

Generation of a functional and durable vascular niche by the adenoviral *E4ORF1* gene

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Edited by Napoleone Ferrara, Genentech, Inc., South San Francisco, CA, and approved September 24, 2008 (received for review June 27, 2008)

Vascular cells contribute to organogenesis and tumorigenesis by producing unknown factors. Primary endothelial cells (PECs) provide an instructive platform for identifying factors that support stem cell and tumor homeostasis. However, long-term maintenance of PECs requires stimulation with cytokines and serum, resulting in loss of their angiogenic properties. To circumvent this hurdle, we have discovered that the adenoviral *E4ORF1* gene product maintains long-term survival and facilitates organ-specific purification of PECs, while preserving their vascular repertoire for months, in serum/cytokine-free cultures. Lentiviral introduction of *E4ORF1* into human PECs (*E4ORF1*⁺ ECs) increased the long-term survival of these cells in serum/cytokine-free conditions, while preserving their *in vivo* angiogenic potential for tubulogenesis and sprouting. Although *E4ORF1*, in the absence of mitogenic signals, does not induce proliferation of ECs, stimulation with VEGF-A and/or FGF-2 induced expansion of *E4ORF1*⁺ ECs in a contact-inhibited manner. Indeed, VEGF-A-induced phospho MAPK activation of *E4ORF1*⁺ ECs is comparable with that of naive PECs, suggesting that the VEGF receptors remain functional upon *E4ORF1* introduction. *E4ORF1*⁺ ECs inoculated in implanted Matrigel plugs formed functional, patent, humanized microvessels that connected to the murine circulation. *E4ORF1*⁺ ECs also incorporated into neo-vessels of human tumor xenotransplants and supported serum/cytokine-free expansion of leukemic and embryonal carcinoma cells. *E4ORF1* augments survival of PECs in part by maintaining FGF-2/FGF-R1 signaling and through tonic Ser-473 phosphorylation of Akt, thereby activating the mTOR and NF- κ B pathways. Therefore, *E4ORF1*⁺ ECs establish an Akt-dependent durable vascular niche not only for expanding stem and tumor cells but also for interrogating the roles of vascular cells in regulating organ-specific vascularization and tumor neo-angiogenesis.

angiogenesis | endothelium | adenovirus | tumor | stem

Endothelial cells (ECs) are not only conduits for delivering oxygen and nutrients but they also provide a permissive niche for the maintenance of organ-specific tissues and tumor cells (1). Within the bone marrow (BM) hematopoietic stem cells interact with sinusoidal ECs and undergo self-renewal or differentiation (1, 2). ECs contribute to self-renewal of neuronal stem cells (3) and promote the growth of leukemic cells (4) and gliomas (5). However, hurdles associated with cultivating functional primary ECs (PECs) in long-term cultures have hindered identification of the molecular pathways involved in the interaction of PECs with stem and tumor cells.

Maintenance of PECs *in vitro* requires utilization of enriched EC growth medium that is supplemented with serum, proangiogenic factors, including VEGF-A, FGF-2, EGF, IGF, and EC growth supplement. Notably, deprivation of PECs for few hours from these growth factors results in a rapid cell death. Therefore, the use of PECs as feeder cells needs to be performed in the presence of EC growth medium, which artificially influences the growth of cocultivated stem and tumor cells, providing a major confounding variable in such studies.

To overcome these hurdles, PECs have been immortalized with hTERT or SV40 large T and polyoma middle T oncogenes. However, activation of proliferative phospho MAPK (pMAPK) signaling pathways in these cell lines has resulted in generation of dysregulated ECs that are either serum-dependent or have acquired transformed phenotypes atypical of PECs. Furthermore, because of a high metabolic rate, immortalized PECs deprive the cocultured stem and tumor cells of nutrients, resulting in cell death.

In search for factors that can support long-term survival of PECs without inducing transformation, we have discovered that the adenoviral (Ad) *E4ORF1* gene, when introduced into PECs (*E4ORF1*⁺ ECs), results in generation of a long-lasting angiogenic state, in which the majority of the provascular functions are preserved. The angiogenic repertoire of *E4ORF1*⁺ ECs is similar to early passaged PECs, thereby providing a permissive experimental platform to investigate organogenesis and tumorigenesis.

The gene products encoded by early region 4 (*E4*) of adenovirus are essential for virus replication, modulating cell cycle, apoptosis, and cell signaling (6). We have shown that the Ad-*E4* gene complex maintains angiogenic properties of PECs, by modulating the migration and apoptosis of PECs (7). Ad *E4* products promote PEC survival via increasing Src kinase and PI3-kinase phosphorylation, and by reducing caspase-3 activity (8). However, the identity of the specific genes transcribed within the *E4* gene complex that regulates angiogenesis up to now has remained elusive.

Indeed, Ad *E4* mRNA contains 7 ORFs, suggesting that *E4* encodes at least 6 gene products (*E4ORF1*–*E4ORF6/7*). Among the known *E4* ORFs, *E4ORF1* primarily affects survival but not cell proliferation (9, 10). Therefore, we hypothesized that *E4ORF1* may modulate survival in ECs without promoting oncogenic transformation.

Here, we show that *E4ORF1* introduction into the human PECs increases the survival of PECs in serum/cytokine-free culture conditions without enforcing cell proliferation. The prosurvival effect of *E4ORF1* is mediated through Akt-PI3-kinase-mTOR and NF- κ B activation without switching on the MAPK pathway, thereby maintaining the angiogenic functions of PECs, including neovascularization *in vivo*. *E4ORF1*⁺ ECs also provide a permissive microenvironment for the growth of leukemic and embryonal carcinoma cells. As such, *E4ORF1* allows for generation of durable PECs that can be maintained long term as intact monolayers, even

Author contributions: M.S., J.L.B., and S. Rafii designed research; M.S., J.M.B., H.K., A.T.H., I.A.W., F.Z., E.L.V., M.K., Y.Z., S.V.S., S. Rabbany, and S. Rafii performed research; M.S., A.T.H., N.R.H., J.L.B., and S. Rafii contributed new reagents/analytic tools; M.S., J.M.B., and S. Rafii analyzed data; and M.S. and S. Rafii wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0805980105/DCSupplemental.

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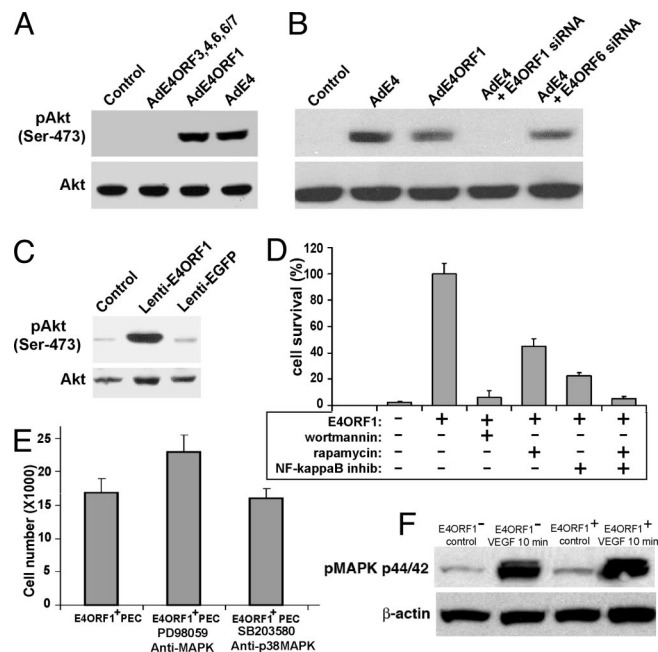


Fig. 2. E4ORF1 tonically activates pAkt in PECs. (A) PECs (HUVECs) were infected with AdE4ORF1 \rightarrow 7 vectors at 100 moi or AdE4ORF1 or AdE4ORF1 at 20 moi in serum/cytokine-free medium for 48 h. Immunoblot of lysates using polyclonal anti-phospho-ser473 Akt antibody (pAkt) (Upper) and total Akt antibody (Akt) (Lower) are shown. (B) PECs were transfected with 20 nM E4ORF1 siRNA, E4ORF6 siRNA or control GFP siRNA for 24 h and then exposed to AdE4ORF1 \rightarrow 7 vectors (100 moi) in serum/cytokine-free medium for 2 days. Western blot analysis using anti-phospho-Akt antibody (Upper) or anti-total Akt antibody (Lower). (C) PECs (HUVECs) were infected with lenti-E4ORF1 or lenti-GFP for 3 days followed by Western blot for pAkt (Upper) or Akt (Lower). (D) Cell viability (MTT assay) of Lenti-E4ORF1 or control HUVECs incubated serum/cytokine-free for 5 days \pm wortmannin A (1 μ M), rapamycin (8 nM), or NK- κ B inhibitor CAPE (2 μ M). (E) Lenti-E4ORF1 HUVECs incubated (serum/cytokine-free) in presence or absence of 10 μ M PD98059 (PD) or SB203580 (SB) for 2 days before cell viability analysis (*P* is not significant). (F) E4ORF1 $^{+}$ and E4ORF1 $^{-}$ ECs stimulated with or without 50 ng/ml of VEGF-A for 10 min followed by Western blot for phospho-MAPK (Upper) or level of β -actin (Lower).

phorylation at Ser-473 (Fig. 2C), as compared with control or to infection with lenti-GFP, indicating that Akt activation is E4ORF1-specific.

To test whether transduction with lenti-E4ORF1 prolongs PEC survival through PI3K, mTOR, or NF- κ B signaling, we treated lenti-E4ORF1-infected ECs (E4ORF1 $^{+}$ ECs) with rapamycin, an inhibitor of the mTOR signaling, wortmannin, an inhibitor of PI3-kinase signaling or caffeic acid phenethyl ester (CAPE), a NK- κ B inhibitor. Wortmannin significantly suppressed the pro-survival effect of E4ORF1, suggesting that PI3-kinase activation of Akt is the primary mechanism by which E4ORF1 supports survival of ECs (Fig. 2D). However, either rapamycin or CAPE only partially inhibited the survival of E4ORF1 $^{+}$ ECs, whereas the combination of rapamycin and CAPE completely blocked the survival of E4ORF1 $^{+}$ ECs under serum/cytokine-free conditions (Fig. 2D). These data suggest that E4ORF1-mediated activation of the PI3-kinase–Akt pathway promotes long-term survival of PECs through recruitment of both mTOR and NF- κ B signaling pathways.

To determine whether E4ORF1 might also activate MAPK, E4ORF1 $^{+}$ ECs were treated with MAPK inhibitors PD98059 (PD for ERK), SB203580 (SB for p38-MAPK), respectively. Neither SB nor PD had significant effects on the survival of E4ORF1 $^{+}$ ECs (Fig. 2E). Indeed, in contrast to the sustained pAkt activation observed in E4ORF1 $^{+}$ ECs, there was no baseline activation of MAPK in E4ORF1 $^{+}$ ECs as seen in control E4ORF1 $^{-}$ ECs (Fig. 2F). Therefore, E4ORF1-mediated prolongation of PEC survival

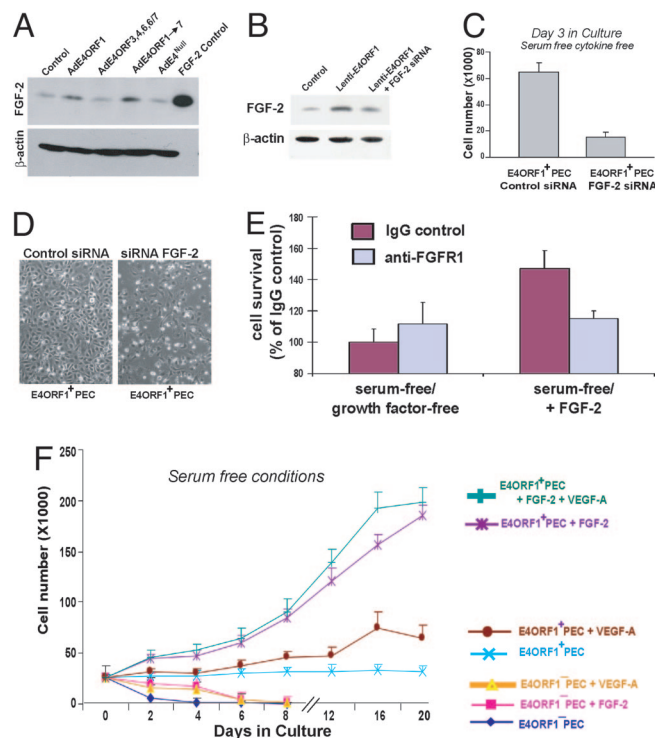


Fig. 3. E4ORF1-mediated activation of FGF-2/FGF receptor pathway supports survival and proliferation of ECs. (A) HUVECs were infected with AdE4ORF1, AdE43,4,6,6/7 at 20 moi, or AdE4ORF1 \rightarrow 7, or AdE4 Null (lacking all E4ORFs) at 100 moi in serum/cytokine-free medium for 48 h. Lysates were analyzed by immunoblot using polyclonal anti-FGF2 and anti- β -Actin antibodies. (B–D) Lenti-E4ORF1 HUVECs were transfected with siRNA against FGF-2 for 48 h. (B) Lysates were analyzed by immunoblot using polyclonal anti-FGF2, anti-FGF receptor-1, and anti- β -actin antibodies. (C) Quantification of ECs in serum/cytokine-free conditions by trypan blue exclusion. (D) Phase-contrast microscopy of E4ORF1 $^{+}$ ECs treated with control siRNA or FGF-2 siRNA, demonstrating detachment and death of PECs treated with siRNA against FGF-2 in serum/cytokine-free conditions. (Magnification: 200 \times .) (E) Lenti-E4ORF1 infected-HUVECs were treated with anti-FGF-R1 antibody or rabbit-IgG in serum/cytokine-free M199 with or without FGF-2 for 5 days and quantitated with the MTT assay. Data are mean \pm SD of 3 experiments. (F) HUVECs were transfected with either lenti-GFP (control, E4ORF1 $^{-}$) or lenti-E4ORF1 (E4ORF1 $^{+}$) enumerated in serum/cytokine-free conditions in the presence and absence of FGF-2 (5 ng/ml) and/or VEGF-A at 10 ng/ml. Data represent mean \pm SD (*n* = 3).

involves Akt activation without stimulating the MAPK pathway. However, stimulation with VEGF-A resulted in a comparable activation of pMAPK observed in E4ORF1 $^{+}$ ECs or control (E4ORF1 $^{-}$ ECs). These data indicate that E4ORF1 $^{+}$ ECs maintained their responsiveness to VEGF-A.

E4ORF1 Increases PEC Survival Through the FGF-2/FGF-R1 Pathway. E4ORF1 $^{+}$ ECs (HUVECs) express FGF receptor 1 (FGF-R1) (Table S1). E4ORF1 $^{+}$ ECs have increased FGF-2 protein expression, when compared with naïve PECs or PECs transduced with E4ORF1 null vectors (Fig. 3A and B). FGF-2 knockdown partially blocked lenti-E4ORF1 increase in FGF-2 or survival of PECs (Fig. 3C and D). Knockdown of FGF-2 by siRNA was confirmed by Western blot analysis and suppressed E4ORF1-induced FGF-2 protein expression (Fig. 3B).

We also demonstrated that a specific mAb to human FGF-R1 (clone H7), selectively blocked the proliferation of E4ORF1 $^{+}$ ECs afforded by exogenous FGF-2 (Fig. 3E). However, inhibition of E4ORF1 $^{+}$ ECs by mAb to FGF-R1, in the absence of exogenous FGF-2, had no major effect on the survival of the ECs, suggesting that intracrine activation of FGF-2/FGF-R1 may play a role in supporting the survival of E4ORF1 $^{+}$ ECs (Fig. 3E). Indeed, although SU5402 (an inhibitor of FGF-R1 tyrosine kinase activity)

blocked expansion of recombinant FGF (rFGF)-treated E4ORF1⁺ ECs, the VEGF-A receptor inhibitor (SU5416) did not significantly affect the survival of E4ORF1⁺ ECs (Fig. S2). These data suggest that E4ORF1 may exert its prosurvival effect in part through activation of the FGF-2/FGF-R1 complex.

To confirm these results, we found that in the absence of any serum or cytokines, E4ORF1⁺ ECs (HUVECs) survive for several weeks without proliferation, maintaining their contact-inhibited status (Fig. 3F). By contrast, in serum/cytokine-free conditions, E4ORF1⁻ ECs underwent apoptosis. Addition of rFGF-2 to serum/cytokine-free cultures of E4ORF1⁺ ECs augmented proliferation of these cells. Although initially rFGF-2 also supported the survival of E4ORF1⁻ ECs, such cells became progressively apoptotic. Addition of recombinant VEGF-A (rVEGF-A) alone or in conjunction with rFGF-2 also increased the expansion of E4ORF1⁺ ECs. These data suggest that E4ORF1⁺ ECs retain their responsiveness to VEGF-A and FGF-2 and that, under serum/cytokine-free conditions, E4ORF1-mediated activation of pAkt supports the survival of E4ORF1⁺ ECs.

E4ORF1 Maintains the Angiogenic Properties of PECs. Similar to FGF-2 induced migration, we found that AdE4ORF1⁺ ECs have retained their capacity to migrate in a wound assay in serum/cytokine-free conditions (Fig. S3). In contrast, uninfected E4ORF1⁻ ECs in serum/cytokine-free medium were unable to migrate, instead undergoing apoptosis. PECs infected with either AdE4ORF1 or lenti-E4ORF1 also maintained their ability to form tubes in serum/cytokine-free conditions, whereas naïve PECs or AdE4ORF3,4,6,6/7-infected ECs (lacking E4ORF1) failed to form stable tubes (Fig. S3). These data indicate that E4ORF1⁺ ECs maintain their angiogenic potential in vitro.

To determine whether E4ORF1⁺ ECs (HUVECs) could survive in vivo and assemble into functional neo-vessels, E4ORF1⁺ ECs and naïve PECs were implanted in the flanks of NOD-SCID mice. Whereas normal PECs were barely detectable 5 days after s.c. injection (Fig. 4A), E4ORF1⁺ ECs survived, established vascular foci (Fig. 4B), and assembled into vascular-type structures containing red blood cells (Fig. 4C and D). Immunostaining with species-specific antibodies revealed labeling of nests of engrafted human PECs surrounded by adjacent mouse vessels (Fig. 4E and F). Consistent with the biochemical profile of E4ORF1⁺ ECs in vitro, FGF-2 expression was detected in the engrafted tissue (Fig. 4G). Staining of these sections with a phospho-Akt-specific antibody shows that E4ORF1⁺ ECs had maintained their Akt activation (Fig. 4H). These data indicate that after transplantation, E4ORF1⁺ ECs retained their Akt-dependent neo-angiogenic potential, forming vascular structures without generating tumors. To interrogate the capacity of E4ORF1⁺ ECs to assemble into functional vessels in the Matrigel implants in the absence of exogenous cytokines, E4ORF1⁺ ECs (transduced with lenti-E4ORF1) or naïve ECs (labeled with lenti-GFP) were inoculated into the flanks of NOD-SCID mice. After 2 weeks, vascular perfusion labeling was performed to assess functional human-derived GFP⁺ neo-vessels. Similar to naïve PECs (Fig. 5A and C), E4ORF1⁺ ECs (Fig. 5B and D) were able to assemble into functional, branching GFP⁺lectin⁺ neo-vessels connecting to the host vessels (Fig. 5C and D). The E4ORF1⁺ ECs also gave rise to long, sprouting GFP⁺lectin⁺ neovessels (Fig. 5E). These data suggest that E4ORF1⁺ ECs maintain their angiogenic repertoire, even in the absence of FGF-2 and VEGF-A supplementation.

E4ORF1⁺ ECs Form Neo-Vessels in Leukemic Xenografts. To further assess the angiogenic properties of E4ORF1⁺ ECs in vivo, we comingled human HL60 leukemia cells with E4ORF1⁺ ECs and inoculated them s.c. in NOD-SCID mice. Whereas normal ECs do not survive within human tumor xenografts (data not shown), the human VE-cadherin⁺ E4ORF1⁺ ECs not only survived for at least 10 days within the HL60 tumors but also formed vessel-like

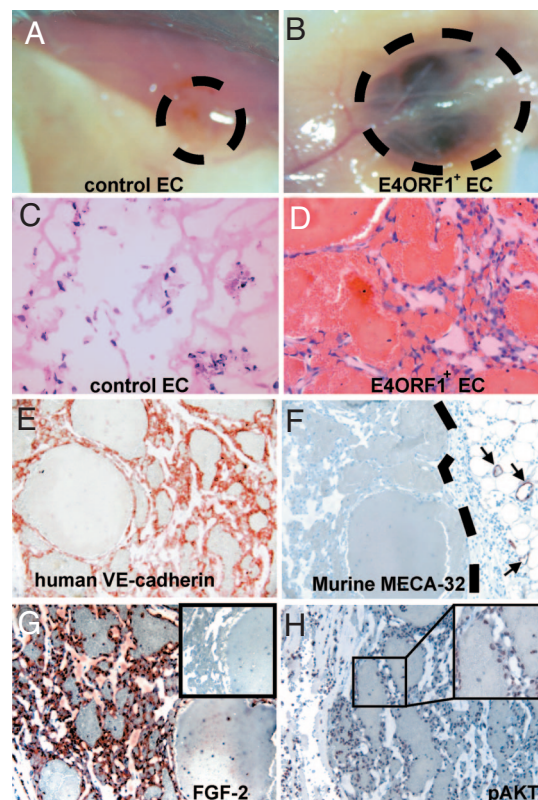


Fig. 4. E4ORF1⁺ ECs maintain their angiogenic potential in vivo. Mice received s.c. inoculations of Matrigel-containing PECs. (A–D) In the absence of E4ORF1, PECs (HUVECs) survived poorly after 5 days, forming barely detectable plugs (A and C), whereas those expressing E4ORF1 formed hemorrhagic lesions, both at the gross level (B) or in histologic sections stained with hematoxylin and eosin (D). (E–H) Immunohistochemistry (red-brown staining) for human ECs (anti-human VE-cadherin) revealed a strong signal in areas containing E4ORF1⁺ PEC (E), whereas mouse ECs (labeled with MECA-32) were detectable only adjacent to the PEC clusters (F, arrows indicate mouse vessels). (G) Abundant FGF-2 expression was present in E4ORF1⁺ HUVECs. Rabbit IgG control is shown in the *Inset*. (H) Presence of anti-phosphoAkt staining (red-brown) in nuclei of E4ORF1⁺ HUVECs was consistent in vitro results showing chronic Akt activation. Boxed area is enlarged and is shown in the *Inset*. (Magnifications: 200 \times .)

structures with a lumen (Fig. 5F and G). Double labeling for mouse (MECA32) and human endothelium (human VE-cadherin) (Fig. 5G) confirmed not only the species specificity of the antibodies but also revealed formation of E4ORF1⁺ vessels within the tumor. These data reveal the potency of E4ORF1 to maintain an angiogenic phenotype in ECs in vivo.

E4ORF1⁺ ECs Support Expansion of Human Leukemic Cells and Embryonal Carcinomas in Serum/Cytokine-Free Conditions. We have shown that angiogenesis is critical for the growth of leukemic HL60 xenografts (4). However, it has been unclear whether PECs in serum/cytokine-free conditions could support the proliferation of HL60 cells. Here, we found that although in serum/cytokine-free conditions HL60 cells underwent apoptosis, E4ORF1⁺ ECs maintained their physical integrity and supported expansion of HL60 cells (Fig. 5H). We have demonstrated that ECs support expansion of leukemic cells without the addition of exogenous cytokines or serum. Notably, in the case of HL60 cells, coculture of these cells in the upper chamber of porous Transwells physically separated from E4ORF1⁺ ECs resulted in failure of the expansion of HL60 (data not shown). However, E4ORF1⁺ ECs supported the expansion of human embryonal carcinoma cells that were plated in the upper chambers of Transwells. These data suggest that in serum/cytokine-free conditions, E4ORF1⁺ ECs could support growth of

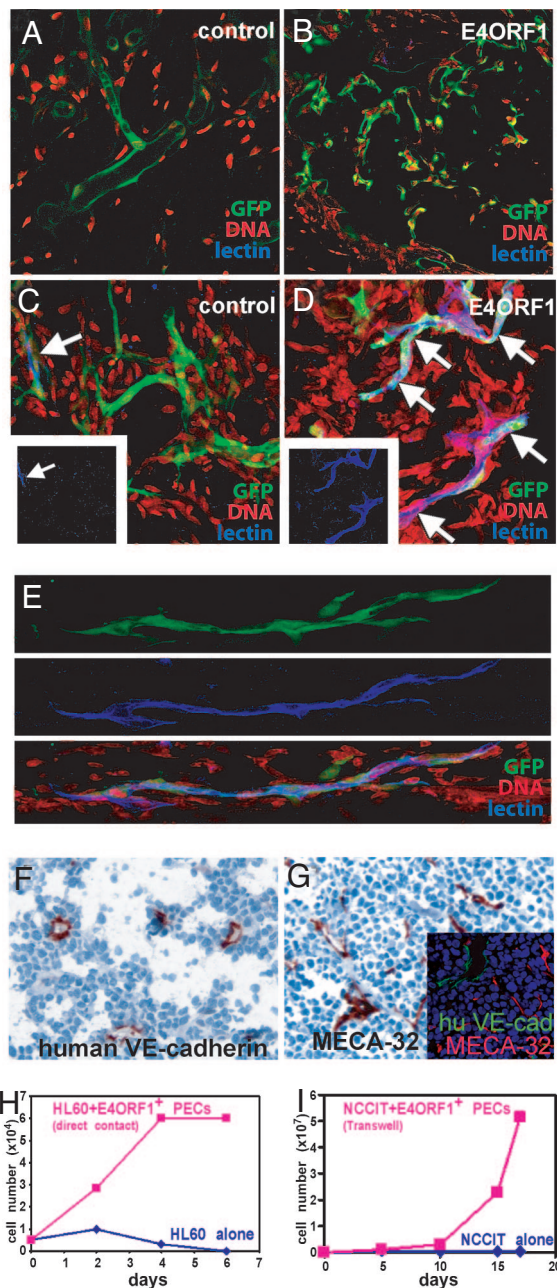


Fig. 5. Generation of functional E4ORF1⁺ ECs capable of tubulogenesis, sprouting, and support of tumor cell growth. (A–E) Mice received s.c. inoculation of 10×10^6 GFP-expressing control PECs (A and C) or GFP-expressing E4ORF1⁺ HUVECs (B, D, and E) and received i.v. human specific-UEA-1 lectin just before death 14 days later. UEA-1 staining (blue signal) denotes vessels that were functional at the time of death. (A and B) Individual confocal slices through areas containing GFP⁺ (green) vessel-like structures. (C–E) Full thickness projections through areas containing functional donor-derived human vessels. The nucleic acid counterstain is red. (F and G) HL60 tumor cells coinoculated with E4ORF1⁺ PECs s.c. and grown for 21 days before harvest. Occasional vessel-like structures with a lumen were labeled by anti-human VE-cadherin (F) within HL60 tumors that had been coinoculated with E4ORF1⁺ HUVECs, although the vast majority of vessels were MECA-32⁺ and of host origin (G). (Inset) Confocal analysis revealed little colocalization of mouse and human markers in chloromas but confirmed specificity of the antibodies. (H and I) Serum/cytokine-free culture of either HL60 leukemia cells (in direct contact) (H) or NCCIT embryonal carcinoma cells (in Transwells) (I) in the presence (pink line) or absence (blue line) of E4ORF1⁺ HUVECs with enumeration at the indicated intervals. (Magnifications: 20 \times objective.)

certain tumors by release of soluble cytokines, in the case of embryonal carcinomas, or perhaps by elaboration of membrane-bound cytokines, in the case of leukemias. Therefore, E4ORF1⁺ ECs establish a durable vascular niche, supporting the expansion of leukemias and solid tumors in the absence of any exogenous growth factor and serum and thereby providing a means to address the mechanisms of cellular cross-talk without previously confounding variables, such as the presence of exogenous growth factors or serum.

Discussion

We have exploited the capacity of the Ad E4 gene to activate cell survival signals to identify factors that can preserve long-term PEC integrity. Of the 6 gene products expressed by the Ad E4 gene complex, we have discovered that the E4ORF1 gene product endows PECs with the capacity to survive and preserves their angiogenic potential in the absence of serum or PEC growth supplements. Although naïve PECs underwent apoptosis within 6 h in cytokine/serum-free conditions, E4ORF1⁺ ECs supported long-term expansion of leukemic cells and embryonal carcinoma cells under serum/cytokine-free conditions, without themselves undergoing apoptosis. E4ORF1 primarily increases the survival of human PECs without promoting MAPK-dependent cell proliferation. Indeed, through tonic stimulation of PI3-kinase–Akt and recruitment of both mTOR and NF- κ B pathways, E4ORF1 supported the survival of ECs without immortalization and without activation the MAPK pathway. E4ORF1 increased the survival of ECs, in part by maintaining the expression of FGF-2 and activation of the FGF-2/FGF-R1 pathway. E4ORF1⁺ ECs sustained the angiogenic profile of ECs and assembled into functional neo-vessels in vitro and in vivo and contributed to angiogenesis in leukemic chloromas. VEGF-A activation of E4ORF1⁺ ECs activated MAPK to the same extent as naïve PECs, suggesting that E4ORF1⁺ ECs retain functionality of VEGF-A receptors. The selective prosurvival effect of E4ORF1 allowed isolation of pure populations of organ-specific PECs from multiple adult organs. As such, E4ORF1 facilitated isolation of organ-specific ECs. The use of E4ORF1⁺ ECs will enable the use of minimal media conditions (i.e., exogenous cytokine/serum-free media) in the study of EC-derived signals in regulating normal and cancer homeostasis.

The mechanisms by which ECs support organogenesis and tumorigenesis are not known (1, 2, 4, 5). To date, the use of immortalized ECs lines has manifested only marginal benefit in elucidating the factors that support tumorigenesis. Furthermore, studies to evaluate the contribution of ECs have been obscured by the presence of stem cell-active cytokines, which can by themselves promote the survival of stem and tumor cells. In fact, it is unclear in published studies (3, 5) whether the proliferation of stem cells was mediated by elaboration of factors by ECs or exogenous factors. Therefore, the unique capacity of E4ORF1 to maintain the neo-angiogenic state of PECs without the otherwise ubiquitous requirement for recombinant cytokines and serum provides for a radically different platform to identify the factors that promote the growth of stem and tumor cells. In addition, the introduction of E4ORF1 allows for purification of organ-specific ECs. These findings enable the study of the heterogeneity of ECs without compromising their neo-angiogenic integrity.

The maintenance of angiogenesis without oncogenic transformation is a feature of the E4ORF1 gene product that is uniquely conferred to human ECs. Although unstimulated E4ORF1⁺ ECs can survive for days, upon stimulation with VEGF-A and/or FGF-2, E4ORF1⁺ ECs proliferate in a contact-inhibited manner. The prosurvival effect of E4ORF1 was mediated through activation of the PI3K–Akt signaling pathway. Inhibition of both mTOR and NF- κ B signaling was essential to completely abrogate the prosurvival effect afforded by the E4ORF1 gene. These results suggest that the E4ORF1 protein augments the survival of PECs primarily by recruiting the PI3-kinase–Akt/mTOR and PI3-kinase–Akt/NF- κ B pathways without oncogenic transformation.

The mechanism by which E4ORF1 sustains tonic activation of the PI3-kinase–Akt pathway remains unknown and may involve interaction with as yet unrecognized signaling chaperones. Studies from murine cells transduced with E4ORF1 suggest that E4ORF1 protein might tonically activate PI3-kinase through interaction with membrane-associated PDZ proteins, including MUPP1 and ZO-2 (9). It remains to be investigated whether E4ORF1 induces Akt activation in PECs through interaction with PDZ proteins.

E4ORF1 activation of the FGF-2/FGF-R1 pathway may also contribute to the survival of PECs. Anti-FGF-R1 mAb was effective in blocking (exogenous) FGF-2-driven proliferation of E4ORF1⁺ ECs. However, anti-FGF-R1 antibody failed to significantly block survival of unstimulated E4ORF1⁺ cells. Thus, the effect of E4ORF1 on the angiogenic process may result from an increase in endogenous FGF-2 expression, activating an autocrine positive feedback loop. Nonetheless, only partial inhibition of E4ORF1-mediated survival of PECs was seen with either knock-down of FGF-2 expression or mAb to the extracellular domain of FGF-R1. These data suggest that FGF-2/FGF-R1 activation plays only a partial role in supporting the survival of E4ORF1⁺ ECs and that other angiogenic pathways might synergize with FGF-2 in supporting the survival of E4ORF1⁺ cells.

In summary, the E4ORF1 Ad gene product, through chronic activation of the PI3-kinase–Akt/mTOR/NF- κ B pathways, enhances survival and maintains the angiogenic repertoire in PECs without cellular transformation even in the absence of growth factors and serum. Further studies are needed to identify the specific proangiogenic factors that interact with the E4ORF1 gene product. E4ORF1⁺ ECs will increase our understanding of the mechanism by which vascular cells regulate homeostasis of normal and cancer stem cells, thereby opening up new avenues of research into the mechanisms of organogenesis and tumorigenesis.

Materials and Methods

Isolation of PECs from Various Tissues. HUVECs and BM ECs were isolated as described (11) and cultured in EC growth medium (M199, 10% FBS, 20 μ g/ml EC growth supplement, and 20 units/ml heparin). Human dermal lymphatic ECs were obtained from Cambrex. Human testicular tissue, cord blood, and BM were obtained through Institutional Review Board-approved protocols and digested enzymatically, and PECs were prepared as described in the SI (12).

Once PECs were transduced with E4ORF1 gene they were then cultured in serum/cytokine-free X-Vivo 20 or EC medium for routine expansion. All experiments described herein were performed with HUVEC-derived E4ORF1⁺ ECs, except as specifically noted.

Construction of Ad Vectors. The Ad vectors used in this study were: (i) AdE41 \rightarrow 7 vector (serotype 5), which expresses the gene products of E4ORF1 to E4ORF6, and E4ORF6/7 but has deletions of the E3 and E1 gene complexes with no transgene in the expression cassette; (ii) AdE4ORF6, which expresses only E4ORF6 and has deletions of E4ORFs 1,2,3,3/4,7; (iii) AdE4ORF1 (H5 dl1004), which expresses only E4ORF1 and has deletions of E4ORFs 2,3,4,5,6/7; (iv) AdE4ORF3,4,3/4,6,6/7 (H5 dl1004), which expresses E4ORFs 3,4,3/4,6,6/7 but has deletions of E4ORF1 and E4ORF2; (v) AdE4ORF4, which expresses only E4ORF4 and has deletions of all other E4ORFs; and (vi) AdE4^{Null}, which lacks the expression of all E4ORFs. The AdE4ORF4, AdE4ORF3,4,3/4,6,6/7, and AdE4ORF1 virus vectors were propagated on W162, a Vero-derived, E4-complementing cell line. AdE41 \rightarrow 7, AdE4^{Null}, and AdE4ORF6 were amplified in 293 cells and purified by cesium chloride centrifugation and dialysis. All Ad vectors had a particle/pfu ratio of 100.

Generation of Lentivirus. The Ad E4ORF1 gene (serotype 5) was cloned into the pCCL-PGK lentivirus vector. Lentiviruses were generated by cotransfecting 15 μ g of lentiviral vector, 3 μ g of pENV/VSU-G, 5 μ g of pRRE, and 2.5 μ g of pRSV-REV in 293T cells (passage 8–10; subconfluent) by the calcium precipitation method. Supernatants were collected 40 and 64 h after transfection.

siRNA Preparation and Transfection. The siRNAs for E4ORF1 (target sequence, 5'-GAAUCAACUGAUGUGUUU-dTdT-3'), E4ORF6 (target sequence, 5'-GCCAAACGCUCAUCAGUGAUA dTdT-3'), and FGF-2 (target sequence, 5'-ACCCU-CACAUCAAGCUACAACUUC A) were designed and synthesized by Invitrogen (Stealth RNAi). Twenty nanomoles of the indicated siRNA preparations control siRNA (scrambled sequence and GFP) was transfected individually into PECs by using Lipofectamine 2000. The transfection efficiency of each duplex siRNA (\approx 80%) was confirmed by using Block-iT Fluorescent Oligo (Invitrogen).

Cell Survival Assay for PECs Treated with Antisurvival Factors. The MTT-based alamarBlue (Invitrogen) reagent was used to assess cell survival. E4ORF1⁺ ECs were plated at a density of 1.5×10^4 cells per well on gelatin-coated 96-well plates and incubated in growth medium overnight. Cells were treated with PI3-kinase inhibitor, wortmannin (1 μ M), mTOR inhibitor rapamycin (8 nM), or NK- κ B inhibitor CAPE (2 μ M) in serum/cytokine-free M199 for 5 days. For the FGF-R1 blocking experiments, E4ORF1⁺ ECs were treated with 10 μ g/ml of FGF-R1 blocking mAb (clone H7; Imclone Systems) or 10 μ g/ml rabbit-IgG in serum/cytokine-free M199 with or without 10 ng/ml FGF-2 and heparin for 5 days. At the day 5, medium was changed to M199 containing alamarBlue reagent, and the fluorescence (Ex530/Em590) was measured after 3 more 1-h incubations.

Western Blot Analysis. Cells were lysed in RIPA buffer and immunoblotted with antibodies to Ser-473-phospho-Akt, total Akt, FGF-2, and β -actin. For measurement of pMAPK, E4ORF1⁻ ECs and E4ORF1⁺ ECs were serum-starved for 6 h and 3 days, respectively, followed by stimulation with 50 ng/ml of VEGF-A for 10 min. pMAPK was detected with polyclonal rabbit anti-pMAPK p44/42 antibody (9101; Cell Signaling). Signal was developed with a ECL Chemiluminescence Kit.

Determination of Contribution of E4ORF1⁺ ECs to Angiogenesis in Vivo and in Vitro. E4ORF1⁺ ECs were inoculated with Matrigel and/or HL60 leukemic cells. For assessment of angiogenesis in vivo E4ORF1⁺ ECs and E4ORF1⁻ ECs (5×10^6 cells) were mixed with Matrigel and implanted in the flanks of 2 groups of mice (Fig. 4). The mice were killed after indicated times and tissue was snap-frozen in OCT (Tissue-Tek). Functionality of donor derived blood vessels was assessed by perfusion labeling as detailed in the SI.

For in vitro proliferation assays human HL60 or embryonal carcinoma cells (NCCIT) from ATCC were cocultured in the presence or absence of E4ORF1⁺ ECs in serum/cytokine-free conditions either in direct cellular contact with E4ORF1⁺ ECs or the upper chambers of Transwells (0.4- μ m pore size) physically separated from the E4ORF1⁺ ECs, which were plated in the lower chamber of the plates. Subsequently, the number of proliferating cancer cells was determined by trypan blue exclusion.

Immunostaining. Rabbit polyclonal anti-human FGF-2 and phospho-Akt (Santa Cruz Biotechnology), goat polyclonal anti-human VE-cadherin I (R&D), MECA-32 rat anti-mouse endothelial (BD Pharmingen), mouse anti-human CD34, anti-human CD31, and rabbit anti-human VWF (Dako) antibodies were used for confirmation of PEC identity by immunostaining.

ACKNOWLEDGMENTS. This work was supported by the Howard Hughes Medical Institute, the Ansary Stem Cell Institute, National Heart, Lung, and Blood Institute Grants HL075234, HL059312, and HL084936 (to S. Rafii) and HL59312 (to N.R.H.), a Memorial Sloan Kettering Cancer Center T32 grant (to M.S.), and the American Association for Cancer Research–Genentech BioOncology Fellowship for Cancer Research on Angiogenesis (to M.S.). M.S. is a New York Stem Cell Foundation Fellow.

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