Fibroblast Growth Factor-2 Is a Downstream Mediator of Phosphatidylinositol 3-Kinase-Akt Signaling in 14,15-Epoxyeicosatrienoic Acid-induced Angiogenesis*

Baolin Zhang, Huiqing Cao, and Gadiparthi N. Rao

From the Department of Physiology, University of Tennessee Health Science Center, Memphis, Tennessee 38163

To determine the efficacy of cytochrome P450 2C9 metabolites of arachidonic acid, viz. 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs), in inducing angiogenesis, we have studied their effects on human dermal microvascular endothelial cell (HDMVEC) tube formation and migration. All four EETs stimulated HDMVEC tube formation and migration in a dose-dependent manner. Because 14,15-EET was found to be slightly more efficacious than 5,6-, 8,9-, and 11,12-EETs in stimulating HDMVEC tube formation and migration, we next focused on elucidation of the signaling mechanisms underlying its angiogenic activity. 14,15-EET stimulated Akt and S6K1 phosphorylation in Src- and phosphatidylinositol 3-kinase (PI3K)-dependent manner in HDMVECs. Inhibition of Src and PI3K-Akt-mTOR signaling by both pharmacological and dominant-negative mutant approaches suppressed 14,15-EET-induced HDMVEC tube formation and migration in vitro and Matrigel plug angiogenesis in vivo. In addition, 14,15-EET induced the expression of fibroblast growth factor-2 (FGF-2) in Src- and PI3K-Akt-dependent and mTOR-independent manner in HDMVECs. Neutralizing anti-FGF-2 antibodies completely suppressed 14,15-EET-induced HDMVEC tube formation and migration in vitro and Matrigel plug angiogenesis in vivo. Together, these results show for the first time that Src and PI3K-Akt signaling via targeting in parallel with FGF-2 expression and mTOR-S6K1 activation plays an indispensable role in 14,15-EET-induced angiogenesis.

Arachidonic acid is an important polyunsaturated fatty acid component of membrane phospholipids and is released acutely in response to a number of agonists, including growth factors, cytokines, and hormones, in various cell types (1–4). Upon release, it is either metabolized via the cyclooxygenase, lipooxygenase, or cytochrome P450 monooxygenase pathway, producing prostaglandins, hydroperoxyeicosatetraenoic acids, and epoxyeicosatrienoic acids (EETs), respectively, or reincorporated into membrane phospholipids via esterification involving sequential actions of arachidonoyl-CoA synthase and arachidonoyl-lyso phospholipid transferase (1, 5). Arachidonic acid and its oxygenative metabolites, collectively known as eicosanoids, are involved in the regulation of important biological activities such as the maintenance of vascular tone (5, 6). In addition, these lipid molecules have been reported to mediate various intracellular signaling events in response to various external cues (7–11). Studies over the past several years also suggest that the cyclooxygenase, lipooxygenase, and cytochrome P450 monooxygenase metabolites of arachidonic acid influence the proliferative capacity of various cell types and play a role in the pathogenesis of numerous types of cancers (12–20). Despite the involvement of eicosanoids in the development of various types of cancers, their mechanisms of actions are poorly understood.

Formation of new capillaries, a process known as angiogenesis, plays an important role in embryonic development and wound healing (21, 22). In addition, angiogenesis is a crucial player in the development of various tumors (21–25). Migration and proliferation of microvascular endothelial cells are essential events of angiogenesis (25). Factors such as fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor that influence endothelial cell migration and proliferation are therefore likely involved in embryonic development and disease processes (21–25). The reports that eicosanoids such as 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), 12(R)-HETE, 11,12-EET, and 14,15-EET induce angiogenesis (14, 26–28) provide clues for elucidation of the possible mechanisms by which these lipid mediators could be involved in the development of various types of cancers. Phosphatidylinositol 3-kinase (PI3K)-Akt-mTOR-S6K1 signaling has been reported to play a major role in mediating cell survival and proliferation and angiogenesis in response to various stimulants, including hypoxia and nitric oxide (29–34). To understand the role of eicosanoids in the development of various types of cancers, we have undertaken a systematic study to identify eicosanoids with potent angiogenic activities and to elucidate the underlying signaling mechanisms. Here, we report that 14,15-EET, the CYP2C9 metabolite of arachidonic acid, stimulates human dermal microvascular endothelial cell (HDMVEC) tube formation and migration in vitro and Matrigel plug angiogenesis in vivo. In addition, we present evidence that 14,15-EET-induced HDMVEC tube formation and migration and Matrigel plug angiogenesis require activation of Src and PI3K-Akt signaling via targeting in parallel with FGF-2 expression and mTOR-S6K1 stimulation.

MATERIALS AND METHODS

Reagents—Aprotinin, dithiothreitol, Drabkin’s reagent, HEPES, leupeptin, phenylmethlysulfonyl fluoride, sodium deoxycholate, and sodium orthovanadate were purchased from Sigma. 5,6-, 8,9-, 11,12-, and 14,15-EETs were bought from Cayman Chemical (Ann Arbor, MI). Growth factor-reduced Matrigel (catalog no. 354250) was obtained from BD Biosciences. LY294002 and PP2 were procured from Calbiochem. Anti-phospho-Akt (catalog no. 9271), anti-phospho-S6K1 (catalog no. 9205), and anti-phospho-Src (catalog no. 2101) antibodies and total anti-Akt (catalog no. 9272) and anti-S6K1 (catalog no. 9202) anti-

* This work was supported by National Institutes of Health Grant HL74860 (to G. N. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Physiology, University of Tennessee Health Science Center, 894 Union Ave., Memphis, TN 38163. Tel.: 901-448-7321; Fax: 901-448-7126; E-mail: grao@physio1.utmem.edu.

2 The abbreviations used are: EETs, epoxyeicosatrienoic acids; FGF-2, fibroblast growth factor-2; HETE, hydroxyeicosatetraenoic acid; PI3K, phosphatidylinositol 3-kinase; HDMVEC, human dermal microvascular endothelial cell; dnAkt, dominant-negative Akt; Ad-GFP, adenooviral vector expressing green fluorescent protein; Ad-dnAkt, adenooviral vector expressing dominant-negative Akt.
bodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-FGF-2 antibodies (catalog no. SC-079) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Neutralizing mouse anti-FGF-2 monoclonal antibodies (catalog no. 05-117) and rabbit anti-Src polyclonal antibodies (catalog no. 05-184) were bought from Upstate Biotechnology, Inc. (Lake Placid, NY). A human FGF-2 enzyme-linked immunosorbent assay kit (catalog no. DFB50) was obtained from R&D Systems (Minneapolis, MN). Rapamycin and wortmannin were bought from BIOMOL (Plymouth Meeting, PA).

Cell Culture—HDMVECs were bought from Clonetics (Walkersville, MD). Cells were grown in endothelial basal medium-2 (catalog no. CC-3156) containing EGM-2 MV SingleQuots (catalog no. CC-4147).

FIGURE 1. EETs induce HDMVEC tube formation and migration. A, quiescent HDMVECs were seeded onto 96-well plates coated with growth factor-reduced Matrigel. Cells were then treated with vehicle or various concentrations of the indicated EETs for 6 h, and tube formation was observed under an inverted microscope. The images were captured with a CCD color camera, and the tube length was measured using NIH Image J (Version 1.31). B, the representative images of HDMVEC tube formation in response to the indicated EETs (0.1 μM) are shown. C, quiescent HDMVECs were added to the upper chamber of the cell culture inserts that were placed in a 24-well plate. Vehicle or various concentrations of the indicated EETs were added to the lower chamber. After 8 h of incubation at 37 °C, the inserts were lifted out from the 24-well plate, and the migrated cells were fixed in methanol, stained with Giemsa-Wright stain, and counted under a light microscope. The values are the means ± S.D. of three separate experiments. *, p < 0.05 versus the control; **, p < 0.01 versus the control.
(Cambrex Bio Science Walkersville, Inc., Walkersville, MD). Cultures were maintained at 37 °C in a humidified 95% air and 5% CO2 atmosphere. Cells were quiesced by incubation in endothelial basal medium-2 for 24 h and used to perform the experiments unless stated otherwise.

**Cell Migration Assay**—The migration assay was performed using a modified Boyden chamber method as described by Nagata et al. (35). The cell culture inserts containing membranes (10-mm diameter and 8.0-μm pore size; Nalge Nunc International, Rochester, NY) were placed in a Costar 24-well tissue culture plate (Corning Inc.). The lower surface of the porous membrane was coated with 0.5% gelatin overnight at 4 °C and then blocked with 0.1% heat-inactivated bovine serum albumin for 1 h at 37 °C. HDMVECs were quiesced for 24 h in endothelial basal medium-2, washed with Hanks’ balanced salt solution, trypsinized, and neutralized with trypsin neutralizing solution (Cambrex Bio Science Walkersville, Inc.). Cells were seeded onto the upper chamber at 1 × 10^5 cells/well. Vehicle, EETs, pharmacological inhibitors, or neutralizing anti-FGF-2 antibodies were added to the lower chamber at the indicated concentrations. To test the effect of dominant-negative Akt (dnAkt) on 14,15-EET-induced HDMVEC migration, cells were infected first with adenoviral vector expressing either green fluorescent protein (Ad-GFP) or dnAkt (Ad-dnAkt) at a multiplicity of infection of 80 and quiesced. Quiescent cells were trypsinized, neutralized, and added to the upper chamber at 1 × 10^5 cells/well. Both the upper and lower chambers contained endothelial basal medium-2. After 8 h of incubation at 37 °C, non-migrated cells were removed from the upper side of the membrane with cotton swabs, and the cells on the lower surface of the membrane were fixed in methanol for 15 min. The membrane was then stained with Giemsa-Wright stain for 10 min (Sigma) and washed once each with 50% and 100% alcohol. Cells were counted in five randomly selected squares/well under a light microscope (Eclipse 50i, Nikon, Tokyo, Japan) and presented as the number of migrated cells/field.

**Matrigel Plug Angiogenesis Assay**—The Matrigel plug assay was performed essentially as described by Medhora et al. (27). C57BL/6 mice (8 weeks old) were lightly anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and injected subcutaneously along the dorsal midline with 0.5 ml of Matrigel premixed with vehicle or 50 mg/kg intraperitoneally) and added to the indicated concentrations. To test the effect of dominant-negative Akt (dnAkt) on 14,15-EET-induced HDMVEC migration, cells were infected first with adenoviral vector expressing either green fluorescent protein (Ad-GFP) or dnAkt (Ad-dnAkt) at a multiplicity of infection of 80 and quiesced. Quiescent cells were trypsinized, neutralized, and added to the upper chamber at 1 × 10^5 cells/well. Both the upper and lower chambers contained endothelial basal medium-2. After 8 h of incubation at 37 °C, non-migrated cells were removed from the upper side of the membrane with cotton swabs, and the cells on the lower surface of the membrane were fixed in methanol for 15 min. The membrane was then stained with Giemsa-Wright stain for 10 min (Sigma) and washed once each with 50% and 100% alcohol. Cells were counted in five randomly selected squares/well under a light microscope (Eclipse 50i, Nikon, Tokyo, Japan) and presented as the number of migrated cells/field.

**Tube Formation Assay**—The tube formation assay was performed as described by Nagata et al. (35). Costar 96-well culture plates (Corning Inc.) were coated with growth factor-reduced Matrigel in a total volume of 20 μl and allowed to solidify for 30 min at 37 °C. HDMVEC were trypsinized, neutralized with trypsin neutralizing solution, and resuspended at 2.5 × 10^5 cells/ml. Two-hundred microliters of this cell suspension was added to each well. Vehicle, EETs, pharmacological inhibitors, or neutralizing anti-FGF-2 antibodies were added to the wells at 50 μM. After 8 h of incubation at 37 °C, non-migrated cells were removed from the upper side of the membrane with cotton swabs, and the cells on the lower surface of the membrane were fixed in methanol for 15 min. The membrane was then stained with Giemsa-Wright stain for 10 min (Sigma) and washed once each with 50% and 100% alcohol. Cells were counted in five randomly selected squares/well under a light microscope (Eclipse 50i, Nikon, Tokyo, Japan) and presented as the number of migrated cells/field.

**Western Blot Analysis**—After appropriate treatments, HDMVECs were rinsed with cold phosphate-buffered saline and frozen immediately in liquid nitrogen. Cells were lysed by thawing in 250 μl of lysis buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate) and scraped into 1.5-ml Eppendorf tubes. After standing on ice for 30 min, the cell lysates were cleared by centrifugation at 12,000 rpm for 20 min at 4 °C. Cell lysates containing equal amounts of protein were resolved by electrophoresis on 0.1% SDS and 10% polyacrylamide gels. The proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond, Amersham Biosciences). After blocking in 10 mM Tris-HCl buffer (pH 8.0) containing 150 mM sodium chloride, 0.1% Tween 20, and 5% (w/v) nonfat dry milk, the membrane was treated

![FIGURE 2. 14,15-EET stimulates phosphorylation of Akt and S6K1 in a time-dependent manner in HDMVECs. Quiescent HDMVECs were treated with vehicle or 0.1 μM 14,15-EET for the indicated times, and cell extracts were prepared. Equal amounts of protein (30 μg) from the control and each treatment were analyzed by Western blotting for phosphorylated Akt (pAkt) and S6K1 (pS6K1) levels using their phospho-specific antibodies. As a loading control, the blots were reprobed with total anti-Akt antibodies.](image2)

![FIGURE 3. Effect of blockade of PI3K and mTOR on 14,15-EET-induced phosphorylation of Akt and S6K1 in HDMVECs. Quiescent HDMVECs were treated with vehicle or 0.1 μM 14,15-EET in the presence and absence of wortmannin (1 μM), LY294002 (10 μM), or rapamycin (50 ng/ml) for 30 min, and cell extracts were prepared. Equal amounts of protein (30 μg) from the control and each treatment were analyzed by Western blotting for phosphorylated Akt (pAkt) and S6K1 (pS6K1) levels using their phospho-specific antibodies. As a loading control, the blots were reprobed with total anti-Akt or anti-S6K1 antibodies.](image3)
with appropriate primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were detected using a chemiluminescence reagent kit (Amersham Biosciences).

**Viral Vectors**—Ad-dnAkt was constructed by Fujio et al. (36) and was generously provided by Dr. Kenneth Walsh (Boston University School of Medicine, Boston, MA) for use in our experiments. To construct Ad-GFP, the GFP DNA fragment was excised from pEGFP-N3 (Clontech) by digestion of the plasmid with SalI and NotI and subcloned into the entry vector pENTR3C (Invitrogen), producing pENTR3C-GFP. pENTR3C-GFP was transformed into Escherichia coli DH5α cells, and the plasmid was amplified. The plasmid was recombined with pAd/CMV/V5-DEST (Invitrogen) as described by the manufacturer, producing plasmid pAd-GFP, and verified by DNA sequencing. pAd-GFP was linearized with PaeI and transfected into human embryonic kidney 293A cells. The resulting adenovirus was further amplified by infection of human embryonic kidney 293A cells and purified by cesium chloride gradient ultracentrifugation (37). Both Ad-GFP and Ad-dnAkt were titrated using a standard plaque assay (37).

**Statistics**—All experiments were repeated three times with similar results. The data on HDMVEC tube formation and migration and Matrigel plug angiogenesis are presented as the means ± S.D. The treatment effects were analyzed by Student’s t test. *p < 0.001 versus the control; **p < 0.001 versus 14,15-EET treatment alone.

**FIGURE 4.** Blockade of PI3K and mTOR suppresses 14,15-EET-induced HDMVEC tube formation and migration. A, quiescent HDMVECs were seeded onto 96-well plates coated with growth factor-reduced Matrigel. Cells were then treated with vehicle or 0.1 μM 14,15-EET in the presence and absence of wortmannin (1 μM), LY294002 (10 μM), or rapamycin (50 ng/ml) for 6 h, and tube formation was observed under an inverted microscope. The images were captured with a CCD color camera, and the tube length was measured using NIH Image J (Version 1.31). B, the representative images of tube formation in response to 0.1 μM 14,15-EET in the presence and absence of wortmannin, LY294002, or rapamycin are shown. C, quiescent HDMVECs were added to the upper chamber of the cell culture inserts that were placed in a 24-well plate. Vehicle or 0.1 μM 14,15-EET was added to the lower chamber in the presence and absence of wortmannin (1 μM), LY294002 (10 μM), or rapamycin (50 ng/ml). After 8 h of incubation at 37 °C, the inserts were lifted out from the 24-well plate, and the non-migrated cells were removed from the upper side of the membrane with a cotton swab. The migrated cells on the lower side of the membrane were fixed in methanol, stained with Giemsa-Wright stain, and counted under a light microscope. The values are the means ± S.D. of three separate experiments. *, p < 0.001 versus the control; **, p < 0.001 versus 14,15-EET treatment alone.
RESULTS

EETs Induce HDMVEC Tube Formation and Migration—To understand the role of the oxygenative metabolites of arachidonic acid in the development of various types of cancers, we have undertaken a systematic study to identify the eicosanoids with potent angiogenic capacities and to elucidate the underlying signaling events. In this study, we focused on studying the effects of various CYP2C9 metabolites of arachidonic acid, viz. 5,6-, 8,9-, 11,12-, and 14,15-EETs, on angiogenesis using in vitro and in vivo model systems. Quiescent HDMVECs were seeded onto 96-well plates coated with growth factor-reduced Matrigel and treated with vehicle or various concentrations of 5,6-, 8,9-, 11,12-, or 14,15-EET for 6 h, and tube formation was measured. All four EETs stimulated HDMVEC tube formation in a dose-dependent manner, with a maximum effect of 2-fold at 0.1 μM (Fig. 1, A and B). Among the four EETs tested, 14,15-EET was found to be slightly more efficacious than 5,6-, 8,9-, and 11,12-EETs in stimulating HDMVEC tube formation. We next determined their effects on HDMVEC migration using a modified Boyden chamber method. Quiescent HDMVECs were added to the upper chamber of the culture inserts that were placed in a 24-well plate, and vehicle or various concentrations of the indicated EET were added to the lower chamber. After incubation at 37 °C for 8 h, the migrated cells on the lower surface of the membrane were fixed, stained with Giemsa-Wright stain, and counted under a light microscope. Simultaneously migrated cells on the lower surface of the membrane were fixed, stained with Drabkin’s reagent, and the hemoglobin was measured. The values are the means ± S.D. of six animals. *, p < 0.01 versus the control; **, p < 0.01 versus 14,15-EET treatment alone.

14,15-EET Activates Akt and S6K1 in HDMVECs—The serine/threonine kinase Akt has been shown to play an important role in mediating angiogenesis in response to various stimulants, including hypoxia and nitric oxide (32–35). We have shown previously that (5S)-HETE, the 5-lipoxygenase metabolite of arachidonic acid, stimulates HDMVEC DNA synthesis via activation of PI3K-Akt-mTOR-S6K1 signaling (38). Therefore, to determine whether PI3K-Akt-mTOR-S6K1 signaling plays a role in angiogenesis induced by 14,15-EET, we studied the time course effect of this eicosanoid on activation of Akt and S6K1 in HDMVECs. Quiescent HDMVECs were treated with vehicle or 0.1 μM 14,15-EET for 30 min, 6 h, and 8 h to measure S6K1 phosphorylation, tube formation, and migration, respectively. A, the levels of phospho-tyrosylated S6K1 (pS6K1) were measured by Western blotting using its phospho-specific antibodies as described in the legend to Fig. 2. The same blot was reprobed sequentially with total anti-S6K1 and anti-Akt antibodies for the purpose of a lane loading control and for the demonstration of overexpression of Akt, respectively. B, shown are the results from the quantitative analysis of the effect of dnAkt on 14,15-EET-induced S6K1 phosphorylation, tube formation, and migration, respectively. C, the representative images of HDMVEC tube formation in response to 0.1 μM 14,15-EET in the presence and absence of adenovirus-mediated expression of dnAkt are shown. D, shown are the results from the quantitative analysis of the effect of dnAkt on 14,15-EET-induced HDMVEC migration. The values are the means ± S.D. of three separate experiments. *, p < 0.01 versus the control; **, p < 0.01 versus 14,15-EET treatment alone.

FGF-2 Mediates 14,15-EET-induced Angiogenesis

FIGURE 5. Blockade of PI3K and mTOR suppresses 14,15-EET-induced angiogenesis in vivo. C57BL/6 mice were injected subcutaneously with 0.5 ml of Matrigel premixed with vehicle or 50 μM 14,15-EET with and without LY294002 (250 μM) or rapamycin (1 μg/ml). Seven days later, the animals were killed, and the Matrigel plugs were harvested from underneath the skin and analyzed for hemoglobin with Drabkin’s reagent. The values are the means ± S.D. of six animals. *, p < 0.01 versus the control; **, p < 0.01 versus 14,15-EET treatment alone.

FIGURE 6. Adenovirus-mediated expression of dnAkt but not GFP suppresses 14,15-EET-induced S6K1 phosphorylation, tube formation, and migration. HDMVECs were infected with either Ad-GFP or Ad-dnAkt at a multiplicity of infection of 80; quiesced; and treated with and without 0.1 μM 14,15-EET for 30 min, 6 h, and 8 h to measure S6K1 phosphorylation, tube formation, and migration, respectively. A, the levels of phospho-tyrosylated S6K1 (pS6K1) were measured by Western blotting using its phospho-specific antibodies as described in the legend to Fig. 2. The same blot was reprobed sequentially with total anti-S6K1 and anti-Akt antibodies for the purpose of a lane loading control and for the demonstration of overexpression of Akt, respectively. B, shown are the results from the quantitative analysis of the effect of dnAkt on 14,15-EET-induced HDMVEC tube formation. C, the representative images of HDMVEC tube formation in response to 0.1 μM 14,15-EET in the presence and absence of adenovirus-mediated expression of dnAkt are shown. D, shown are the results from the quantitative analysis of the effect of dnAkt on 14,15-EET-induced HDMVEC migration. The values are the means ± S.D. of three separate experiments. *, p < 0.01 versus the control; **, p < 0.01 versus 14,15-EET treatment alone.
Fibroblast Growth Factor-2 (FGF-2) Mediates 14,15-EET-induced Angiogenesis

Blockade of Activation of Akt or S6K1 Inhibits 14,15-EET-induced Angiogenesis in Vitro—To determine the role of PI3K-Akt-mTOR-S6K1 signaling in 14,15-EET-induced angiogenesis, we next studied the effects of its inhibitors, viz. wortmannin and LY294002 (39, 40). Quiescent HDMVECs were treated with vehicle or 0.1 μM 14,15-EET in the presence and absence of wortmannin (1 μM) or LY294002 (10 μM) for 30 min, and cell extracts were prepared and analyzed for Akt and S6K1 phosphorylation. Wortmannin and LY294002 completely inhibited 14,15-EET-stimulated Akt and S6K1 phosphorylation (Fig. 3). These results suggest that Akt and S6K1 phosphorylation stimulated by 14,15-EET are dependent on activation of PI3K. To understand the order in which 14,15-EET activates PI3K-Akt-mTOR-S6K1 signaling pathway, we also studied the effect of rapamycin, a specific inhibitor of mTOR (mammalian target of rapamycin) (41), which is an immediate downstream effector of Akt and upstream regulator of S6K1 (42). As shown in Fig. 3, although having no effect on Akt phosphorylation, rapamycin (50 ng/ml) inhibited S6K1 phosphorylation induced by 14,15-EET. These results indicate that 14,15-EET activates PI3K-Akt-mTOR-S6K1 signaling in this order in HDMVECs.

Fibroblast Growth Factor-2 (FGF-2) Mediates 14,15-EET-induced Angiogenesis in Vivo—To determine the role of PI3K-Akt-mTOR-S6K1 signaling in 14,15-EET-induced angiogenesis, we next studied the effects of wortmannin, LY294002, and rapamycin on the angiogenic potential of the Matrigel plugs. All three inhibitors significantly attenuated 14,15-EET-induced HDMVEC tube formation and migration (Fig. 4, A–C). These inhibitors suppressed, to some extent, basal tube formation and migration as well (Fig. 4, A–C). Because inhibition of PI3K-Akt-mTOR-S6K1 signaling for a period of 8 h had no noticeable effect on cell survival as measured by trypan blue dye exclusion assay (43), the decreases in tube formation and migration of HDMVECs in the presence of wortmannin, LY294002, and rapamycin appear to be independent of their effects on apoptosis. To relate the effects of 14,15-EET on tube formation and migration to in vivo angiogenesis, we next determined its capacity to induce Matrigel plug angiogenesis in mice. Mice were injected underneath the skin with growth factor-reduced Matrigel containing either vehicle or 50 μM 14,15-EET; 7 days later, the animals were killed, and the plugs were retrieved and analyzed for hemoglobin content using Drabkin’s reagent. As shown in Fig. 5, 14,15-EET induced angiogenesis by ~2-fold. To understand the role of PI3K-Akt-mTOR-S6K1 signaling in 14,15-EET-induced in vivo angiogenesis, we tested the effects of LY294002 and rapamycin. LY294002 (250 μM) or rapamycin (1 μg/ml) with and without 50 μM 14,15-EET was added to the Matrigel and injected into the mice underneath the skin. Seven days later, the Matrigel plugs were retrieved and analyzed for hemoglobin. Both
LY294002 and rapamycin significantly inhibited 14,15-EET-induced angiogenesis (Fig. 5).

Adenovirus-mediated Expression of dnAkt Suppresses 14,15-EET-induced HDMVEC Tube Formation and Migration in Vitro and Matrigel Plug Angiogenesis in Vivo—To confirm the role of PI3K-Akt-mTOR-S6K1 signaling in 14,15-EET-induced angiogenesis, we also used a dominant-negative mutant approach. HDMVECs were infected with Ad-GFP or Ad-dnAkt at a multiplicity of infection of 80; quiesced for 24 h; treated with vehicle or 0.1 μM 14,15-EET for the desired time period; and tested for S6K1 phosphorylation, tube formation, and migration as described above. Adenovirus-mediated expression of dnAkt but not GFP completely blocked 14,15-EET-induced S6K1 phosphorylation (Fig. 6A). Similarly, adenovirus-mediated expression of dnAkt but not GFP also blocked 14,15-EET-induced HDMVEC tube formation and migration (Fig. 6, B–D). To test the effect of dnAkt on 14,15-EET-induced in vivo angiogenesis, Ad-GFP or Ad-dnAkt (1 × 10⁸ plaque-forming units) with and without 50 μM 14,15-EET was mixed with growth factor-reduced Matrigel and injected into mice underneath the skin. Two weeks later, the Matrigel plugs were retrieved and assayed for hemoglobin content as described above. Adenovirus-mediated expression of dnAkt but not GFP substantially blocked Matrigel plug angiogenesis induced by 14,15-EET (Fig. 7).

FGF-2 Acts as a Downstream Mediator of Akt in 14,15-EET-induced HDMVEC Tube Formation and Migration in Vitro and Matrigel Plug Angiogenesis in Vivo—We have previously demonstrated that (5S)-HETE induces FGF-2 expression in HDMVECs in a manner that requires activation of PI3K (38). To understand the possible mechanism(s) by which PI3K-Akt-mTOR-S6K1 signaling is involved in 14,15-EET-induced angiogenesis, we next tested its effect on FGF-2 expression. 14,15-EET (0.1 μM) induced FGF-2 expression in a time-dependent manner, with a maximum effect of 3-fold at 30 min of treatment and return to basal levels thereafter (Fig. 8A). In addition, the PI3K inhibitor LY294002 (10 μM), but not the mTOR inhibitor rapamycin (50 ng/ml), attenuated 14,15-EET-induced increases in FGF-2 levels (Fig. 8B). Adenovirus-mediated expression of dnAkt also blocked 14,15-EET-induced increases in FGF-2 levels (Fig. 8C). To determine whether 14,15-EET-induced increases in the cellular levels of FGF-2 lead to its release and, if so, its dependence on Akt activation, we next studied the effect of dnAkt on its levels in the medium. Consistent with its effect on FGF-2 expression, 14,15-EET induced the release of FGF-2 by ~3-fold

FIGURE 9. Neutralizing anti-FGF-2 antibodies suppress 14,15-EET-induced HDMVEC tube formation and migration in vitro and Matrigel plug angiogenesis in vivo. A, quiescent HDMVECs were seeded onto 96-well plates coated with growth factor-reduced Matrigel. Cells were then treated with vehicle or 0.1 μM 14,15-EET in the presence and absence of 2 μg/ml neutralizing anti-FGF-2 antibodies (Ab) for 6 h, and tube formation was observed under an inverted microscope. The images were captured with a CCD color camera, and the tube length was measured using NIH Image J (Version 1.31). B, the representative images of HDMVEC tube formation in response to 0.1 μM 14,15-EET in the presence and absence of neutralizing anti-FGF-2 antibodies are shown. Vehicle or 0.1 μM 14,15-EET was added to the lower chamber in the presence and absence of 2 μg/ml neutralizing anti-FGF-2 antibodies. After 8 h of incubation at 37 °C, the inserts were lifted out from the 24-well plate, and the non-migrated cells were removed from the upper side of the membrane with a cotton swab. The migrated cells on the lower side of the membrane were fixed in methanol, stained with Giemsa-Wright stain, and counted under a light microscope. C, 57BL/6 mice were injected subcutaneously with 0.5 ml of Matrigel premixed with vehicle or 50 μM 14,15-EET with and without 20 μg/ml neutralizing anti-FGF-2 antibodies (for each group, n = 6). One week later, the animals were killed, and the Matrigel plugs were harvested from underneath the skin and analyzed for hemoglobin with Drabkin’s reagent. The values are the means ± S.D. of three separate experiments. *p < 0.01 versus the control; **p < 0.01 versus 14,15-EET treatment alone.

LY294002 and rapamycin significantly inhibited 14,15-EET-induced in vivo angiogenesis (Fig. 5).

Adenovirus-mediated Expression of dnAkt Suppresses 14,15-EET-induced HDMVEC Tube Formation and Migration in Vitro and Matrigel Plug Angiogenesis in Vivo—To confirm the role of PI3K-Akt-mTOR-S6K1 signaling in 14,15-EET-induced angiogenesis, we also used a dominant-negative mutant approach. HDMVECs were infected with Ad-GFP or Ad-dnAkt at a multiplicity of infection of 80; quiesced for 24 h; treated with vehicle or 0.1 μM 14,15-EET for the desired time period; and tested for S6K1 phosphorylation, tube formation, and migration as described above. Adenovirus-mediated expression of dnAkt but not GFP completely blocked 14,15-EET-induced S6K1 phosphorylation (Fig. 6A). Similarly, adenovirus-mediated expression of dnAkt but not GFP also blocked 14,15-EET-induced HDMVEC tube formation and migration (Fig. 6, B–D). To test the effect of dnAkt on 14,15-EET-induced in vivo angiogenesis, Ad-GFP or Ad-dnAkt (1 × 10⁸ plaque-forming units) with and without 50 μM 14,15-EET was mixed with growth factor-reduced Matrigel and injected into mice underneath the skin. Two weeks later, the Matrigel plugs were retrieved and assayed for hemoglobin content as described above. Adenovirus-mediated expression of dnAkt but not GFP substantially blocked Matrigel plug angiogenesis induced by 14,15-EET (Fig. 7).

FGF-2 Acts as a Downstream Mediator of Akt in 14,15-EET-induced HDMVEC Tube Formation and Migration in Vitro and Matrigel Plug Angiogenesis in Vivo—We have previously demonstrated that (5S)-HETE induces FGF-2 expression in HDMVECs in a manner that requires activation of PI3K (38). To understand the possible mechanism(s) by which PI3K-Akt-mTOR-S6K1 signaling is involved in 14,15-EET-induced angiogenesis, we next tested its effect on FGF-2 expression. 14,15-EET (0.1 μM) induced FGF-2 expression in a time-dependent manner, with a maximum effect of 3-fold at 30 min of treatment and return to basal levels thereafter (Fig. 8A). In addition, the PI3K inhibitor LY294002 (10 μM), but not the mTOR inhibitor rapamycin (50 ng/ml), attenuated 14,15-EET-induced increases in FGF-2 levels (Fig. 8B). Adenovirus-mediated expression of dnAkt also blocked 14,15-EET-induced increases in FGF-2 levels (Fig. 8C). To determine whether 14,15-EET-induced increases in the cellular levels of FGF-2 lead to its release and, if so, its dependence on Akt activation, we next studied the effect of dnAkt on its levels in the medium. Consistent with its effect on FGF-2 expression, 14,15-EET induced the release of FGF-2 by ~3-fold
FGF-2 Mediates 14,15-EET-induced Angiogenesis

FIGURE 10. Src mediates 14,15-EET-induced Akt and S6K1 phosphorylation, FGF-2 expression and release in HDMVECs, and HDMVEC tube formation and migration. A and B, quiescent HDMVECs were treated with vehicle or 0.1 μM 14,15-EET for the indicated times (A) or in the presence and absence of PP2 (1 μM) for 30 min (B), and cell extracts were prepared. Equal amounts of protein (30 μg) from the control and each treatment were analyzed by Western blotting for phosphorylated Src (pSrc), phosphorylated Akt (pAkt), phosphorylated S6K1 (pS6K1), and FGF-2 levels using their specific antibodies. As a loading control, the blots were reprobed with total anti-Src or anti-Akt antibodies. C, the conditions were the same as described for B, except that the FGF-2 released into the medium was measured by enzyme-linked immunosorbent assay. D and E, the conditions were the same described for C, except that HDMVEC tube formation and migration were measured as described in the legend to Fig. 1. The values are the means ± S.D. of three separate experiments. *, p < 0.01 versus the control; **, p < 0.01 versus 14,15-EET treatment alone.

compared with the control, and FGF-2 release was substantially inhibited by overexpression of dnAkt (Fig. 8D). To understand the role of transcriptional and translational events in 14,15-EET-induced expression of FGF-2, we also tested the effect of actinomycin D and cycloheximide, potent and selective inhibitors of RNA polymerase II and protein synthesis, respectively. Both actinomycin D (5 μg/ml) and cycloheximide (10 μg/ml) suppressed 14,15-EET-induced FGF-2 expression (Fig. 8E), suggesting a role for transcriptional events in this effect. To test whether FGF-2 acts in an autocrine manner in 14,15-EET-induced angiogenesis, the effect of neutralizing anti-FGF-2 antibodies on the responsiveness of HDMVECs to 14,15-EET-induced tube formation and migration was studied. Neutralizing anti-FGF-2 antibodies (2 μg/ml) blocked 14,15-EET-induced HDMVEC tube formation and migration by ∼70% (Fig. 9, A–C). Addition of neutralizing anti-FGF-2 antibodies (20 μg/ml) to the Matrigel before it was injected subcutaneously into mice also reduced 14,15-EET-induced in vivo angiogenesis by 60% (Fig. 9D).

Several studies have shown that Src mediates Akt activation at the level of PI3K in response to a variety of stimulants (44, 45). In addition, the ability of 14,15-EET to activate Src has been demonstrated in renal epithelial cells (15). Therefore, to determine the upstream mechanisms of Akt and S6K1 activation by 14,15-EET in HDMVECs, we studied the role of Src. 14,15-EET stimulated the phosphorylation of Src at Tyr416 in a time-dependent manner, with a maximum effect of 2.4-fold at 30 min of treatment and return to basal levels thereafter (Fig. 10A). Furthermore, as shown in Fig. 10 (B–E), PP2, a potent and selective inhibitor of Src (46), blocked 14,15-EET-induced Src, Akt, and S6K1 phosphorylation and FGF-2 expression and release in HDMVECs and the tube formation and migration capacity of these cells.

DISCUSSION

The important findings of this study are as follows. 1) Among the four CYP2C9 products of arachidonic acid tested, 14,15-EET was found to be slightly more efficacious than 5,6-, 8,9-, and 11,12-EETs in inducing HDMVEC tube formation and migration. 2) 14,15-EET stimulated phosphorylation of Akt and S6K1 in a time-dependent manner in HDMVECs. 3) Wortmannin and LY294002 (two specific inhibitors of PI3K) suppressed both Akt and S6K1 phosphorylation, whereas rapamycin (a specific inhibitor of mTOR) blocked only S6K1 phosphorylation induced by 14,15-EET, suggesting that this eicosanoid possesses the ability to activate PI3K-Akt-mTOR-S6K1 signaling in this order in HDMVECs. 4) Pharmacological blockade of activation of PI3K-Akt-mTOR-S6K1 signaling inhibited 14,15-EET-induced HDMVEC tube formation and migration in vitro and Matrigel plug angiogenesis in vivo. 5) Adenovirus-mediated expression of dnAkt also completely suppressed 14,15-EET-induced HDMVEC tube formation and migration in vitro and Matrigel plug angiogenesis in vivo. 6) 14,15-EET induced FGF-2 expression in a time- and PI3K-Akt-dependent and mTOR-independent manner. 7) Neutralizing anti-FGF-2 antibodies blocked 14,15-EET-induced HDMVEC tube formation and migration in vitro and Matrigel plug angiogenesis in vivo. 8) 14,15-EET activated Src as measured by its tyrosine phosphorylation, and its inhibition attenuated 14,15-EET-induced Akt and S6K1 phosphorylation and FGF-2 expression and release in HDMVECs and the tube formation and migration capacity of these cells. Together, these observations demonstrate that 14,15-EET induces angiogenesis by activation of Src and PI3K-Akt sig-
naling via targeting in parallel with FGF-2 expression and mTOR-S6K1 stimulation.

Many studies over the past several years have indicated that the cyclooxygenase, lipooxygenase, and cytochrome P450 monooxygenase metabolites of arachidonic acid stimulate growth in several cancer cell types (12–20). Despite the indications that these lipid molecules are involved in the development of various types of cancers, very little is known with regard to possible mechanisms of their actions. Both the Jak and Src families of non-receptor protein-tyrosine kinases play an important role in receptor tyrosine kinase and G protein-coupled receptor agonist-induced cell growth regulation and tumor progression (47, 48). We (38) and others (15) have shown previously that, like receptor tyrosine kinase and G protein-coupled receptor agonists, 14,15-EET and the 5-lipoxygenase metabolite of arachidonic acid, (5S)-HETE, activate Src and Jak-2 non-receptor protein-tyrosine kinases in mediating their growth effects in renal epithelial cells and HDMVECs, respectively. It has also been demonstrated that 11,12-EET and the 12-lipooxygenase metabolite of arachidonic acid, 12(S)-HETE, activate Akt to stimulate growth in endothelial and prostate cancer cells, respectively (17, 49). In addition, 14,15-EET has been shown to stimulate Akt in mediating renal epithelial cell survival (50). Although these observations clearly reveal the ability of some of the eicosanoids, including 14,15-EET, to stimulate cell proliferation and to activate a few signaling events associated with these cellular responses, the underlying mechanisms are unclear. Src plays an important role in transducing cell differentiation, survival, and proliferative signals in response to activation of various types of receptors and has been implicated in the development of numerous cancers (51, 52). In addition, it has been shown that Src mediates Akt activation at the level of PI3K in response to a number of stimulants (44, 45, 53). The fact that 14,15-EET stimulates Src and inhibition of Src suppresses 14,15-EET-induced Akt and S6K1 phosphorylation suggests that Src acts upstream of and mediates the activation of Akt in response to this eicosanoid.

Endothelial cell migration and proliferation are critical events for angiogenesis (25). In addition, angiogenesis plays an important role in tumor survival and progression (21–25). A large number of studies have demonstrated a role for Akt in mediating angiogenesis in response to several stimulants, including hypoxia and nitric oxide (33–35). Toward understanding the role of eicosanoids in the development of various types of cancers, some recent studies from other laboratories have reported that lipooxygenase and cytochrome P450 monooxygenase metabolites of arachidonic acid such as 12(S)-HETE, 12(R)-HETE, 11,12-EET, and 14,15-EET induce angiogenesis (14, 26–28). These reports thus provide clues for possible actions of eicosanoids by which they could influence tumor progression, although the mediators of their effects are less clear. Toward this end, while confirming 14,15-EET as a potent neovascularization lipid molecule, this study unravels the potential mechanism(s) underlying its angiogenic effects. In particular, our results demonstrate for the first time that activation of Src and PI3K-Akt signaling is essential for 14,15-EET-induced angiogenesis. Although a large number of studies have demonstrated a role for Akt in angiogenesis (32–35), its downstream effector molecules that are crucial in the expression of new blood vessels are less understood. Some reports have shown that Src facilitates vascular endothelial growth factor expression toward mediating angiogenesis (54, 55). In this study, we have identified FGF-2 as one of the target molecules of Src and PI3K-Akt signaling in the mediation of angiogenesis at least in response to 14,15-EET. Furthermore, because both actinomycin D and cycloheximide suppressed 14,15-EET-induced FGF-2 expression, the actions of Src and PI3K-Akt signaling on this response appear to be at the transcriptional level.

If PI3K-Akt-dependent FGF-2 expression is sufficient in facilitating 14,15-EET-induced angiogenesis, then why does rapamycin alone enhance FGF-2 expression but block angiogenesis? FGF-2 contains an internal ribosome entry site in its 5′-untranslated mRNA (56). Some pathophysiological conditions such as hyperglycemia have been reported to enhance FGF-2 expression via its internal ribosome entry site-mediated cap-independent translation (56). Therefore, suppression of mTOR by rapamycin may facilitate internal ribosome entry site-mediated cap-independent translation of FGF-2 to overcome the cellular stress caused by inhibition of global cap-dependent protein synthesis. However, enhanced cap-independent translation of FGF-2 in the absence of mTOR activation may not be sufficient to trigger the production of other factors, including the downstream mediators of FGF-2, that are needed for angiogenesis, as global cap-dependent protein synthesis is suppressed under these conditions. Inhibition of mTOR-mediated cap-dependent protein synthesis by rapamycin has also been reported to enhance internal ribosome entry site-mediated cap-independent synthesis of cyclin D1 and c-Myc, molecules that play an important role in cell cycle progression (57). On the other hand, the inhibitory effects of rapamycin on 14,15-EET-induced angiogenesis may be due to its actions on FGF-2 signaling subsequent to FGF-2 induction and release by 14,15-EET. In summary, our results demonstrate for the time that 14,15-EET-induced angiogenesis requires activation of Src and PI3K-Akt signaling via targeting in parallel with FGF-2 expression and mTOR-S6K1 stimulation.

Acknowledgments—We thank Dr. Kenneth Walsh for providing adenovirus harboring dominant-negative Akt and Dr. Nagahara Dronadula for preparing the figures.

REFERENCES

1. Piomelli, D. (1993) *Curr. Opin. Cell Biol.* 5, 274–280
2. Dethlefsen, S. M., Shepro, D., and D’Amore, P. A. (1994) *Exp. Cell Res.* 212, 262–273
3. Gronich, J., Komineczkowski, M., Goll, M. H., Nemenoff, R. A., and Sedor, J. R. (1994) *J. Clin. Investig.* 93, 1224–1233
4. Rao, G. N., Lassegue, B., Alexander, R. W., and Griendling, K. K. (1994) *Biochem. J.* 299, 197–201
5. Smith, W. L. (1989) *Biochem. J.* 259, 315–324
6. Lin, L., Balazsy, M., Pagano, P. J., and Naselli, A. J. (1994) *Circ. Res.* 74, 197–205
7. Golobic, M., Tanaka, K., Dobrowolski, S., Wood, D., Tsai, M. H., Marshall, M., Tamaro, I., and Stacey, D. W. (1991) *EMBO J.* 10, 2887–2903
8. Tsai, M. H., Roubeshub, M., Dobrowolski, S., Yu, C. L., Gibbs, J. B., and Stacey, D. W. (1991) *Mol. Cell. Biol.* 11, 2785–2793
9. Peppeleneboch, M. P., Tertoolen, L. G., Hage, W. J., and DeLaat, S. W. (1993) *Cell* 74, 565–575
10. Rao, G. N., Baas, A. S., Glasgow, W. C., Eling, T. E., Runge, M. S., and Alexander, R. W. (1994) *J. Biol. Chem.* 269, 32586–32591
11. Gruber, M. N., Alfonso, A., and Gill, D. L. (1996) *J. Biol. Chem.* 271, 883–888
12. Postoak, D., Nystuen, L., King, L., Ueno, M., and Beckman, B. S. (1990) *Annu. Rev. Physiol.* 52, 899–920
13. Glasgow, W. C., Afshari, C. A., Barrett, J. C., and Eling, T. E. (1992) *J. Biol. Chem.* 267, 10771–10779
14. Nie, D., Helman, G. G., Goddles, T., Tang, K., Pierson, C., Grignon, D. J., and Honn, K. V. (1998) *Cancer Res.* 58, 4047–4051
15. Chen, J. K., Capdevila, I., and Harris, R. C. (2000) *J. Biol. Chem.* 275, 13789–13792
16. Romano, M., Catalano, A., Nutini, M., D’Urbano, E., Crescenzoni, C., Clarizia, J., Libner, R., Davi, G., and Procopio, A. (2001) *FASEB J.* 15, 2326–2336
17. Potente, M., Fischtshler, B., Busse, R., and Fleming, I. (2003) *J. Biol. Chem.* 278, 29619–29625
18. Shappell, B. S., Olson, S. J., Hannah, S. E., Manning, S., Roberts, R. L., Masumori, N., Jisaka, M., Boeglin, W. E., Vater, Y., Dave, D. S., Shook, M. F., Thomas, T. Z., Funk, C. D., Brash, A. R., and Matsuski, R. J. (2003) *Cancer Res.* 63, 2256–2267
19. Badawi, A. F., Elder, M. B., Liu, Y., Ross, E. A., and Badr, M. Z. (2004) *Cancer Res.* 64, 1181–1189
20. Pozzi, A., Yan, X., Macias-Perez, I., Wei, S., Hata, A. N., Breyer, R. M., Morrow, J. D.,...
FGF-2 Mediates 14,15-EET-induced Angiogenesis

and Capdevila, J. H. (2004) J. Biol. Chem. 279, 29797–29804
21. Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Van den Hoff, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., and Nagy, A. (1996) Nature 380, 435–439
22. Hanahan, D., and Folkman, J. (1996) Cell 86, 353–364
23. Carmeliet, P., Gonzalez, A. M., Carceller, F., and Baird, A. (1991) Circ. Res. 69, 360–369
24. O'Brien, E. R., Garvin, M. R., Dev, R., Stewart, D. K., Hinojosa, T., Simpson, J. B., and Schwartz, S. M. (1994) Am. J. Pathol. 145, 883–894
25. Folkman, J. (1995) Nat. Med. 1, 27–31
26. Mezentsev, A., Seta, F., Dunn, M. W., Ono, N., Falck, J. R., and Schwartzman, M. L. (2002) J. Biol. Chem. 277, 18670–18676
27. Medhora, M., Daniels, J., Mundey, K., Fisslthaler, B., Busse, R., Jacobs, E. R., and Harder, D. R. (2003) Am. J. Physiol. 284, H215–H224
28. Michaelis, U. R., Fisslthaler, B., Medhora, M., Harder, D., Fleming, I., and Busse, R. (2003) FAESB J. 17, 770–772
29. Klippel, A., Kavagnaugh, W. M., Pot, D., and Williams, L. T. (1997) Mol. Cell. Biol. 17, 338–344
30. Peso, L. D., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997) Science 278, 687–689
31. Blume-Jensen, P., Janknecht, R., and Hunter, T. (1998) Mol. Cell. Biol. 18, 5726–5737
32. Skinner, H. D., Zheng, J. Z., Fang, J., Agani, F., and Jiang, B. H. (2004) J. Biol. Chem. 279, 45643–45651
33. Nakata, D., Mogi, M., and Walsh, K. (2003) J. Biol. Chem. 278, 31000–31006
34. Fujii, Y., Gao, K., Mano, T., Mitsuuchi, Y., Tets, J. R., and Walsh, K. (1999) Mol. Cell. Biol. 19, 5073–5082
35. Berker, K. L. (1988) BioTechniques 6, 616–629
36. Zeng, Z. Z., Yellaturu, C. R., Neeli, I., and Rao, G. N. (2002) J. Biol. Chem. 277, 41213–41219
37. Myers, M. G., Jr., Grammer, T. C., Wang, L. M., Sun, X. J., Pierce, J. H., Blenis, J., and White, M. F. (1994) J. Biol. Chem. 269, 28783–28789
38. Vlahos, C. I., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) J. Biol. Chem. 269, 5241–5248
39. Bierer, B. E., Mattila, P. S., Standaert, R. F., Herzenberg, L. A., Burakoff, S. J., Crabtree, G., and Schreiber, S. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9231–9235
40. von Manteuffel, S. R., Dennis, P. B., Pullen, N., Gingras, A. C., Sonenberg, N., and Thomas, G. (1997) Mol. Cell. Biol. 17, 5426–5436
41. Wang, B., Cao, H., and Zeng, G. N. (2002) J. Biol. Chem. 277, 41213–41219
42. Myers, M. G., Jr., Grammer, T. C., Wang, L. M., Sun, X. J., Pierce, J. H., Blenis, J., and White, M. F. (1994) J. Biol. Chem. 269, 28783–28789
43. Vlahos, C. I., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) J. Biol. Chem. 269, 5241–5248
44. Bierer, B. E., Mattila, P. S., Standaert, R. F., Herzenberg, L. A., Burakoff, S. J., Crabtree, G., and Schreiber, S. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9231–9235
45. von Manteuffel, S. R., Dennis, P. B., Pullen, N., Gingras, A. C., Sonenberg, N., and Thomas, G. (1997) Mol. Cell. Biol. 17, 5426–5436
46. Zhang, B., Cao, H., and Zeng, G. N. (2002) J. Biol. Chem. 277, 41213–41219
47. Vlahos, C. I., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) J. Biol. Chem. 269, 5241–5248
48. Bierer, B. E., Mattila, P. S., Standaert, R. F., Herzenberg, L. A., Burakoff, S. J., Crabtree, G., and Schreiber, S. L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 9231–9235
49. von Manteuffel, S. R., Dennis, P. B., Pullen, N., Gingras, A. C., Sonenberg, N., and Thomas, G. (1997) Mol. Cell. Biol. 17, 5426–5436
50. Lu, Y., Yu, Q., Liu, J. H., Zhang, J., Wang, H., Koul, D., McMurray, J. S., Fang, X., Yung, W. K. A., Siminovitch, K. A., and Mills, G. B. (2003) J. Biol. Chem. 278, 40057–40066
51. Proietti, C., Salatino, M., Rosenblit, C., Carnevale, R., Pecci, A., Kornbluh, A. R., Molinolo, A. A., Frahm, L., Charreau, E. H., Schillaci, R., and Elizalde, P. V. (2005) Mol. Cell. Biol. 25, 4826–4840
52. Rane, S. G., and Reddy, E. P. (2002) Oncogene 21, 3334–3338
53. Parsons, S. J., and Parsons, J. T. (2004) Oncogene 23, 7906–7909
54. Pidgeon, G. P., Kandouz, M., Meram, A., and Honn, K. V. (2002) Cancer Res. 62, 2721–2727
55. Chen, J. K., Capdevila, J., and Harris, R. C. (2001) Mol. Cell. Biol. 21, 6322–6331
56. Izhizar, R., and Parsons, S. J. (2004) Cancer Cell 6, 209–214
57. Schlesinger, J. (2000) Cell 100, 293–296
58. Nijhuis, E., Lamers, J. W. J., Koenderman, L., and Cofer, P. J. (2002) J. Leukocyte Biol. 71, 115–124
59. Fukuda, R., King, B., and Semenza, G. L. (2003) Cancer Res. 63, 2330–2334
60. Sounni, N. E., Roghi, C., Chaobrantsu, V., Ianssen, M., Munaut, C., Maquioi, E., Galvez, B. G., Gilles, C., Franke, N., Murphy, G., Foidart, J. M., and Noel, A. (2004) J. Biol. Chem. 279, 13564–13574
61. Teshima-Kondo, S., Kondo, K., Prado-Lourenco, L., Gonzalez-Herrera, I. G., Kakuta, K., Bayard, F., Arna, J. F., and Prats, C. A. (2004) FASEB J. 18, 1583–1585
62. Shi, Y., Sharma, A., Wu, H., Lichtenstein, A., and Ger, J. (2005) J. Biol. Chem. 280, 10964–10973