1). Fas ligand (FasL) is a type II mem-

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
tance of FLIP regulation by Akt signaling in angiogenesis and tumorigenesis, nothing is known about the downstream effectors of Akt signaling that control FLIP expression.

FOXO3a, also referred to as FKHRL-1, is a recently discovered member of the family of forkhead transcription factors that are phosphorylated by Akt (17, 18). Phosphorylation leads to cytoplasmic retention and impairment of FOXO3a nuclear transcriptional activity. FOXO3a has been shown to induce apoptosis in neuronal cell lines and fibroblasts by up-regulation of Fas ligand expression and activation of the death receptor pathway (17). However, Dijkers et al. (19) recently showed that the “intrinsic” mitochondrial pathway, rather than the “extrinsic” Fas-mediated pathway, can promote apoptotic cell death in hematopoietic cells following activation of FOXO3a signaling.

The role of forkhead transcription factors in endothelial cell biology has not been described previously. Thus, an understanding of how this pathway regulates endothelial cell viability may provide novel insights into the maintenance of endothelial cell integrity during vascular disease. In this study, we investigated the role of FOXO3a in Akt/PKB-mediated FLIP regulation and apoptosis in endothelial cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—Human umbilical vein endothelial cells (HUVEC, Cambrex) passage 3–6 were used in this study and were cultured in endothelial growth medium (EGM, Cambrex) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin for experiments using serum stimulation or EBM + 1% penicillin/streptomycin with 0% FBS for serum starvation. Typically, HUVEC were grown to subconfluence in 1.5% gelatin-coated 10-cm dishes, 6-well plates, or...

---

**Fig. 1. Growth factor stimulation and Akt signaling determines cellular distribution of FOXO3a in endothelial cells.** HUVEC were cultured on Lab-Tek chamber slides in EGM with 10% FBS (serum stimulation) (A) or EBM (serum deprivation) (B). Some cultures were transduced with Ad-myr-Akt 40 m.o.i. (C) or Ad-dnAkt 40 m.o.i. (D) in serum-enriched media. After fixation, immunofluorescence staining for endogenous FOXO3a was performed (primary antibody anti-FKHRL-1, and secondary antibody Alexa®-Fluor 588). Upper left panels depict phase contrast; lower left panels show nuclear staining (DAPI); upper middle panels indicate FOXO3a staining (rhodamine); and lower middle left panels illustrate merged DAPI and FOXO3a images. Upper and lower right panels depict higher magnification images of cells from FOXO3a-stained or FOXO3a- and DAPI-merged images from the middle panels that are indicated by the white arrows. E, quantification of immunofluorescence staining patterns for endogenous FOXO3. Data are expressed as mean ± S.E. for four separate experiments (n = 100 cells per experiment).
slide chambers. Infection with the adenoviral vectors was carried out overnight. For Western blot analysis cells were harvested at 16 h post-infection. To examine the role of FOXO3a signaling on apoptosis, cells were infected with the indicated vectors overnight; medium was changed, and HUVEC were harvested 48 h post-infection. For anoikis studies, cell culture plates were coated with 30 mg/ml Poly-HEMA® (poly-2-hydroxyethylmethacrylate, Sigma), dried overnight, and rinsed twice with PBS before use. Cells were grown to subconfluency, detached with 0.05% trypsin, and incubated on Poly-HEMA-coated dishes for the times indicated.

**Adenoviral Constructs**—The hemagglutinin-tagged human FOXO3a triple mutant and wild-type sequence cDNA (from Michael E. Greenberg, Harvard Medical School) was subcloned into a shuttle vector pAdTrack-CMV, which contains green fluorescent protein (GFP) under the control of a separate cytomegalovirus (CMV) promoter (from Bert Vogelstein, The Johns Hopkins Oncology Center) (Fig. 3A). The FOXO3a-AAA triple mutant (TM-FOXO3a) is not phosphorylatable because three phosphorylation sites, Thr-32, Ser-253, and Ser-315, were replaced by alanine residues (17). The dominant-negative form (dn-FOXO3a) was constructed by deletion of the transactivation domain from the C terminus. The shuttle vector containing the FOXO3a cDNA was linearized and co-transformed into Escherichia coli with the adenoviral backbone plasmid pAdEasy-1. The resultant recombinant adenoviral DNA with FOXO3a cDNA was transfected into a packaging cell line (293 cells) to produce the recombinant adenoviral vectors. For experiments with these reagents, control cultures were infected with an adenoviral vector expressing only the GFP transgene (Ad-GFP) prepared by the same system (20). The Ad-Tet-FLIP, Ad-Tet-LacZ encoding /H9252-galactosidase, and Ad-rtTA vectors have been described previously (4). The adenoviral vector Ad-CMV-rtTA encodes a chimeric transcription factor composed of a mutant tetracycline repressor fused to a VP16 trans-activator under control of the CMV promoter/enhancer. Ad-TET-FLIP encodes for a FLAG-tagged protein of FLIP downstream of seven consecutive tetracycline-responsive elements. Replication-defective adenovirus vectors expressing dominant-negative and constitutively active forms of murine Akt tagged with the hemagglutinin epitope were constructed as described previously (21). The dominant-negative Akt mutant (Ad-dnAkt) has alanine residues substituted for threonine at...
position 308 and serine at position 473. The constitutively active Akt (Ad-myAkt) has the c-Src myristoylation sequence fused in-frame to the N terminus of the wild-type Akt coding sequence that targets the fusion protein to the membrane. Like the Akt vectors, adenoviral-β-galactosidase expresses the lacZ gene from the CMV promoter (22). The adenovirus expressing CrmA (Ad-CrmA) was generated by homologous recombination in human embryonic 293 kidney cells from the 1.4-kbp CrmA cDNA. The resulting shuttle plasmid was rescued into recombinant adenovirus as described previously (22). All viral constructs were purified by CsCl ultracentrifugation. Titration was performed by means of the plaque assay.

**Immunofluorescence Staining**—HUVEC were cultured in Lab-Tek® cover glass chambers (Nalge Nunc International) using endothelial basal medium (EBM) for serum starvation or EGM + 10% FBS for serum stimulation and transduced with the adenoviral vectors as indicated. Following a 12–16-h incubation period, cells were washed three times with warm PBS and fixed with 3.7% paraformaldehyde for 20 min at room temperature. After another washing step with PBS, cells were permeabilized using 0.2% Triton X-100 in PBS for 20 min at room temperature. Blocking was carried out using PBS solution containing 10% fetal calf serum, 0.1N NaN₃, and 0.1% Triton X-100. First antibody temperature. Blocking was carried out using PBS solution containing permeabilized using 0.2% Triton X-100 in PBS for 20 min at room temperature. After another washing step with PBS, cells were washed with PBS twice before incubating in lysis buffer consisting of 50 mM Tris-HCl, pH 7.2, 2 mM EDTA, 1% Nonidet P-40, 0.05% SDS, and 250 mM NaCl, 10 mM β-glycerol phosphate, 1 mM vanadate. One tablet of protease inhibitor mixture (Complete Mini, Roche Applied Science) was added just prior to use. After scraping, whole cell lysates were put on ice for 30 min, and the protein concentration was determined by using the BCA protein assay kit (Pierce). 20–50 μg of protein was fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membrane was blocked using 0.05% T-PBS (1× PBS and 0.05% Tween 20) containing 3% dry milk for 30 min and incubated with the primary antibody overnight at 4 °C. Following a wash step in 0.3% T-PBS containing 1.5% dry milk, the membrane was blocked with 3% bovine serum albumin. The second antibody was applied for 1 h at room temperature. After washing the membrane in 0.05% T-PBS for 1 h, the immune complexes were detected by chemiluminescent detection reagents ECL or ECL-Plus (Amersham Biosciences). To probe the membrane, Restore Western blot Stripping Buffer (Pierce) was used. Primary antibodies used in this study are as follows: anti-FKHRL-1 (rabbit polyclonal IgG, Upstate) 1:1000; anti-phospho-FKHRL-1 (Ser-253) (rabbit polyclonal IgG, Upstate) 1:500; anti-phospho-FKHRL-1 (Thr-32) (rabbit polyclonal IgG, Upstate) 1:500; anti-human-FLIP (NF6, M. Peter, Chicago) 1:20; and anti-FA (Roche Applied Science) 1:4000. As secondary antibodies, antimouse IgG HRP (horseradish peroxidase) (Promega) or anti-rabbit IgG HRP (Promega) was used at a dilution of 1:2000. Immunoblots were quantified using NIH-Image® software.

**RNase Protection Assay**—Changes in RNA expression of FLIP were determined using the RiboQuant™ MultiProbe RNase Protection Assay System according to the instructions of the manufacturer (Pharmingen). HUVEC were grown in 10-cm dishes to subconfluency and infected with the viral vectors at m.o.i. 40. After infection for 16 h, RNA was extracted using the Ultraspec™ RNA isolation system (Biotec). Cells were homogenized with 1 ml of Ultraspec RNA reagent, and RNA was extracted by adding 0.2 ml of chloroform. Following centrifugation at 4 °C and 12,000 × g for 15 min, the aqueous phase was transferred to another tube, and precipitation was carried out with isopropanol (1:1, v/v). After centrifugation for 10 min at 12,000 × g at 4 °C isolated RNA was washed with 75% ethanol. Total RNA was then quantified, and equal amounts were further processed in the protection assay. The probe synthesis was carried out at 37 °C for 1 h by using the RNase protection assay template set (hAPO-3b) and [α-32P]UTP for labeling the product. The reaction was terminated with 2 μl of DNase, and the radioactive RNA probes were
isolated. Hybridization was carried out for 12–16 h at 56°C by mixing 20 μg of target RNA dissolved in 8 μl of hybridization buffer with 2 μl of RNA probes. Following RNA and protein digestion, hybridized target RNA was isolated, run by electrophoresis on a 5% acrylamide gel, and visualized with autoradiography films.

Cell Viability Assays—Cell viability was determined by the MTT assay (Roche Applied Science) as suggested by the manufacturer. HUVEC were then seeded in 96-well plates and infected with the indicated adenoviral vectors. At 24 and 48 h post-infection, MTT solution (tetrazolium salt, final concentration 0.5 mg/ml) was added for 4 h at 37°C. Formazan salt crystals were then solubilized by adding the solubilization solution and incubated overnight in a humidified atmosphere (e.g. 37°C, 5% CO₂). The solubilized formazan product was spectrophotometrically quantified at a wavelength of 550 nm on a microplate reader.

In Vitro Caspase-8 Activity Assay—Caspase-8 activity was measured by using the caspase-8 activity colorimetric assay kit (R & D Systems) according to the directions of the manufacturer. HUVEC were grown in 10-cm dishes and at the time of subconfluence infected with the adeno-
viral vectors overnight. 24 h after infection cells were collected by trypsinization and centrifuged by 200 g for 10 min. 50 l of lysis buffer was added to the cell pellet. Protein concentration was determined using the BCA protein assay kit (Roche Applied Science). Whole cell lysates of \(1 \times 10^6\) cells were incubated with 5 mmol/liter dithiothreitol and the caspase-8 substrate IETD-p-nitroanilide in reaction buffer for 2 h at 37 °C in 5% CO2. Cleavage of the substrate was determined by an increase in absorbance at 405 nm using a spectrophotometer. At least five independent experiments were performed in each group. Controls consisted of non-induced cells, i.e. adenoviral infection with GFP as well as omitting either substrate or cell lysate. Results were expressed as fold increase in caspase activity compared with the GFP group.

**RESULTS**

**FOXO3a Expression and Regulation in HUVEC**

FOXO3a expression was detected in HUVEC cultures by immunofluorescence staining (Fig. 1). Under conditions of serum-stimulation, FOXO3a was predominantly detected in the cytoplasm (Fig. 1A). In contrast, serum starvation for 24 h led to a predominantly nuclear staining pattern (Fig. 1B). These results illustrate that FOXO3a is present in endothelial cells and that this transcription factor is regulated by growth factor signaling.

**Statistical Analysis**—All data were compared by Student’s t test using Stat View 4.5 (Abacus software). Data are expressed as mean ± S.E. for the number of independent experiments indicated. A p < 0.05 was considered to be significant.
negative Akt (Ad-dnAkt) or constitutively active Akt (Ad-myrAkt). Transduction at 40 m.o.i. led to greater than a 99% transduction efficiency (data not shown). Expression of constitutively active Akt promoted the cytoplasmic localization of FOXO3a that appeared more pronounced than in the serum-stimulated cultures (Fig. 1C). In contrast, transduction with dominant-negative Akt strongly promoted FOXO3a localization in the nucleus (Fig. 1D). In quantitative analyses of these cultures, 5.7 ± 1.5% of cells scored for nuclear FOXO3a localization following transduction with Ad-myrAkt, and 99.0 ± 1.0% of cells scored for nuclear localization following transduction with Ad-dnAkt (Fig. 1E). Collectively, these data in HUVEC are consistent with mitogen-mediated FOXO3a regulation that has been reported in neuronal cells and fibroblasts, and they are in accordance with the proposed mechanism of Akt-mediated FOXO3a phosphorylation (17).

Anoikis, cell death that results from a lack of proper cell-matrix attachments (24), is an important regulator of endothelial cell viability during an angiogenic response (25). The disruption of adhesive interactions between endothelial cells and the extracellular matrix leads to an inactivation of Akt (13), down-regulation of FLIP (26), and activation of caspase-8 (27). Therefore, FOXO3a translocation was assessed in HUVEC under conditions that promote anoikis. Rapid FOXO3a translocation to the nucleus could be detected in suspension culture (Fig. 2A). Quantitative analyses of these cultures revealed that FOXO3a translocation was time-dependent, with 36.2 ± 3.5% of cells displaying nuclear FOXO3a by 3 h and 80.5% by 12 h (Fig. 2B).

To study the function of FOXO3a in HUVEC, replication-defective adenoviral vectors were constructed that express the wild-type human FOXO3a (Ad-WT-FOXO3a) or a mutant form that has alanine residues substituted for serine residues at positions 253 and 315 and the threonine residue at position 32 (Fig. 3A). The vector expressing the triple mutant form is referred to as Ad-TM-FOXO3a, and it is constitutively active because it cannot be inactivated by phosphorylation (4, 17). A third vector expresses the portion of the FOXO3a cDNA that encodes the DNA binding and regulatory phosphorylation domains but lacks the trans-activation domain. The vector expressing the truncated cDNA is referred to as dn-FOXO3a, and it functions in a dominant-negative manner because it can bind to DNA but cannot activate transcription. HUVEC cultures were transduced with Ad-WT-FOXO3a, Ad-TM-FOXO3a, or Ad-dn-FOXO3a and stimulated with serum. All vectors expressed FOXO3a protein, but the phosphorylation of regulatory residues was only detected in cultures transduced with wild-type or the truncated FOXO3a by Western blot analysis (Fig. 3B).

Immunofluorescence staining of the transgene products was performed to validate the FOXO3a-expressing adenoviral vectors. HUVEC transduced with Ad-WT-FOXO3a and cultured in
the presence of high serum (10% FBS) showed a predominantly cytoplasmic pattern of transgene-encoded FOXO3a expression (Fig. 4A). For these experiments, immunofluorescence conditions were such that only the virally encoded transgene product was detected with little or no signal from endogenous FOXO3a. Co-transduction of Ad-WT-FOXO3a and Ad-myrAkt led to complete exclusion of FOXO3a from the nucleus (Fig. 4, B and E), whereas co-transfection of Ad-WT-FOXO3a and Ad-dnAkt led to a predominantly nuclear staining pattern of FOXO3a (Fig. 4, C and E). In contrast, an almost exclusively nuclear staining pattern was observed with the TM-FOXO3a-expressing adenovirus, which was independent of serum concentration (Fig. 4D and data not shown). Furthermore, the nuclear localization of TM-FOXO3a was not influenced by transduction with Ad-dnAkt nor Ad-myrAkt (Fig. 4E).

FOXO3a Regulates FLIP Expression—Akt has been shown to up-regulate FLIP expression, contributing to cellular survival (11). To investigate the role of FOXO3a in FLIP regulation, subconfluent HUVEC were transduced with increasing concentrations of the adenoviral FOXO3a vectors. Following Ad-TM-FOXO3a gene transfer, significant decreases in FLIP protein levels were observed compared with GFP-transduced cells (Fig. 5A). The decrease in FLIP expression was dose-dependent with statistically significant reductions occurring with as little as 10 m.o.i. of the Ad-TM-FOXO3a vector (Fig. 5B). FLIP levels are reduced in HUVECs subjected to serum deprivation (see Ref. 11 and data not shown). Under these conditions of cellular stress, transduction with dominant-negative FOXO3a led to a dose-dependent increase in FLIP expression (Fig. 5, A and B). The expressions of both long and short forms of FLIP were affected by these manipulations.

Additional experiments were performed with the adenoviral vector encoding wild-type FOXO3a. Serum-stimulated HUVEC transduced with Ad-WT-FOXO3a, which leads to the cytoplasmic localization of the transgene-encoded protein (Fig. 4A), had no detectable effect on FLIP expression at any dose analyzed (Fig. 5, C and D). Thus, the down-regulation of FLIP was completely dependent upon the FOXO3a residues that are phosphorylated by Akt. However, in serum-deprived cultures, transduction of wild-type FOXO3a led to a modest but statistically significant decrease in FLIP expression that was dependent on the dose of the WT-FOXO3a vector. Under conditions of serum deprivation, both endogenous (Fig. 1) and transgene-encoded (data not shown) FOXO3a translocate to the nucleus. Although wild-type FOXO3a increased apoptosis under these conditions, further studies primarily used the TM-FOXO3a mutant in serum-stimulated cultures because this combination provided a much higher signal-to-background ratio.

To test whether FOXO3a participates in the Akt-FLIP signaling axis, subconfluent HUVEC were transduced with adenoviral vectors encoding constitutively active Akt (myrAkt) in the presence or absence of TM-FOXO3a (Fig. 6). Alternately, cells were transduced with adenoviral vectors expressing dnAkt in the presence or absence of dn-FOXO3a. Consistent with previous findings (11), MyrAkt promoted FLIP expression, and this effect was most pronounced in low mitogen media. Conversely, dnAkt repressed FLIP expression. TM-FOXO3a down-regulated FLIP in the MyrAkt-transduced cultures, and dn-FOXO3a reversed the down-regulation of FLIP by dnAkt. Weaker signals for FLIPs were also noted in some of these Western immunoblots (Fig. 6).

Finally, RNase protection assays were employed to assess whether the FOXO3a-induced down-regulation of FLIP occurred, at least in part, at the level of mRNA (Fig. 7A). Serum-stimulated HUVEC cultures were transduced with adenoviral vectors expressing GFP, WT-FOXO3a, or TM-FOXO3a. Under these culture conditions, only TM-FOXO3a significantly reduced the level of FLIP mRNA expression (Fig. 7B). In serum-deprived cells, transduction with dn-FOXO3a led to a modest, but reproducible, increase in FLIP mRNA levels (Fig. 7).

FOXO3a Promotes Activation of Caspase-8—A reduction in intracellular FLIP levels is predicted to result in the autocatalytic activation of caspase-8. Thus, caspase-8 activity was measured in HUVEC lysates to test the functional significance of FLIP down-regulation by constitutively active FOXO3a (Fig. 8A). Compared with control, transduction with Ad-TM-FOXO3a led to significant elevation of caspase-8 enzymatic activity. This activation was dependent on the dose of Ad-TM-FOXO3a (data not shown). In contrast, transduction with Ad-WT-FOXO3a had no effect on caspase-8 activity at any dose under conditions of serum stimulation (Fig. 8), showing that the activation of caspase-8 by FOXO3a is dependent upon the
three residues that are phosphorylated by Akt. Furthermore, the activation of caspase-8 by the triple mutant FOXO3a could be significantly diminished by adenovirus-mediated overexpression of the caspase-8 inhibitor CrmA (Fig. 8A) or by incubation with the caspase-8 inhibitor Z-IETD-fmk (data not shown). Activation of caspase-8 could be reversed by adenoviral overexpression of FLIP, suggesting that caspase-8 activation by FOXO3a is mediated by the down-regulation of endogenous FLIP. A binary adenoviral expression system for FLIP was employed for these experiments, which produces higher levels of transgene expression than conventional adenoviral vectors (11, 28). Finally, co-transduction with CrmA inhibited the down-regulation of FLIP by TM-FOXO3a (Fig. 8B), indicating the involvement of caspase activation in FLIP regulation. The effect of CrmA was modest but reproducible (41% increase, $p < 0.05$, $n = 4$). Long exposures of these immunoblots to film revealed faint bands corresponding to FLIPs and the putative 43-kDa FLIP cleavage band (49, 50). Of note, cells transduced with CrmA (Fig. 8B), or the caspase-8 inhibitor Z-IETD-fmk (data not shown), expressed lower levels of the 43-kDa species.

**FOXO3a Gene Transfer Diminishes Cell Viability in Endothelial Cells**—Forkhead transcription factors are reported to have cytotoxic as well as cytoprotective effects in mammalian cells (17, 29, 30). To investigate the action of FOXO3a on endothelial cell viability, serum-stimulated HUVEC cultures were transduced with Ad-GFP or Ad-TM-FOXO3a, and MTT assays, which measure the mitochondrial function, were performed at 24 and 48 h after transduction. As depicted in Fig. 9, TM-FOXO3a gene transfer diminished cell viability, and this effect was dependent on the dose of Ad-TM-FOXO3a. The toxic effect was also time-dependent and appeared between 24 and 48 h post-transduction, which is in agreement with the time course of FLIP down-regulation (Fig. 9). Transduction of endothelial cells with a control vector encoding GFP did not alter cell viability relative to non-transduced cells.

To analyze further the role of FOXO3a on endothelial cell viability, FACS analysis of hypodiploid DNA was performed (Fig. 10A). Consistent with the results of the MTT assay, transduction with Ad-TM-FOXO3a, and MTT assays, which measure the mitochondrial function, were performed at 24 and 48 h after transduction. As depicted in Fig. 9, TM-FOXO3a gene transfer diminished cell viability, and this effect was dependent on the dose of Ad-TM-FOXO3a. The toxic effect was also time-dependent and appeared between 24 and 48 h post-transduction, which is in agreement with the time course of FLIP down-regulation (Fig. 9). Transduction of endothelial cells with a control vector encoding GFP did not alter cell viability relative to non-transduced cells.

**Forkhead Regulation in Endothelial Cells**—Forkhead transcription factors are reported to have cytotoxic as well as cytoprotective effects in mammalian cells (17, 29, 30). To investigate the action of FOXO3a on endothelial cell viability, serum-stimulated HUVEC cultures were transduced with Ad-GFP or Ad-TM-FOXO3a, and MTT assays, which measure the mitochondrial function, were performed at 24 and 48 h after transduction. As depicted in Fig. 9, TM-FOXO3a gene transfer diminished cell viability, and this effect was dependent on the dose of Ad-TM-FOXO3a. The toxic effect was also time-dependent and appeared between 24 and 48 h post-transduction, which is in agreement with the time course of FLIP down-regulation (Fig. 9). Transduction of endothelial cells with a control vector encoding GFP did not alter cell viability relative to non-transduced cells.

This data provided correlative evidence suggesting that FLIP down-regulation contributes to endothelial cell apoptosis.

**Fig. 7.** FOXO3a gene transfer reduces FLIP RNA abundance. A, representative autoradiography film exposure from RNA protection assay of isolated RNA 16 h following Ad-FOXO3a gene transfer. Ad-TM-FOXO3a gene transfer diminished FLIP mRNA abundance, whereas WT-FOXO3a had no effect under conditions of serum stimulation. In contrast, transduction of HUVEC with Ad-dn-FOXO3a under serum deprivation led to a significant increase in FLIP-RNA abundance. C, control. B, quantitative analysis of three independent experiments. Results are expressed as mean ± S.E. *, $p < 0.05$ GFP (Serum–) versus dn-FOXO3a; **, $p < 0.01$, GFP (Serum–) versus TM-FOXO3a.
To provide causal evidence that FOXO3a-mediated down-regulation of FLIP is a key feature of endothelial cell apoptosis, HUVEC cultures were co-transduced with Ad-TM-FOXO3a and low levels of the adenoviral vector that expresses exogenous FLIP. Transduction with FLIP significantly reduced apoptosis that was induced by the triple mutant FOXO3a vector (Fig. 10C). Although the reduction in apoptosis was statistically significant relative to Ad-TM-FOXO3a alone, apoptosis was not reduced to control levels ($p < 0.01$). Furthermore, no further reduction in apoptosis could be observed by increasing the dose of the Ad-Tet-FLIP vector (data not shown). Similarly, a partial reduction in Ad-TM-FOXO3a apoptosis occurred when cells were transduced with an adenoviral vector expressing the CrmA caspase-8 inhibitor (Fig. 10C). Increasing the level of Ad-CrmA from 50 to 100 m.o.i. did not lead to a further reduction in the level of apoptosis (data not shown). Furthermore, partial inhibition of apoptosis was also achieved when Ad-TM-FOXO3a-transduced cultures were incubated with the caspase-8 inhibitor Z-IETD-fmk (data not shown).

Other studies have shown that FOXO3a can promote cell death by activating the intrinsic apoptotic pathway that may be due to its ability to up-regulate pro-apoptotic molecules such as Bim (11). Therefore, HUVEC cultures were transduced with an adenoviral vector encoding dominant-negative caspase-9 (Ad-DN-caspase-9), and cell viability was assessed by FACS (Fig. 10C). Partial protection was achieved with 20 m.o.i., and higher doses of virus did not lead to a further reduction in apoptosis. Incubation with the caspase-9 inhibitor Z-LEHD-fmk also partially protected HUVEC from apoptosis induced by TM-FOXO3a (data not shown). To test whether the protective effects of caspase-8 and caspase-9 inhibition were additive, Ad-TM-FOXO3a-infected HUVEC were co-transduced with CrmA and DN-caspase-9. This combination led to a nearly complete suppression of apoptosis, indicating that FOXO3a activates both the extrinsic and intrinsic pathways of cell death in HUVEC (Fig. 10C).

**DISCUSSION**

It is well established that Akt signaling regulates several critical steps in angiogenesis, including endothelial cell survival, migration, and capillary-like structure formation (14, 31). During the process of neovascularization, endothelial cell survival is determined by the balance of exogenous pro-angiogenic and anti-angiogenic stimuli. We have shown previously (11) that Akt-mediated regulation of FLIP expression confers pro-angiogenic survival signals to endothelial cells. FLIP functions to inhibit the extrinsic apoptotic pathway that can be activated by anti-angiogenic stimuli (32, 33). Forkhead transcription factors have been identified as downstream targets of the Akt protein kinase (17, 34, 35). Thus, we investigated the role of the forkhead protein FOXO3a in controlling endothelial cell viability. We show that FOXO3a is expressed in endothelial cells and that it is regulated by Akt signaling. We also show that FOXO3a controls endothelial cell viability through a novel mechanism involving the regulation of FLIP expression (Fig. 11).

Following activation of Akt, FOXO3a is phosphorylated at three Akt consensus sequence sites, and this leads to the cytoplasmic retention of this transcription factor (36). In endothelial cell cultures, serum stimulation induced cytoplasmic accu-
mulation of FOXO3a, whereas serum starvation promoted its translocation to the nucleus. Transduction with constitutively active Akt led to nuclear exclusion of FOXO3a, whereas transduction with dominant-negative Akt resulted in its nuclear accumulation in the serum-stimulated cultures. The effects of matrix detachment on FOXO3a localization were also examined because disruption of integrin-matrix interactions leads to diminished Akt activation and anoikis in endothelial cells (13, 23). FOXO3a was found to accumulate rapidly in the nucleus after the placement of endothelial cells in suspension culture. Collectively, these data suggest that FOXO3a is regulated by Akt signaling in endothelial cells and that it accumulates in the nucleus under conditions that promote apoptosis.

To assess the functional significance of FOXO3a transduction to the nucleus, endothelial cells were transduced with adenoviral vectors expressing constitutively active, dominant-negative, or wild-type FOXO3a. The constitutively active vector expresses a non-phosphorylatable mutant of FOXO3a. This protein accumulated in the nucleus and was not subject to regulation by Akt or mitogens. Expression of non-phosphorylatable FOXO3a in serum-stimulated endothelial cells led to profound apoptotic cell death in a dose- and time-dependent manner. Apoptosis was indicated by diminished mitochondrial function and the formation of hypodiploid DNA and pyknotic nuclei. Apoptosis was also associated with the activation of caspase-8, suggesting the induction of the extrinsic cell death pathway. In contrast, wild-type FOXO3a did not affect viability in serum-stimulated cultures, a condition that leads to FOXO3a localization in the cytoplasm. However, overexpression of wild-type FOXO3a significantly increased apoptosis when endothelial cell cultures were deprived of serum leading to FOXO3a translocation to the nucleus. Finally, transduction with dominant-negative FOXO3a protected cells from apoptosis induced by serum deprivation.

The mechanisms by which forkhead factors kill cells appear to be highly cell type-specific. In cerebellar neurons, fibroblasts, and Jurkat cells the transient overexpression of non-phosphorylatable FOXO3a induces apoptotic cell death by the up-regulation of Fas ligand expression and activation of the death receptor pathway (17). It has also been shown that forkhead transcription factors positively regulate the expression of TRAIL/Apo2L, another death receptor ligand, in myeloma cells (37). In contrast, it has been reported that cytokine withdrawal induced apoptosis of hematopoietic cells involves FOXO3a induction of apoptotic cell death independent of ligand-mediated activation of death receptors (38). In the latter study it was shown that neither cytokine withdrawal nor FOXO3a activation resulted in caspase-8 cleavage or increased Fas ligand promoter activity. Instead, FOXO3a induced transcriptional up-regulation of the pro-apoptotic Bcl-2 family member protein Bim by loss of mitochondrial membrane integrity, cytochrome c release, and caspase activation. Thus, FOXO3a can also promote death by activating the intrinsic apoptosis pathway in some cell types.

Our data indicate a new mechanism by which FOXO3a signaling induces apoptosis. It was found that FOXO3a down-
regulated FLIP which is required to maintain endothelial cell viability (Fig. 11). FOXO3a-mediated regulation of FLIP was shown by multiple lines of evidence. Increased FOXO3a signaling, either with constitutively active or wild-type FOXO3a, down-regulated FLIP protein levels in a dose-dependent manner. TM-FOXO3a also down-regulated FLIP mRNA expression. Conversely, dominant-negative FOXO3a increased FLIP protein and mRNA expression. Further evidence for the FOXO3a-FLIP regulatory interaction was obtained by assessing the functional significance of FLIP down-regulation on endothelial cell viability. Repletion of FLIP in endothelial cells expressing constitutively active FOXO3a completely suppressed caspase-8 activation and partially protected cells from apoptosis. The functional significance of the extrinsic apoptosis pathway in FOXO3a toxicity to endothelial cells was further indicated by the finding that transduction of the caspase-8 inhibitor CrmA also blocked caspase-8 activation and partially repressed apoptosis.

Although these data provide causal evidence for the importance of FLIP and the extrinsic pathway downstream from FOXO3a, the finding that FLIP or CrmA transduction only partially protects endothelial cells from apoptosis suggests that intrinsic apoptosis regulatory pathways may also participate in FOXO3a toxicity. The observation that transduction with dominant-negative caspase-9 partially protects endothelial cells from FOXO3a-mediated toxicity provides further support for this hypothesis. Moreover, the observation that the protective effects of caspase-8 and caspase-9 inhibition are additive suggests that FOXO3a-induced toxicity in endothelial cells is mediated by the combination of intrinsic and extrinsic cell death pathways (Fig. 11).

Under normal conditions, endothelial cells are highly resistant to death receptor-mediated apoptosis (3). Endothelial cells express endogenous FasL on their cell surface and can be genetically modified to express high levels of Fas ligand in vitro, with no effect on viability, or in transgenic mice with no detectable pathological or developmental abnormalities (3, 5, 30). Presumably, endothelial cells can survive high levels of Fas ligand expression because they express abundant FLIP (9). However, endothelial cells become susceptible to Fas-mediated cell death under conditions of serum deprivation or following exposure to stimuli that lead to Akt inactivation (11). For example, oxidized lipids, which inactivate Akt (40), promote FLIP down-regulation and induce Fas-mediated apoptosis in endothelial cells (9, 10). It has also been reported that anoikis is associated with caspase-8 cleavage (27) and FLIP down-regulation (26). Because Akt inactivation is an essential step in anoikis (13), FOXO3a regulation of FLIP may contribute to activation of the extrinsic apoptosis pathway under these conditions. Thus, the Akt-FOXO3a-FLIP regulatory axis described here may be an important regulator of endothelial cell viability in response to multiple environmental stimuli.

The mechanism of forkhead action on FLIP expression is not known. Forkhead transcription factors can function as activators of transcription (17), but not exclusively so. For example, trans-repression has been reported for FoxA2, -C2, -D2, -D3, and -G1 (41–44), and the Caenorhabditis elegans forkhead protein LIN 31 can act either as a repressor or activator (45). Furthermore, the Akt-regulated forkhead protein FOX4 has been shown to activate the transcription of the BCL-6 transcriptional repressor which induces apoptosis (46). In endothelial cells, activation of FOXO3a was found to decrease FLIP mRNA raising the possibility that this regulation may occur at the transcriptional level. However, the FLIP promoter has not been characterized. It is also possible that this regulation involves a protein-protein association between FOXO3a and a transcriptional regulator that is bound to the FLIP promoter, as suggested recently (47, 48) for FOXO3a-mediated repression of the cyclin D promoter. In addition, FLIP down-regulation can also result from caspase activation by FOXO3a. A caspase-3/10 cleavage site in the p12-caspase domain (LEVQD amino acids 373–377) of the FLIP protein has been described (49), and FLIP cleavage has been noted in this and other studies (50). Although the role of caspase-dependent FLIP cleavage has not been clarified, it is conceivable that it participates in a positive feedback loop that increases caspase activation during the apoptotic response. In support of this hypothesis, we found that the FOXO3a-mediated reductions in FLIP protein expression and cell viability were diminished by caspase-8 inhibition. Therefore, it is possible that two mechanisms contribute to FLIP down-regulation by FOXO3a, one at the level of protein and the other at the level of mRNA.

In conclusion, we show that the forkhead transcription factor FOXO3a is regulated by Akt in endothelial cells and that it promotes apoptosis by negatively regulating the expression of FLIP. FLIP down-regulation was accompanied by caspase-8 activation, and restoration of intracellular FLIP levels reversed caspase-8 activation and inhibited endothelial cell apoptosis. These results provide further insights into the roles of FLIP and FOXO3a in endothelial cell viability, and they suggest that these molecules may be significant in controlling the balance between blood vessel growth and regression.

REFERENCES

1. Walsh, K., Smith, R. C., and Kim, H. S. (2000) Circ. Res. 87, 184–188
2. Nagata, S., and Golstein, P. (1995) Science 267, 1449–1456
3. Sata, M., Suhara, T., and Walsh, K. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 369–376
4. Suhara, T., Kim, H.-S., Kirshenbaum, L. A., and Walsh, K. (2002) Mol. Cell. Biol. 22, 680–691
