Astrocyte elevated gene-1 (AEG-1) functions as an oncogene and regulates angiogenesis

Luni Emdad[a,1], Seok-Geun Lee[b,c,1], Zhao Zhong Su[b], Hyun Yong Jeon[b], Habib Boukerche[d], Devanand Sarkar[b,c,e], and Paul B. Fisher[b,c,e,2]

[a]Department of Neurosurgery and Oncological Sciences, Mount Sinai School of Medicine, New York, NY 10029; [b]Department of Human and Molecular Genetics, [c]Virginia Commonwealth University Massey Cancer Center, [e]Virginia Commonwealth University Institute of Molecular Medicine, Virginia Commonwealth University, School of Medicine, Richmond, VA 23298; and [d]EA 4174, University Lyon 1, French National Institute for Health and Medical Research, France

Communicated by George J. Todaro, Targeted Growth, Inc., Seattle, WA, October 7, 2009 (received for review March 17, 2009)

Astrocyte-elevated gene-1 (AEG-1) expression is increased in multiple cancers and plays a central role in Ha-*ras*-mediated oncogenesis through the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. Additionally, overexpression of AEG-1 protects primary and transformed human and rat cells from serum starvation-induced apoptosis through activation of PI3K/Akt signaling. These findings suggest, but do not prove, that AEG-1 may function as an oncogene. We now provide definitive evidence that AEG-1 is indeed a transforming oncogene and show that stable expression of AEG-1 in normal immortal cloned rat embryo fibroblast (CREF) cells induces morphological transformation and enhances invasion and anchorage-independent growth in soft agar, two fundamental biological events associated with cellular transformation. Additionally, AEG-1-expressing CREF clones form aggressive tumors in nude mice. Immunohistochemistry analysis of tumor sections demonstrates that AEG-1-expressing tumors have increased microvessel density throughout the entire tumor sections. Overexpression of AEG-1 increases expression of molecular markers of angiogenesis, including angiopoietin-1, matrix metalloprotease-2, and hypoxia-inducible factor 1-α. In vitro angiogenesis studies further demonstrate that AEG-1 promotes tube formation in Matrigel and increases invasion of human umbilical vein endothelial cells via the PI3K/Akt signaling pathway. Tube formation induced by AEG-1 correlates with increased expression of angiogenesis markers, including Tie2 and hypoxia-inducible factor-α, and blocking AEG-1-induced Tie2 with Tie2 siRNA significantly inhibits AEG-1-induced tube formation in Matrigel. Overall, our findings demonstrate that aberrant AEG-1 expression plays a dominant positive role in regulating oncogenic transformation and angiogenesis. These findings suggest that AEG-1 may provide a viable target for directly suppressing the cancer phenotype.

angiogenesis-related molecules | tumor progression

Cancer development and acquisition of malignant potential are multifactor and multistep processes that occur in a temporal manner during tumor progression (1, 2). Significant components of these processes in the evolving neoplastic cell include development of growth signal autonomy, insensitivity to growth-inhibitory signals, evasion from apoptosis, unlimited replicative potential, and aberrant angiogenesis, as well as tissue invasion that can ultimately culminate in tumor dissemination (1–3). An important defining element in controlling growth in both primary and metastatic tumors is new blood vessel formation (angiogenesis). The importance of angiogenesis in the growth of solid tumors is well established (3, 4). Indeed, tumor size is restricted to a few cubic millimeters if it is not able to attract new blood vessels. Increased intratumoral microvascular density relative to normal tissue is observed in tumors of different tissues including the brain, colon, and breast (5–7). Production of new vessels by the developing tumor and distant metastases results from the amplification of large quantities of pro-angiogenic molecules by both the tumor and host cells and reflects a net balance between positive and negative regulators of angiogenesis (8–10). These observations emphasize that any genetic modification(s) in a cancer cell that culminates in expansion of tumor growth and metastasis will be inexorably linked to angiogenesis.

Astrocyte elevated gene (AEG)-1 was cloned by rapid subtraction hybridization as a gene induced in primary human fetal astrocytes (PHFA) infected with HIV-1 or treated with tumor necrosis factor-α (TNF-α) (11). Intriguingly, expression analysis revealed that AEG-1 was significantly elevated in subsets of breast carcinoma, melanoma, and malignant glioma cell lines compared to their normal cellular counterparts (12). Its expression was also elevated in adult astrocytes transformed by SV40-T antigen, human telomerase reverse transcriptase (hTERT), and oncogenic Ha-*ras* that displayed an aggressive glioma-like phenotype (12). AEG-1 synergized with oncogenic Ha-*ras* to enhance soft agar colony formation of SV40-T antigen-induced immortalized human melanocytes (FM516-SV) as well as in PHFA. Interestingly, AEG-1 itself is a downstream target of Ha-*ras* and plays an important role in mediating the growth-promoting effects of Ha-*ras* (13). Overexpression of AEG-1 also augmented the anchorage-independent growth of HeLa cells and human glioma cell lines and increased their migration and invasion properties (14, 15). Conversely, inhibition of AEG-1 by siRNA significantly inhibited migration and invasion of malignant glioma cells and prostate cancer cells and in vivo lung metastasis of breast cancer cells (16–19). In PHFA, FM516-SV, and cloned rat embryo fibroblasts (CREF), AEG-1 protects from serum starvation-induced apoptosis by activating the PI3K/Akt signaling pathway indicating, but not definitively proving, that AEG-1 might function as an oncogene (20). Inhibition of AEG-1 in prostate cancer cells downregulated Akt activation and lead to upregulation of forkhead box (FOXO) 3a activity resulting in apoptosis (17). Other studies identified AEG-1 homologs in rat and mouse, named Lyric/3D3 and Metadherin, respectively (18, 21, 22), and provided evidence for its involvement in cancer progression and metastasis. In total, these observations indicate that AEG-1 may represent an essential gene regulating multiple signaling and biochemical pathways leading to cell transformation and tumor progression in diverse target cells.

A number of questions remain concerning the potential role(s) of AEG-1 in regulating the cancerous state and precisely what phenotypes it impacts on. These include foremost the biological consequences of elevated AEG-1 expression in normal cells in vitro and in vivo and its potential definitive role, if any, in angiogenesis, which as indicated above is a hallmark of

Author contributions: L.E., S.-G.L., D.S., and P.B.F. designed research; L.E., S.-G.L., Z.Z.S., and H.Y.J. performed research; L.E., S.-G.L., Z.Z.S., H.B., D.S., and P.B.F. contributed new reagents/analytic tools; L.E., S.-G.L., Z.Z.S., H.Y.J., H.B., D.S., and P.B.F. analyzed data; and L.E., S.-G.L., and P.B.F. wrote the paper.

The authors declare no conflict of interest.

[1]L.E. and S.-G.L. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: pbfisher@vcu.edu.

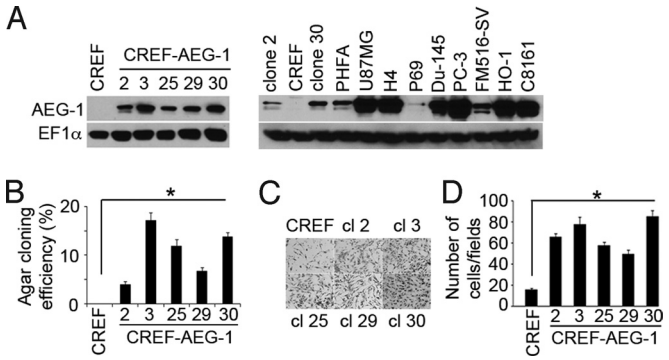

Fig. 1. Effects of stable overexpression of AEG-1 in CREF cells on colony formation in soft agar and cell invasion. (*A*) CREF cells were stably transfected with either the empty pcDNA3.1 vector or the AEG-1 expression vector, respectively. CREF-AEG-1 clones were selected for expression of AEG-1. (*Left*) Expression of AEG-1 protein by stably transfected CREF-AEG-1 cells is shown by Western blot analysis. EF1α was used as an internal control to ascertain equal loading. (*Right*) Expression of AEG-1 protein in stably transfected CREF-AEG-1 cells (clone 2 and 30) and normal immortal CREF and a series of normal human cells (PHFA, P69, FM516-SV) and corresponding human tumor cells (U87MG, H4, DU-145, PC-3, HO-1, C8161) by Western blot analysis. EF1α was used as an internal control for protein loading. (*B*) A total of 1×10^5 cells were seeded in 0.4% agar on 0.8% base agar. Two weeks later, colonies >0.1-mm were counted under a dissection microscope. *, $P < 0.05$ vs. CREF. (*C*) Cells (5×10^4) were seeded onto the upper chamber of a Matrigel invasion chamber system in the absence of serum. Twenty-four hours after seeding, the filters were fixed, stained, and photographed. (*D*) Quantitation of the invasion assay. The data expressed in the graph is the mean ± SE of three independent experiments. *, $P < 0.05$ vs. CREF.

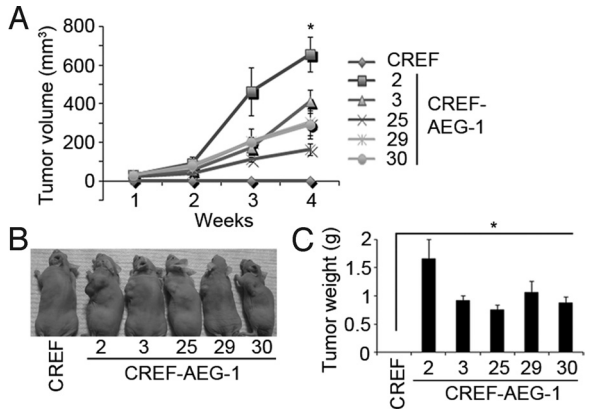

Fig. 2. Overexpression of AEG-1 in CREF cells induces tumorigenesis in vivo. (*A*) 2×10^6 cells of each cell line were subcutaneously injected into the flank of each nude mouse. Tumor volumes were measured at the indicated time points. Results are expressed as means ± SE ($n = 5$ per group). CREF did not form tumors in nude mice. The average tumor volume in cubic millimeters of five animals ± SD. Unpaired two-tailed Student's *t* test ($P < 0.01$). (*B*) Photograph showing tumor growth in nude mice. (*C*) Tumor weights were measured after sacrifice of the mice 4 weeks after injection. The data represent mean ± SE in each group (*, $P < 0.01$ vs. CREF).

cancer progression and metastasis. In this study, we demonstrate that AEG-1 can function as an oncogene, which when expressed at physiological levels in normal immortal CREF cells results in morphological transformation, enhanced invasion, anchorage-independent growth in agar, and acquisition of tumorigenic potential when injected into athymic nude mice. Additionally, sections of AEG-1-overexpressing tumors showed enhanced CD31 expression indicating that CREF-AEG-1 tumors are highly vascularized. In vitro angiogenesis assays with human umbilical vein endothelial cells (HUVECs) revealed that overexpression of AEG-1 significantly increased tube formation in Matrigel through PI3K/Akt signaling. Furthermore, overexpression of AEG-1 in HUVECs and malignant glioma cells modulated angiogenic regulators including Tie2 and hypoxia inducible factor 1 (HIF1)-α. In total, the present studies now confirm that AEG-1 is indeed an oncogene and also a direct regulator of angiogenesis by upregulating key components in the process of blood vessel formation.

Results

AEG-1 Induces Invasion and Anchorage-Independent Growth in Normal Immortal CREF Cells. Anchorage-independent growth and invasion are two crucial events in tumor initiation and progression. Aberrant expression of *AEG*-1 modulates these phenotypes in HeLa, malignant glioma, prostate cancer, neuroblastoma, and hepatocellular carcinoma cells (14, 15, 17, 23, 24). AEG-1 also synergizes with Ha-*ras* to augment the transformed phenotype in FM-516-SV and PHFA cells (12). However, the oncogenic potential of AEG-1 as a single gene in nontransformed cells remained to be determined. To explore this possibility, CREF cells were engineered to stably express AEG-1 (Fig. 1). We used CREF cells for this study because they can be morphologically transformed by single oncogenes, including Ha-*ras*, Ad E1A, v-*Src*, human papilloma virus type 18, and v-*Raf*, or high molecular weight human tumor-derived DNA (25, 26), and can therefore serve as a sensitive barometer for monitoring oncogene functions (25–27). As shown in Fig. 1, parental CREF do not form macroscopic colonies in soft agar (Fig. 1*B*) and are minimally invasive (Fig. 1 *C* and *D*), whereas AEG-1-transformed clones all grow with variable efficiencies in soft agar and are highly invasive. Expression levels of AEG-1 positively correlated with agar cloning efficiency and invasive abilities of CREF-AEG-1 stable clones, suggesting a central role of AEG-1 in development of the transformed state. A concern when examining the function of transfected genes in cells is their level of expression and how this relates to true endogenous gene expression in untransfected cells. A comparison of the level of AEG-1 protein expressed endogenously in a series of human cancers and normal human cells indicated that the level of expression of AEG-1 in stable CREF-AEG-1 clone 2 and clone 30 is comparable or lower than observed in normal immortal human astrocyte, prostate, and melanocyte cells (Fig. 1*A*). In contrast, AEG-1 expression is significantly lower in these transformed CREF clones than in human cancers, including malignant gliomas, prostate cancers, and melanomas. These findings further support genuine oncogenic functions attributed to expression of human AEG-1 at physiologically relevant levels in CREF cells.

AEG-1 as a Single Gene Promotes Tumor Formation When Expressed in CREF Cells. To evaluate oncogenic potential of cells expressing AEG-1 in vivo, nude mice were s.c. injected with CREF-AEG-1 clones (Fig. 2). Inoculation of nude mice with CREF-AEG-1 cells (five independent clones of CREF genetically engineered to express elevated levels of AEG-1) resulted in the formation of aggressive and highly vascularized tumors (Figs. 2 and 3*A*). In contrast, as previously observed (26, 28), injection of nude mice with parental CREF cells did not generate any visible tumors. These in vivo results document that AEG-1 can function as an oncogene when expressed as a single genetic element at physiologically pertinent levels in immortal normal rat embryo cells resulting in an aggressive tumorigenic phenotype in nude mice.

AEG-1 Expression Directly Correlates with Increased Expression of Angiogenesis Markers and Elevated Angiogenesis. On the basis of the morphology (high vascularization) of tumors in nude mice induced by CREF-AEG-1 cells (Fig. 3*A*), we focused on angio-

MEDICAL SCIENCES

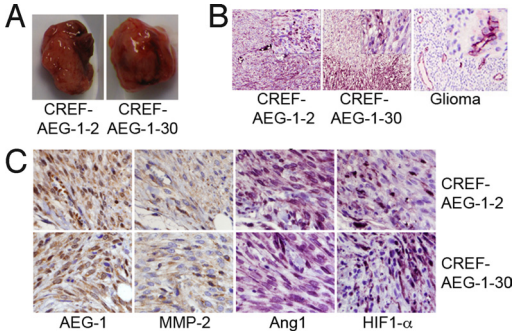

Fig. 3. Histochemical analysis of tumors derived from nude mice injected with AEG-1 stable overexpressing CREF clone 2 and 30. (*A*) Representative examples of tumors derived from CREF-AEG-1 clone 2 and 30. (*B*) The sections of CREF-AEG-1 clone 2 and 30 xenografts and human glioma tissue were immunostained for the endothelial cell marker CD31. Immunohistochemical analysis demonstrated robustly increased blood vessel density in CREF-AEG-1 tumor sections (*inserts*, high magnification view). (*C*) Serial sections of formalin-fixed, paraffin-embedded tumors were stained with a rabbit polyclonal AEG-1, a rabbit polyclonal MMP-2, a rabbit polyclonal Ang1, and a mouse monoclonal HIF1-α antibodies. Signals were developed with DAB chromogen (brown, AEG-1 and MMP-2) or Vector VIP (purple, Ang1 and HIF1-α) and counterstained with hematoxylin.

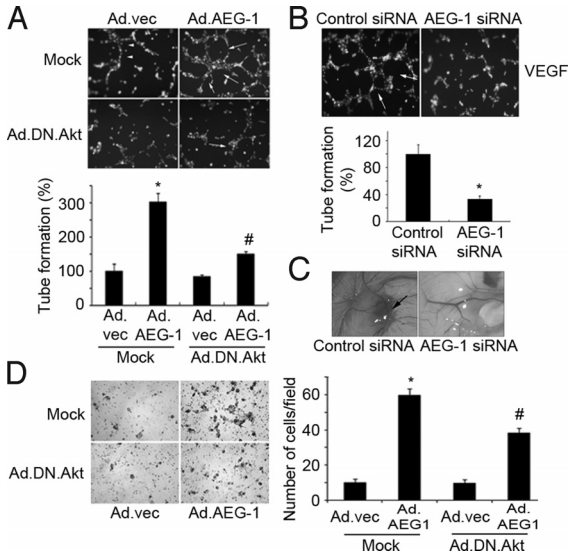

Fig. 4. AEG-1 promotes angiogenesis. (*A*) AEG-1 induces tube formation on Matrigel through the PI3K/Akt signaling pathway. HUVECs were infected with Ad.*vec* or Ad.*AEG*-1 (25 PFU/cell) in combination with Ad.DN.*Akt* (25 PFU/cell). One day after infection, cells (5×10^4), which were labeled with a fluorescent dye, calcein AM, were seeded onto Matrigel and tube formation was assayed after 16 h by fluorescence microscopy. *Upper*, cells that were infected with Ad.*AEG*-1 clearly showed an increase in tube formation on Matrigel (arrows), whereas the Ad.*vec*-treated cells were a poor inducer of tube-like structures (arrowheads). (*Lower*) Graphical presentation of tube formation assay, data expressed in the graph is the mean ± SE of three independent experiments. *, $P < 0.05$ vs. Ad.*vec*-infected cells; #, $P < 0.05$ vs. Ad.*AEG*-1-infected cells. (*B*) HUVECs were treated with either control siRNA or *AEG*-1 siRNA, plated on Matrigel and stimulated with VEGF (10 ng/mL). Tube formation was assayed after 16 h by fluorescence microscopy. *, $P < 0.05$ vs. control siRNA treated cells. (*C*) Inhibition of *AEG*-1 by siRNA inhibits angiogenesis in in vivo CAM assays. CAMs of 9-day-old chicken embryos were injected with either *control* siRNA or *AEG*-1 siRNA-transfected H4 glioma cells. Angiogenesis in yolk sac was monitored 1 week after inoculation, and representative fields were photographed. (*D*) HUVECs were infected with Ad.*vec* or Ad.*AEG-1* (25 PFU/cell) in combination with Ad. DN.*Akt* (25 PFU/cell). One day after infection, cells (5×10^4) were seeded onto the upper chamber of a Matrigel invasion chamber system in the absence of serum. Twenty-four hours after seeding, the filters were fixed, stained, and photographed. (Right panel) Graphical representation of the invasion assay. The data expressed in the graph is the mean ± SE of three independent experiments. *, $P < 0.05$ vs. Ad.*vec*-infected cells, #$P <$ 0.05 vs. Ad.*AEG*-1-infected cells.

genesis as a possible process underlying the increased in vivo aggressiveness of these transformed cells resulting from enhanced AEG-1 expression. Tumors were isolated, formalin-fixed, and paraffin-embedded sections were made. Staining for CD31 demonstrated the presence of microvessels indicative of increased angiogenesis (Fig. 3*B*). AEG-1 staining was most prominent in the peri-nuclear region of the cells, with only a minority of tumor cells also showing nuclear staining (Fig. 3*C*). Additional immunohistochemical analysis revealed that enhanced expression of AEG-1 in tumor sections augmented expression of specific angiogenesis molecules including angiopoietin-1 (Ang1), matrix metalloprotease (MMP)-2, and HIF1-α (Fig. 3*C*), further supporting a potential role of AEG-1 in tumor angiogenesis.

AEG-1 Functions as a Potent Inducer of Angiogenesis. Histochemical analysis of the CREF-AEG-1 tumors indicated potential angiogenic activities of AEG-1, which we investigated using in vitro angiogenesis assays. Tube formation is an important parameter of endothelial function in angiogenesis that can readily be evaluated and quantified in vitro. HUVECs cultured on Matrigel rapidly align, extend processes into the matrix, and finally form capillary-like structures surrounding a central lumen. Within 16 h after seeding on Matrigel, HUVECs start to form tubular structures (Fig. 4*A*). Forced expression of AEG-1 by a replication incompetent adenovirus (Ad.*AEG*-1) significantly enhanced tube formation, with an increased number of intracellular contacts and overall complexity of the network (Fig. 4*A*). Conversely, treatment of HUVECs with *AEG*-1 siRNA significantly inhibited VEGF-induced tube formation in Matrigel (Fig. 4*B*). Next we used a chicken chorioallantoic membrane (CAM) angiogenesis model to study whether siRNA knockdown of AEG-1 would be a feasible approach to suppress tumor-induced angiogenesis in vivo. H4 glioma cells, previously shown to express elevated levels of *AEG*-1, were treated with either control or *AEG*-1 siRNA and seeded on fresh, 9-day-old CAMs. One week after seeding the cells, the CAMs were harvested. CAMs seeded with control siRNA-treated H4 cells caused outgrowth capillary neovascularization near the tumor core region (Fig. 4*C*, left). On the other hand, CAMs seeded with *AEG*-1 si-RNA-treated H4 glioma cells exhibited little neovascularization (Fig. 4*C*, right).

Previous studies have linked many AEG-1-induced cellular changes with PI3K/Akt signaling (13, 20). PI3K/Akt regulates a number of processes associated with cancer progression including angiogenesis (29). To analyze the potential importance of PI3K/Akt signaling in AEG-1-mediated angiogenesis, we used a recombinant replication-incompetent adenovirus expressing a dominant-negative Akt (Ad.DN.*Akt*). AEG-1-mediated endothelial cell tube formation was significantly inhibited by Ad.DN.*Akt* (Fig. 4*A*), suggesting that the PI3K/Akt pathway is an integral component of this process.

Since endothelial cell migration and invasion are important steps in angiogenesis, we tested the effect of forced expression of AEG-1 on the invasive ability of HUVECs. As shown in Fig. 4*D*, overexpression of AEG-1 significantly enhanced the invasive ability of HUVECs, which was conversely blocked by inhibition of the PI3K/Akt pathway. Taken together, these results suggest that AEG-1 is a potent inducer of angiogenesis, and PI3K/Akt signaling is a necessary event in AEG-1-induced angiogenesis.

AEG-1 Modulates Angiogenic Regulators. To further explore the mechanism(s) that mediate the pro-angiogenic activities of

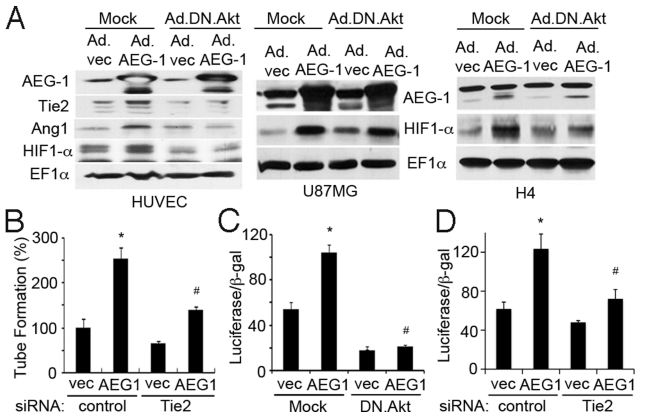

Fig. 5. AEG-1 enhances expression of angiogenesis-associated genes and promotes VEGF promoter activity. (*A*) HUVECs, U87 and H4 glioma cells were infected with the indicated virus as in Fig. 4. Forty-eight hours after infection, total cellular extracts were prepared in RIPA buffer. Equal amounts of proteins were separated on 8–12% SDS-PAGE, transferred onto nitrocellulose membrane and probed with a chicken polyclonal AEG-1, a rabbit polyclonal Tie2 and Ang1, and a mouse monoclonal HIF1-α antibodies. EF1α was used as a control to confirm equal protein loading. (*B*) HUVECs were transfected with either control siRNA or Tie2 siRNA and then infected with Ad.vec or Ad.*AEG-1* (25 PFU/cell). One day after infection, cells (5×10^4) were seeded onto Matrigel, and tube formation was assayed after 16 h. The data expressed in the graph is the mean ± SE of three independent experiments. *, $P < 0.05$ vs. Ad.*vec*-infected cells; #, $P < 0.05$ vs. Ad.*AEG*-1-infected cells. (*C*) H4 cells were infected with the indicated virus as in Fig. 4. The next day cells were transfected with pGL3/VEGF promoter and pSV-β-gal vectors, and 48 h later, luciferase and β-gal activity were determined as described in *Materials and Methods*. The data expressed in the graph is the mean ± SE of three independent experiments. *, $P < 0.05$ vs. Ad.*vec*-infected cells; #, $P < 0.05$ vs. Ad.*AEG*-1-infected cells. (*D*) One day after infection with the indicated virus, H4 cells were transfected with pGL3/VEGF promoter and pSV-β-gal and either control or Tie2 siRNA. The data in the graph is the mean ± SE of three independent experiments. *, $P < 0.05$ vs. Ad.*vec*-infected cells; #, $P < 0.05$ vs. Ad.*AEG*-1-infected cells.

AEG-1, we investigated the effects of AEG-1 on Tie2 and HIF1-α expressions, which are commonly associated with angiogenesis. As shown in Fig. 5*A*, overexpression of AEG-1 significantly augmented the expressions of Tie2 and HIF1-α in HUVECs. Additionally, overexpression of AEG-1 significantly augmented the expression of HIF1-α in U87 and H4 human malignant glioma cells. This modulation of angiogenic regulators by AEG-1 was abrogated by co-expression of a dominant negative Akt, further supporting the involvement of PI3K/Akt signaling in AEG-1-induced angiogenesis. To test the specific role of Tie2 in the angiogenic activities of AEG-1, HUVECs were transfected with either control or Tie2 siRNA and infected with Ad.*AEG*-1 or an Ad vector not containing the *AEG*-1 gene (Ad.*vec*) as a control. Treatment with Tie2 siRNA significantly abrogated AEG-1-induced tube formation in Matrigel (Fig. 5*B*), indicating that Tie2 plays a significant role in AEG-1-induced angiogenesis. Since vascular endothelial growth factor (VEGF) is a known inducer of angiogenesis and the Tie2 and VEGF receptor (VEGFR) pathways seem to work in a complementary and coordinated fashion during vascular development, we examined the effects of AEG-1 overexpression on VEGF promoter activity. As shown in Fig. 5 *C* and *D*, aberrant expression of AEG-1 in H4 cells modestly enhanced VEGF promoter activity, which was ablated by co-expression of a dominant-negative Akt or Tie2 siRNA, indicating the role of the PI3K/Akt pathway and Tie2 in the AEG-1-mediated VEGF induction.

Discussion

Cancer is a continuous process culminating in transformed cells that develop both qualitatively and quantitatively distinct properties as a tumor grows and evolves (30, 31). An initial step in conversion of a cell from normal to transformed can frequently involve the activation of a positive acting oncogene that significantly alters cellular phenotype (30, 31). Additionally, it is now considered almost axiomatic that tumor progression is manifested by the ability of the cancer cell to grow in an unrestrained manner and establish secondary colonies, both of which require a well-developed set of blood vessels, i.e., angiogenesis, to sustain the growth of the primary and secondary metastatic tumors (32, 33). In the last few years, multiple lines of evidence have suggested that AEG-1, a unique and highly conserved protein, plays a significant role in the progression of multiple cancers and metastasis. In the present study, we provide definitive evidence that AEG-1 can function directly as an oncogene and is also a potent inducer of angiogenesis.

Previous studies have demonstrated that AEG-1 can protect PHFA, rat embryo fibroblasts, and SV40-immortalized human melanocytes from serum-starvation-induced apoptosis (20). AEG-1 alone modestly increases soft agar growth of PHFA, but can synergize with Ha-*ras* to significantly augment anchorage-independent growth (12). In addition, we now demonstrate that AEG-1 as a single genetic element can significantly augment anchorage-independent growth in normal immortal CREF cells (Fig. 1*B*) and can induce aggressive tumor growth in nude mice (Fig. 2). Tumors developing from injection with CREF-AEG-1 clones are highly vascularized and express elevated levels of CD31 (Fig. 3*B*), a marker of angiogenesis. CREF-AEG-1 tumors also display high levels of additional angiogenic markers, including Ang1, MMP-2, and HIF1-α (Fig. 3*C*). Moreover, we have recently shown that overexpression of AEG-1 in nontumorigenic human hepatocellular carcinoma cells also results in highly aggressive and vascularized tumors (24). These findings provide support for the hypothesis that AEG-1 might be a positive regulator of angiogenesis. This is indeed the case, since we now show that overexpression of AEG-1 in HUVECs significantly enhances tube formation in Matrigel (Fig. 4*A*); conversely, knockdown of AEG-1 using siRNA significantly abrogates VEGF-induced tube formation in Matrigel (Fig. 4*B*).

To elucidate the precise molecular mechanism underlying AEG-1 function as an angiogenesis inducer, we focused on expression of Tie2, HIF1-α, and Ang1 in AEG-1-overexpressing HUVECs, U87, and H4 malignant glioma cells. Ang1 and Ang2 function as ligands for Tie2, a tyrosine kinase receptor predominantly expressed in vascular endothelial cells. Ang1 specifically induces tyrosine phosphorylation of Tie2, which results in the activation of multiple activities related to angiogenesis such as endothelial cell migration, tube formation, sprouting, and survival (34, 35). Upregulation of Ang1 in high-grade gliomas, non-small cell lung carcinomas, and ovarian, breast, and gastric carcinomas strongly correlate with tumor malignancy (36–40). Furthermore, overexpression of Ang1 in HeLa, GS9L, and in some established glioma cell lines has been reported to increase tumor growth (41–43). Increased Tie2 levels were found in the vasculature of a number of human tumors, including breast cancer, non-small cell lung cancer, hepatocellular carcinoma, prostate cancer, hemangioma, and astrocytoma, and these levels were found to correlate with increasing malignancy (37, 44–47). Recent studies indicate that Tie2 plays a significant role in angiogenesis occurring in cancer (44–47). Suppression of Tie2 signaling (47–49) using specific inhibitors, such as soluble dominant-negative receptors, an antisense oligonucleotide, RNA aptamers, RNA interference, or a short synthetic peptide, promotes antitumor effects by restraining tumor angiogenesis. In these contexts, modulation of Tie2 and Ang1 by AEG-1 (Fig. 5*A*) provides a mechanistic model by which AEG-1 regulates tumor angiogenesis (Fig. 6). We also show that AEG-1 enhances HIF-1α expression in HUVECs, U87, and H4 human malignant glioma cells (Fig. 5*A*). HIF-1 is expressed in hypoxic tumor cells

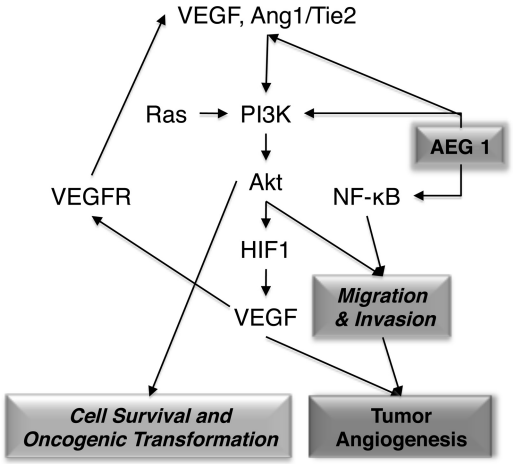

Fig. 6. A hypothetical model of the signal transduction pathways involved in AEG-1-mediated oncogenic transformation and angiogenesis. PI3K is activated by AEG-1, and AEG-1 induces VEGF and Tie-2/Ang. PI3K is also activated by growth factors and angiogenesis inducers such as VEGF and angiopoietins. Akt is an essential downstream target of PI3K for mediating angiogenic signals. Akt activation increases HIF1 expression, which in turn increases VEGF transcriptional expression. Additionally AEG-1 activates NF-κB, which is involved in increased invasion and migration and thus indirectly facilitates tumor angiogenesis. VEGF and VEGFR can form an autocrine loop to regulate tumor angiogenesis.

and transactivates various hypoxia-responsive genes, which confer malignant properties to tumors including apoptosis-resistance and enhanced tumor growth, invasion, and metastasis (50). In addition, HIF-1 activates pro-angiogenic cytokines such as VEGF and platelet-derived growth factor, which increase the proliferation and re-growth of tumor blood vessels (50, 51). We now show that AEG-1 also activates the *VEGF* promoter in malignant glioma cells (Fig. 5*C*), which is in agreement with observations from our group in stable hepatocellular carcinoma cells overexpressing AEG-1 that display elevated levels of VEGF, placental growth factor (PIGF) and fibroblast growth factor α (FGFα) compared to parental hepatocellular carcinoma cells (24).

Our results also reveal the central contribution of PI3K/Akt signaling in AEG-1-induced angiogenesis. Ang1 regulates endothelial cell survival through the PI3K/Akt pathway (52). Overexpression of a dominant-negative Akt construct inhibits Ang1-mediated endothelial cell survival (53), supporting a critical role of Akt in Tie2/Ang-mediated endothelial cell survival. Additionally, both Ang1- and VEGF-induced endothelial cell survival are partially regulated through the PI3K/Akt pathway. In these contexts, our results firmly establish PI3K/Akt signaling as one of the primary mediators of AEG-1-induced angiogenesis in both normal HUVECs and malignant glioma cells. The Ras/MEK signaling pathway is another major pathway for regulating angiogenesis. Thus, our observation of an approximately 50% reduction in tube formation by inhibition of the PI3K/Akt pathway in AEG-1 overexpressing HUVECs (Fig. 4) indicates that other signaling pathways might partially contribute to AEG-1-induced increased angiogenesis. Future studies are required to clarify the complex mechanisms involved in this phenomenon.

In conclusion, this study evaluates the direct oncogenic potential of AEG-1 as a single gene in nontransformed normal cells. Our data show that AEG-1 plays a crucial role in both oncogenic transformation and angiogenesis, which are essential components in tumor cell development, growth, and progression to metastasis (54). In the contexts of angiogenesis, we define important changes induced by AEG-1 in both HUVECs and malignant glioma cells, namely upregulation of Ang1 and Tie2, as potential factors underlying pro-angiogenic activity of AEG-1. We further document the importance of PI3K/Akt signaling in mediating pro-angiogenic action of AEG-1. The present studies provide mechanistic insights into AEG-1 function and support the development of therapeutic strategies that target this gene (or its downstream mediators of transformation and angiogenesis) by a genetic (antisense or siRNA) or pharmacological (small-molecule) inhibitor approach to develop an effective rational target-based strategy for the therapy of multiple cancers (54).

Materials and Methods

Cell Lines and Culture Conditions. CREF is a specific clone of Fischer F2408 rat embryo fibroblast cells (55). CREF-AEG-1 clone 2, 3, 25, 29, and 30 are hygromycin-resistant stable clones obtained after transfection of CREF cells with pcDNA3.1-AEG-1 and selected for growth in 100 μg/mL hygromycin (Invitrogen). Normal human cells, PHFA, P69 (SV40-immortalized normal human prostate epithelial cells), and FM516-SV, have been described in ref. 20. Human tumor cells, U87MG and H4 (malignant glioma); DU-145 and PC-3 (prostate carcinoma); and HO-1 and C8161 (metastatic melanoma cells), have been described (12, 15). Cells resistant to hygromycin were maintained in medium containing 50 μg/mL hygromycin. HUVEC cells were purchased from Cambrex and maintained according to manufacturer's instructions.

Xenograft Studies in Athymic Nude Mice. Xenograft studies in nude mice with CREF, CREF-AEG-1 clone 2, 3, 25, 29, and 30 (CREF stably transfected with the human *AEG*-1 cDNA) were performed as described (23, 24). Four weeks after injection, mice were killed, tumors were weighed and measured, and tumors were fixed in 10% neutral buffered formalin for the immunohistochemical studies. Statistical significance between the groups was determined by unpaired two-tailed Student's t test. A P value of < 0.05 was considered significant.

Immunohistochemistry. Formalin-fixed tumors were embedded in paraffin, sectioned, and mounted on glass slides (55). Immunohistochemical staining was performed with anti-rabbit AEG-1, Ang1, and MMP-2, and anti-mouse CD31 and HIF1-α antibodies as described in ref. 24.

Recombinant Adenovirus Constructs and siRNAs. The recombinant replication-incompetent Ad.*AEG-1* and Ad.DN.*Akt* and the control and AEG-1 siRNAs were described (13, 20). Tie2 siRNA was purchased from Santa Cruz Biotechnology. The transfection of siRNA was performed as described in ref. 13.

Invasion Assays. Invasion assays were performed by using 24-well BioCoat cell culture inserts with an 8-μ-porosity polyethylene terepthylate membrane coated with Matrigel basement membrane matrix (100 μg/cm^2) as described in ref. 15. The invasive ability of the cells was determined by counting of the cells that had migrated to the lower side of the filter with a microscope (magnification, $\times$100). Experiments were assayed in triplicate, and at least 10 fields were counted in each experiment.

Anchorage-Independent Growth Assay in Soft Agar. Anchorage-independent growth assays were performed by seeding 1×10^5 cells in 0.4% Noble agar on an 0.8% agar base layer, both of which contained growth medium. Colonies were counted (>0.1-mm) 2 weeks after seeding, and the data from triplicate determinations were expressed as mean $\pm$ SD.

Western Blot Analysis. Whole-cell lysates were prepared, and Western blot analysis was done as described in ref. 14. The primary antibodies used were anti-AEG-1 (chicken polyclonal, 1:2,000), anti-HIF1-α (mouse monoclonal, 1:500; AbCam), anti-EF1α (mouse monoclonal, 1:1,000; Upstate Biotechnology), anti-Tie2, and anti-Ang1 (rabbit polyclonal, 1:500; Santa Cruz Biotechnology).

Capillary-Like Tube Formation Assay. The formation of tube-like structures by HUVECs on Matrigel (Chemicon) was performed as described in ref. 56. The adenovirus-transduced or siRNA-transfected HUVECs (pretreated with calcein-AM) were seeded on coated plates at 5×10^4 cells/well in EGM containing 2% FBS and incubated at 37 °C for overnight. Cells were observed using a Fluorescence microscope (Nikon). Images were captured with a video graphic system. The degree of network formation was quantified using Image analyzer (National Institutes of Health Image).

Chorioallantoic Membrane Assay. To detect in vivo tubule formation, we performed CAM assays as described in ref. 57. H4 glioma cells treated with either control or AEG-1 siRNA were seeded on the CAM surface of 9-day-old chick embryos. One week after inoculation, the neovasculature was examined and photographed.

VEGF-Promoter Assay. H4 cells were seeded at 1×10^5/well in 12-well plates 24 h before infection. The adenovirus-transduced or siRNA-transfected cells were cotransfected with 2 μg pGL3/VEGF promoter vector (55) and 0.5 μg pSV-β-galactosidase (β-gal) vector (Promega), and luciferase and β-gal assays were performed as described in ref. 14.

Statistical Analysis. All of the experiments were performed at least three times. The results are expressed as mean $\pm$ SD. Statistical comparisons were made using an unpaired two-tailed Student t test. A P value<0.05 was considered as significant.

ACKNOWLEDGMENTS. The present study was supported in part by National Institutes of Health grant nos. CA134721, CA097318, and NS31492 (to P.B.F.) and awards from the Goldhirsh Foundation for Brain Tumor Research and the Dana Foundation (to D.S.). D.S. is the Harrison Endowed Scholar in the Virginia Commonwealth University (VCU) Massey Cancer Center. P.B.F. holds the Thelma Newmeyer Corman Endowed Chair in Cancer Research at the VCU Massey Cancer Center and is a Samuel Waxman Cancer Research Foundation Investigator.

1. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* 100:57–70.
2. Hahn WC, Weinberg RA (2002) Modelling the molecular circuitry of cancer. *Nat Rev Cancer* 2:331–341.
3. Fidler IJ, Ellis LM (1994) The implications of angiogenesis for the biology and therapy of cancer metastasis. *Cell* 79:185–188.
4. Folkman J (1992) The role of angiogenesis in tumor growth. *Semin Cancer Biol* 32:65–71.
5. Wesseling P, van der Laak JA, Link M, Teepen HL, Ruiter DJ (1998) Quantitative analysis of microvascular changes in diffuse astrocytic neoplasms with increasing grade of malignancy. *Hum Pathol* 29:352–358.
6. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM (1995) Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 55:3964–3968.
7. Weidner N, Semple JP, Welch WR, Folkman J (1991) Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N Engl J Med* 324:1–8.
8. Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1:27–31.
9. Liotta LA, Stetler-Stevenson WG (1991) Tumor invasion and metastasis: An imbalance of positive and negative regulation. *Cancer Res* 51:5054s–5059s.
10. Kumar R, Fidler IJ (1998) Angiogenic molecules and cancer metastasis. *In Vivo* 12:27–34.
11. Su ZZ, et al. (2002) Identification and cloning of human astrocyte genes displaying elevated expression after infection with HIV-1 or exposure to HIV-1 envelope glycoprotein by rapid subtraction hybridization, RaSH. *Oncogene* 21:3592–3602.
12. Kang DC, et al. (2005) Cloning and characterization of HIV-1-inducible astrocyte elevated gene-1, AEG-1. *Gene* 353:8–15.
13. Lee SG, Su ZZ, Emdad L, Sarkar D, Fisher PB (2006) Astrocyte elevated gene-1 (AEG-1) is a target gene of oncogenic Ha-ras requiring phosphatidylinositol 3-kinase and c-Myc. *Proc Natl Acad Sci USA* 103:17390–17395.
14. Emdad L, et al. (2006) Activation of the nuclear factor kappaB pathway by astrocyte elevated gene-1: Implications for tumor progression and metastasis. *Cancer Res* 66:1509–1516.
15. Sarkar D, et al. (2008) Molecular basis of nuclear factor-kappaB activation by astrocyte elevated gene-1. *Cancer Res* 68:1478–1484.
16. Emdad L, et al. (2007) Astrocyte elevated gene-1: Recent insights into a novel gene involved in tumor progression, metastasis and neurodegeneration. *Pharmacol Ther* 114:155–170.
17. Kikuno N, et al. (2007) Knockdown of astrocyte elevated gene-1 inhibits prostate cancer progression through upregulation of FOXO3a activity. *Oncogene* 26:7647–7655.
18. Brown DM, Ruoslahti E (2004) Metadherin, a cell surface protein in breast tumors that mediates lung metastasis. *Cancer Cell* 5:365–374.
19. Hu G, et al. (2009) MTDH activation by 8q22 genomic gain promotes chemoresistance and metastasis of poor-prognosis breast cancer. *Cancer Cell* 15:9–20.
20. Lee SG, et al. (2008) Astrocyte elevated gene-1 activates cell survival pathways through PI3K-Akt signaling. *Oncogene* 27:1114–1121.
21. Sutherland HG, Lam YW, Briers S, Lamond AI, Bickmore WA (2004) 3D3/lyric: A novel transmembrane protein of the endoplasmic reticulum and nuclear envelope, which is also present in the nucleolus. *Exp Cell Res* 294:94–105.
22. Britt DE, et al. (2004) Identification of a novel protein, LYRIC, localized to tight junctions of polarized epithelial cells. *Exp Cell Res* 300:134–148.
23. Lee SG, et al. (2009) Astrocyte elevated gene-1 contributes to the pathogenesis of neuroblastoma. *Oncogene* 28:2476–2484.
24. Yoo BK, et al. (2009) Astrocyte elevated gene-1 regulates hepatocellular carcinoma development and progression. *J Clin Invest* 119:465–477.
25. Su ZZ, Shi Y, Fisher PB (1997) Subtraction hybridization identifies a progression elevated gene PEG-3 with sequence homology to a growth arrest and DNA damage inducible gene. *Proc Natl Acad Sci USA* 94:9125–9130.
26. Su ZZ, Olsson CA, Zimmer SG, Fisher PB (1992) Transfer of a dominant-acting tumor-inducing oncogene from human prostatic carcinoma cells to cloned rat embryo fibroblast cells by DNA-transfection. *Anticancer Res* 12:297–304.
27. Fisher PB, Babiss LE, Weinstein IB, Ginsberg HS (1982) Analysis of type 5 adenovirus transformation with a cloned rat embryo cell line (CREF). *Proc Natl Acad Sci USA* 79:3527–3531.
28. Babiss LE, et al. (1986) Mutations in the E1a gene of type 5 adenovirus alter the tumorigenic properties of transformed cloned rat embryo fibroblast cells. *Proc Natl Acad Sci USA* 83:2167–2171.
29. Shiojima I, Walsh K (2002) Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ Res* 90:1243–1250.
30. Vogelstein B, Kinzler KW (2004) Cancer genes and the pathways they control. *Nat Med* 10:789–799.
31. Fisher PB (1984) Enhancement of viral transformation and expression of the transformed phenotype by tumor promoters, in *Tumor Promotion and Cocarcinogenesis In Vitro, Mechanisms of Tumor Promotion*, ed Slaga TJ (CRC Press, Boca Raton, FL), pp 57–123.
32. Carmeliet P (2000) Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 6:389–395.
33. Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. *Nature* 407:249–257.
34. Papapetropoulos A, et al. (1999) Direct actions of angiopoietin-1 on human endothelium: Evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors. *Lab Invest* 79:213–223.
35. Koblizek TI, Weiss C, Yancopoulos GD, Deutsch U, Risau W (1998) Angiopoietin-1 induces sprouting angiogenesis in vitro. *Curr Biol* 9:529–532.
36. Stratmann A, Risau W, Plate KH (1998) Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. *Am J Pathol* 153:1459–1466.
37. Takahama M, et al. (1999) Enhanced expression of Tie2, its ligand angiopoietin-1, vascular endothelial growth factor, and CD31 in human non-small cell lung carcinomas. *Clin Cancer Res* 5:2506–2510.
38. Martoglio AM, et al. (2000) Changes in tumorigenesis- and angiogenesis-related gene transcript abundance profiles in ovarian cancer detected by tailored high density cDNA arrays. *Mol Med* 6:750–765.
39. Shirakawa K, et al. (2001) Absence of endothelial cells, central necrosis, and fibrosis are associated with aggressive inflammatory breast cancer. *Cancer Res* 61:445–451.
40. Wang J, et al. (2005) Expressions and clinical significances of angiopoietin-1, -2 and Tie2 in human gastric cancer. *Biochem Biophys Res Commun* 337:386–393.
41. Shim WS, et al. (2002) Angiopoietin 1 promotes tumor angiogenesis and tumor vessel plasticity of human cervical cancer in mice. *Exp Cell Res* 279:299–309.
42. Machein MR, et al. (2004) Angiopoietin-1 promotes tumor angiogenesis in a rat glioma model. *Am J Pathol* 165:1557–1570.
43. Zadeh G, Koushan K, Pillo L, Shannon P, Guha A (2004) Role of Ang1 and its interaction with VEGF-A in astrocytomas. *J Neuropathol Exp Neurol* 63:978–989.
44. Tanaka S, et al. (2002) Tie2 vascular endothelial receptor expression and function in hepatocellular carcinoma. *Hepatology* 35:861–867.
45. Caine GJ, Blann AD, Stonelake PS, Ryan P, Lip GY (2003) Plasma angiopoietin-1, angiopoietin-2 and Tie-2 in breast and prostate cancer: A comparison with VEGF and Flt-1. *Eur J Clin Invest* 33:883–890.
46. Yu Y, Varughese J, Brown LF, Mulliken JB, Bischoff J (2001) Increased Tie2 expression, enhanced response to angiopoietin-1, and dysregulated angiopoietin-2 expression in hemangioma-derived endothelial cells. *Am J Pathol* 159:2271–2280.
47. Zadeh G, et al. (2004) Targeting the Tie2/Tek receptor in astrocytomas. *Am J Pathol* 164:467–476.
48. Lin P, et al. (1997) Inhibition of tumor angiogenesis using a soluble receptor establishes a role for Tie2 in pathologic vascular growth. *J Clin Invest* 100:2072–2078.
49. Lin P, et al. (1998) Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2. *Proc Natl Acad Sci USA* 95:8829–8834.
50. Semenza GL (2003) Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 3:721–732.
51. Semenza GL (2002) HIF-1 and tumor progression: Pathophysiology and therapeutics. *Trends Mol Med* 8:S62–S67.
52. Fujikawa K, et al. (1999) Role of PI 3-kinase in angiopoietin-1-mediated migration and attachment-dependent survival of endothelial cells. *Exp Cell Res* 253:663–672.
53. Papapetropoulos A, et al. (2000) Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. *J Biol Chem* 275:9102–9105.
54. Sarkar D, Emdad L, Lee SG, Yoo BK, Su ZZ, Fisher PB (2009) Astrocyte elevated gene-1 (AEG-1): Far more than just a gene regulated in astrocytes. *Cancer Res* 69:8529–8535.
55. Su ZZ, et al. (1999) PEG-3, a nontransforming cancer progression gene, is a positive regulator of cancer aggressiveness and angiogenesis. *Proc Natl Acad Sci USA* 96:15115–15120.
56. Rocnik EF, Liu P, Sato K, Walsh K, Vaziri C (2006) The novel SPARC family member SMOC-2 potentiates angiogenic growth factor activity. *J Biol Chem* 281:22855–22864.
57. Pfeifer A, Kessler T, Silletti S, Cheresh DA, Verma IM (2000) Suppression of angiogenesis by lentiviral delivery of PEX, a noncatalytic fragment of matrix metalloproteinase 2. *Proc Natl Acad Sci USA* 97:12227–12232.