Src Family Kinases Promote Vessel Stability by Antagonizing the Rho/ROCK Pathway*§

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Src family kinases (SFKs) are one of the signaling enzymes that contribute to angiogenesis, but their precise input to the various phases of the angiogenic program has not been defined. Using an in vitro model system, we discovered that SFKs promoted the formation of tubes and prevented their regression. They suppressed regression by activating the ERK pathway that antagonized the Rho/ROCK pathway, which was essential for regression. These studies reveal that SFKs contribute to several phases of the angiogenic program and identify the downstream effectors by which SFKs stabilize tubes.

The angiogenic program consists of a deliberately orchestrated series of cellular events by which new vessels arise from pre-existing ones. Misregulated angiogenesis underlies major human diseases such as cancer, age-related macular degeneration, and proliferative diabetic retinopathy (1, 2). Hence there is an acute need to identify angiomodulators and the relationships between them. The greatest advances in this regard have been the discovery of growth factors such as vascular endothelial growth factor (VEGF) (3) and anti-VEGF-based therapies (4).

The realization that effectively silencing VEGF is likely to have undesirable side effects (5, 6) has catalyzed research to identify signaling enzymes that govern the various phases of the angiogenic program. For instance, the Rho/ROCK pathway is essential for the remodeling/regression phase of the angiogenic program (7, 8). The ERK pathway antagonizes the Rho/ROCK pathway and thereby stabilizes vessels (8). Furthermore, inhibiting the ERK pathway within tumor vessels results in reduced angiogenesis and tumor growth (8). Thus elucidating the signaling enzymes that regulate individual phases of the angiogenesis program is a proven approach to guide development of new anti-angiogenic therapies.

Although regression of vessels is in certain cases the inevitable consequence of growth factor withdrawal (9), this is not always the case (10). In the postnatal developing mouse eye, hyaloid vessels regress within the first 2 postnatal weeks, and this regression is correlated with macrophage-induced apoptosis of the endothelial cells (11). Moreover, extracellular factors can induce regression. For instance, angiopoietin 2 induces vessel regression, provided that the action of VEGF is blocked (12). Furthermore, several laboratories discovered that signaling enzymes such as PLCγ, Rho/ROCK, and the ERK pathway govern vessel regression (7, 8, 13). Together these studies indicate that vessels regress for multiple reasons. In some cases regression results from the withdrawal of the pro-angiogenic growth factors, whereas in other situations regression is the deliberate response directed by specific signaling enzymes (10).

SFKs have been found previously to contribute to angiogenesis (14, 15). We extended these findings by identifying the phases of the angiogenic program to which SFKs contribute. We report that SFKs promote tube formation and antagonize regression. The underlying mechanism appears to involve SFK-dependent activation of ERK, which antagonizes Rho and thereby suppresses regression. These findings suggest that SFKs participate in controlling vessel stability.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Anti-mouse and anti-rabbit antibodies conjugated to hors eradish peroxidase were obtained from Amersham Biosciences. Rabbit polyclonal anti-phospho-Akt antibody, anti-Akt antibody, anti-phospho-ERK antibody, and anti-ERK antibody were obtained from Cell Signaling Technology (Beverly, MA). Anti-phospho-myosin light chain (MLC) 2 antibody and anti-MLC 2 antibody were the gifts from Dr. Alexey M. Belkin (University of Maryland, Baltimore). The anti-SFK antibody, which recognizes multiple SFKs, was a mouse monoclonal antibody that was described previously (16). Rabbit polyclonal anti-phospho-SFK (Y418) antibody was purchased from BIOSOURCE and recognizes the appropriately phosphorylated forms of Src, Fyn, and Yes. The RasGAP and platelet-derived growth factor receptors (PDGFR) antibodies were crude polyclonal rabbit antisera that were described previously (16, 17). Cell-permeable C3 enzyme was purchased from Cytoskeleton (Denver, CO). PD98059 was purchased from Cell Signaling Technology. SU6656 and Y27632 were purchased from Calbiochem. The BB isoform of PDGF (PDGF-B; referred to as “PDGF” throughout) was purchased from R & D Systems (Minneapolis, MN). All other chemicals and reagents were obtained from Sigma unless otherwise indicated.
**SFKs Block Tube Regression by Antagonizing Rho**

**Cell Culture**—Primary cultures of bovine retinal endothelial cells (BRECs) were obtained as described previously (18, 19) and were maintained in EBM (Clonetics, Walkersville, MD) supplemented with 10% horse serum (Clonetics), 80 units/ml penicillin/streptomycin C (Irvine Scientific, Santa Ana, CA), and 12 μg/ml bovine brain extract (Clonetics). The cells were maintained in tissue culture dishes that had been coated with 50 μg/ml bovine fibronectin. For all experiments, cells were used between passages 7 and 10.

**Tube Formation Assay**—The tube formation assay was performed as described previously (19). The average tube length was routinely 15–30 mm. Each experimental condition was assayed in triplicate, and all experiments were repeated on at least three independent occasions.

**Construction of α/β Chimeric PDGFR Mutants**—The tyrosine phosphorylation sites that permit association of SFKs with the PDGFRβ receptor (PDGFRβ(Tyr-579 and -581 in the juxtamembrane domain) are also required for maximal PDGF-stimulated kinase activity (20, 21). In contrast, the corresponding Tyr residues in the PDGFRα receptor (PDGFRα) could be mutated to phenylalanine without compromising the kinase activity of the PDGFRα (22). Like PDGFRα, the kinase activity of a chimeric PDGFR(α/β) was unaffected by phenylalanine substitution of these two Tyr residues (23). The chimera consisted of the extracellular, transmembrane, and juxtamembrane domains from the PDGFRα, and the remainder of the intracellular portion was PDGFRβ (24). The α/β F7 chimera receptor has tyrosines 572, 574, 740, 751, 771, 1009, and 1021 mutated to phenylalanine. Consequently, it is unable to recruit or activate SFKs, PI3K, RasGAP, SHP2, and PLCγ. The α/β SFK+ receptor is the same as α/β F7, except that the Tyr residues necessary for engaging SFKs (572 and 574) have been repaired. Similarly, the Tyr residues necessary for recruitment of PI3K (740 and 751) were restored in the α/β PI3K+ chimera. The α/β SFK/PI3K+ chimera contained the Tyr residues necessary for recruiting both SFKs and PI3K. The chimeric α/β PDGFR cDNAs were subcloned into the retroviral vector pLXSN2 and transfected into 293GPG cells. The virus-containing medium was collected for 5 days, concentrated (25,000 g, 90 min, 4 °C), and used as described previously (25). Cells were infected and selected on the basis of proliferation in the presence of G418 (1 mg/ml). The expression level of the chimeric receptor has tyrosines 572, 574, 740, 751, 771, 1009, and 1021 mutated to phenylalanine.

**R388A and D386N Src Mutants**—The murine Src R388A and Src D386N cDNAs were kindly provided by Dr. Philip A. Cole (The Johns Hopkins University, Baltimore) (26). They were subcloned into the pLXSN vector and stably expressed in BRECs as described above. The expression level of the mutants is shown in supplemental Fig. S1A. Association of SFKs with the chimeric receptors was dependent on tyrosines 572 and 574 (supplemental Fig. S1B).

**Western Blot Analysis and Immunoprecipitation**—For Western blot analysis, 2 × 10⁶ BRECs were plated into a 10-cm tissue
culture plate, incubated for at least 18 h in culture medium, and serum-starved for at least 18 h in medium containing 0.2% horse serum. Total cell lysates were prepared by adding lysis buffer containing 20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM dithiothreitol, 0.5% sodium dodecyl sulfate, 1% Nonidet P-40, and protease inhibitors (2 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride, and 10 mM sodium fluoride) and incubating for 1 h on ice. After centrifugation, the supernatants were collected, and the protein concentration was determined. 10–30 μg of proteins were separated on 10% SDS-polyacrylamide gels, and Western blot analysis was performed as described previously (19). To re-probe a blot, the blot was first stripped by incubating for 30 min at 60 °C in a buffer containing 6.25 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol and then reprobed with the desired primary antibody.

To analyze cells that had been plated in a collagen gel, the gel was dissolved by a collagenase treatment (collagenase type I-S, 281 units/well for 20–30 min at 37 °C), and the liberated cells were rinsed with ice-cold phosphate-buffered saline and lysed as described previously (13).

**Preparation of Retinal Explants**—The vessel outgrowth procedure was a modification of a previously published protocol (19, 27). Briefly, a collagen gel mixture was prepared as described for the tube formation assay, and 400 μl was placed into each well of a 24-well plate and incubated at 37 °C for 2 h to allow the gel to solidify. Whole retinas were dissected from enucleated eyes and spread out in a Petri dish containing phosphate-buffered saline. The retinas were cut into pieces of ~1 mm diameter. Individual explants were added to 200 μl of a freshly prepared collagen gel mixture and placed on top of the solidified collagen gel. The plates were incubated at 37 °C for 2 h to permit solidification of the newly added collagen. At the end of this incubation, 500 μl of medium supplemented with 10% horse serum, 12 μg/ml bovine brain extract, and 25 ng/ml mouse VEGF (R & D Systems) was added. The explants were incubated at 37 °C for the indicated duration, during which time they were periodically photographed through an Eclipse TE300 inverted phase microscope (Nikon). Images were captured using Adobe Photoshop, and the data were imported as a TIFF file into NIH Image. The total vessel length
in each explant was measured, and the data were imported into Microsoft Excel.

Statistics—The Student’s t test was used to assess statistical significance.

RESULTS AND DISCUSSION

We previously reported that PI3K was required for tube formation in an in vitro angiogenesis assay (13). These studies relied in part on the Tyr-740/Tyr-751 PDGFRβ that recruits and activates two signaling enzymes known to be involved with angiogenesis, PI3K and SFKs. In this study we used molecular and pharmacological approaches to assess the role of SFKs in tube formation and regression.

SFKs Promoted Tube Formation—We first examined tube formation. As shown in Fig. 1A, the chimera that was unable to recruit either SFK or PI3K (α/β F7) failed to form tubes in response to endogenously produced PDGF (13). Although boosting the level of PDGF to 10 ng/ml improved the response, it was never very strong for these cells. The low, endogenous level of PDGF was also insufficient to induce tube formation in cells expressing the α/β SFK+ chimera (which activates SFKs (23) (supplemental Fig. S1B)). However, tubes formed readily when the concentration of PDGF was increased (Fig. 1A). Cells expressing the chimera that activated PI3K (α/β PI3K+) formed tubes in both experimental settings, and the response was greater in the presence of the higher concentration of PDGF (Fig. 1A). These studies indicate that both SFKs and PI3K were capable of promoting tube formation and that in the context of the PDGFR, PI3K was more effective than SFKs.

Although α/β SFK+ recruits and activates SFK, it is possible that other proteins engaged by this receptor were responsible for inducing tube formation. To address this possibility, we tested the effect of inhibiting the activity of SFKs on tube formation. Tube formation was suppressed by an SFK inhibitor (SU6656) in cells expressing the α/β SFK+ chimera but not in cells expressing the α/β PI3K+ chimera (Fig. 1B). We conclude that SFK activity was required downstream of the α/β SFK+ chimera for tube formation.

Endothelial cells typically express Src, Fyn, and Yes (28), and both Fyn and Yes were readily detectable in BRECs, whereas Lyn was not (supplemental Fig. S1C). Because we did not have an Src-specific antibody, we were unable to determine whether Src was expressed in these cells. PDGF activated SFKs in the α/β SFK+ chimera-expressing BRECs within the expected time frame (supplemental Fig. S1D). Thus it appears that Fyn and/or Yes were among the SFK family members that were promoting tube formation.

Finally, we investigated the mechanism by which SFKs induced tube formation. Because several reports indicate that SFKs can activate the PI3K/Akt pathway (29–31), we tested if SFKs activated Akt. Indeed, SFKs increased phospho-Akt without recruiting PI3K to chimeric PDGFR (supplemental Fig. S2, A and B). Furthermore, SFK-dependent tube formation was attenuated by treatment with a PI3K inhibitor (LY294002) (supplemental Fig. S2C). These results suggest that SFKs promoted tube formation by activating PI3K/Akt.

SFKs Attenuated Tube Regression—After tubes form, they regress despite daily media changes and the continual presence of PDGF. To assess the role of SFKs in tube regression, we examined the effect of SFK inhibition on tube formation. Tube formation was suppressed by an SFK inhibitor (SU6656) in cells expressing the α/β SFK+ chimera but not in cells expressing the α/β PI3K+ chimera (Fig. 1B). We conclude that SFK activity was required downstream of the α/β SFK+ chimera for tube formation.

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FIGURE 3. Bar, 100 μm. D), cells expressing the indicated chimeras were stimulated with PDGF (1 ng/ml) for 5 min. Total cell lysates were subjected to a Western blot using an anti-phospho-MLC2-serine-19) antibody. The blot was then stripped and re-probed with an anti-MLC2 antibody. The ratio of the phospho-MLC2/MLC2 signal was quantified and presented as the mean ± S.D. from all three experiments. The difference between p-MLC2 in α/β SFK/Pi3K^- expressing cells was statistically significant (p < 0.05). The fold stimulation (+/- PDGF) is shown below the MLC2 blot. E, α/β SFK/Pi3K^- cells were subjected to a tube assay in the presence of a ROCK inhibitor (Y27632; 1 μM) and/or Src inhibitor (SU6656; 10 μM). The day 2 data are presented. The difference between SU6656 and SU6656 + Y27632 was statistically significant (*, p = 0.0001). F, same as E, except that the cells were parental BRECs, and tube formation was driven by exogenously added VEGF-A (2.5 ng/ml). The difference between SU6656 and SU6656 + Y27632 at day 2 was statistically significant (*, p < 0.0001). G, retinal explants were isolated from wild-type mice (C57BL/6;129 mixed background, n = 4 per group) and plated in a collagen sandwich gel in the presence of 25 ng/ml mouse VEGF. On day 14 (once the vessels had growth to 50% of the length attained at day 25), SU6656 (10 μM) and/or Y27632 (1 μM) was added. On day 25, the total length of the vessels was quantified, and the percentage of disorganized vessels was determined. Disorganized vessels are ones that formed normally but then underwent dissolution when the cells migrated out of the vessel. The percentage of disorganized vessels is presented in the table. The difference between SU6656 and Y27632 + SU6656 was statistically significant (p < 0.01). The panels show representative data from explants derived from four mice; bar, 100 μm. The right-hand panel in the top row is a close up showing the morphology of a disorganized vessel; bar, 100 μm.

of serum and pro-angiogenic factors (13). We used four complementary approaches to test whether SFKs influenced regression. First, we compared regression of tubes organized from cells expressing chimeras that activated SFKs (α/β SFK^-) or Pi3K (α/β Pi3K^-) or both Pi3K and SFKs (α/β SFK/Pi3K^-) in the presence of high PDGF (which was needed to observe tube formation by the α/β SFK^- expressing cells). Fig. 2A shows that the α/β Pi3K^- tubes regressed, whereas tubes formed with cells expressing chimeras that activated SFKs (α/β SFK/Pi3K^-) did not. Second, we observed that pharmacologically inhibiting SFKs promoted regression of the α/β SFK/Pi3K^- tubes (Fig. 2B). Third, we considered whether SFKs also prevented regression when driven by VEGF acting through endogenous VEGF receptors. Indeed, pharmacologically inhibiting SFKs enhanced regression (Fig. 2C). Fourth, we tested if activating Src would prevent regression of VEGF-driven tubes. For these experiments, we stably expressed a point mutant of Src that can be activated by imidazole (R388A) (26). Characterization of these cells indicated that imidazole activated Src in a time- and dose-dependent manner in the R388A-expressing cells, but not in cells expressing D386N that is not responsive to imidazole (Fig. 2D and E). Importantly, activation of Src prevented regression of VEGF-induced tubes (Fig. 2F). Taken together these data indicate that SFKs antagonized regression of tubes.

Src Blocked Tube Regression by Antagonizing Rho/ROCK Pathway

—We next investigated the mechanism by which SFKs suppressed regression. A recent study indicates that Src can antagonize RhoA (32). A second publication reported that Rho is required for tube destabilization/regression (8). Therefore, we hypothesized that SFKs attenuated tube regression by blocking the RhoA/Rho-associated coiled-coil containing serine/threonine protein kinase (ROCK) pathway (Fig. 5).
The first step in testing our hypothesis was to determine whether the RhoA/ROCK pathway was required for regression in our system. Indeed, we found that blocking RhoA or ROCK prevented regression of tubes in the VEGF- and PDGF-driven models (Fig. 3, A–C). Thus, the Rho/ROCK pathway was required for regression in our systems as in those used by other groups (7, 8).

Next we tested if SFKs antagonize the Rho/ROCK pathway by measuring phosphorylation of MLC 2 at Ser-19, which depends at least in part on ROCK activity (33). Indeed, PDGF stimulation (1 ng/ml, 5 min) of phospho-MLC2 was reduced by measuring phosphorylation of MLC2 at Ser-19, which depends at least in part on ROCK activity (33). Indeed, PDGF stimulation (1 ng/ml, 5 min) of phospho-MLC2 was reduced in the α/β SFK−/− cells (Fig. 3D). Furthermore, although PDGF stimulated phosphorylation of MLC2 in the α/β PI3K−/− cells, enabling the chimera to also activate SFKs (α/β SFK/PI3K−/−) reduced MLC2 phosphorylation (Fig. 3D). These results indicate that SFKs inhibited ROCK activity and support our hypothesis that SFKs attenuate regression by antagonizing the RhoA/ROCK pathway (Fig. 5).

If SFKs suppressed regression by inhibiting the Rho/ROCK pathway, then the SFK inhibitor should be unable to promote regression of tubes when the Rho/ROCK pathway is blocked. We found that the ability of SU6656 to promote regression was substantially diminished in ROCK inhibitor Y27632-treated tubes (Fig. 3, E and F). These findings suggest that SFKs attenuate regression by antagonizing the RhoA/ROCK pathway.

We also tested whether the relationship between the SFK-Rho/ROCK pathways was relevant beyond the in vitro tube assay. To this end we used a retinal organ culture (19) in which vessels grew out of retinal explants in response to VEGF. Outgrowth was first detected on day 10 and continued for at least 1 month. The SFK inhibitor (added at day 14) promoted the disorganization of vessels, which was characterized by the migration of cells out of the vessels and vessel dissolution (Fig. 3G). The SU6656-induced disorganization of vessels was diminished in cultures that were also treated with Y27632 (Fig. 3G). These findings indicate that the SFK-Rho/ROCK relationship appears to regulate the stability of vessels in the retinal explant setting as it does in the in vitro tube assay.

ERK Was the Liaison between SFKs and the Rho/ROCK Pathway—To identify the intermediary between SFKs and the Rho/ROCK pathway, we considered ERK because of previously published reports. Marshall and co-workers (8) demonstrated that ERK regulates Rho in the context of vessel stability/regression. Furthermore, SFKs can activate ERK and thereby promote endothelial cell proliferation and motility (34). Because of these publications we hypothesized that SFKs act through ERK to block Rho/ROCK and thereby prevent tube regression (Fig. 5).

A prerequisite for our hypothesis is that SFKs activate ERK in our model. Chimeras capable of recruiting SFKs (α/β SFK+/− and α/β SFK/PI3K−/−) activated ERK better than the corresponding chimeras that did not recruit SFKs (α/β F7 and α/β PI3K−/−) (Fig. 4, A and B). Furthermore, pharmacologically inhibiting SFKs reduced ERK activation in both the basal and PDGF-stimulated settings (Fig. 4C). These results reveal that active SFKs were required for ERK activity.

If SFKs prevented regression through an ERK-dependent pathway, then blocking the ERK pathway should destabilize tubes. Indeed, although vehicle-treated α/β SFK−/− tubes were stable, those that received PD98059 regressed (Fig. 4D). The experiment in Fig. 4D also shows that formation of tubes was much less dependent on the ERK pathway because tube formation was only modestly affected by inhibition of the ERK pathway, and only at the highest dose (50 μM) of PD98059 (Fig. 4D). Finally, if the Rho/ROCK pathway were...
downstream of ERK, then blocking the Rho/ROCK pathway would protect tubes from PD98059-induced regression. Indeed, we found that the ability of PD98059 to promote regression was substantially diminished in ROCK inhibitor Y27632-treated tubes (Fig. 4E).

Taken together our findings support the following model regarding how SFKs prevent tube regression (Fig. 5). Activation of SFKs augments the ERK pathway, which antagonizes the Rho/ROCK pathway that is essential for tube regression.

Recent studies demonstrating that ROCK phosphorylates and activates PTEN in leukocytes (35) suggest that the Rho/ROCK pathway may prevent regression by boosting the output of PI3K, which is a known mechanism for suppressing regression (13). However, in our experimental setting, the phospho-Akt level was not changed by Rho/ROCK inhibitors (supplemental Fig. S4). These findings indicate that tube regression by Rho/ROCK pathway was independent of the PI3K pathway.

A recent paper from Marshall and co-workers (8) provides evidence for the relationship between ERK and Rho/ROCK pathways in tumor vessels. They found that inhibiting the ERK pathway (by expressing dominant negative MEK1) reduced angiogenesis in tumors, and this phenomenon was reversed by treatment with Y27632. The concordance between these in vivo studies and our in vitro findings indicates that the in vitro model can be used to learn how the angiogenesis is regulated in vivo.

As compared with PI3K, SFKs were less capable of driving tube formation in both the PDGF- and VEGF-driven models. Thus although SFKs are capable of promoting the formation of tubes, it appears that PI3Ks drive the bulk of this portion of the angiogenic program. Curiously, SFKs-driven tube formation involves activation of Akt, and thereby implicates Akt as the downstream effector for both the SFK- and PI3K-driven pathways. However, because PI3K drives tube formation more potently than SFKs, signaling enzymes other than Akt maybe contributing to the tube response.

In contrast to their feeble ability to promote the formation of tubes, SFKs fully prevented regression. Our working hypothesis is that SFKs activate the ERK pathway and thereby antagonize the Rho/ROCK pathway that promotes regression (Fig. 5). Curiously, we found that PLCγ, which promoted regression, had little effect on ERK activation (13). Instead, PLCγ-induced regression by reducing the level of PtdIns-4,5-P₂. Thus it appears that enzymes that promote and suppress regression interface with distinct components of the putative intrinsic regression pathway.

In addition to the ERK/Rho mechanism to stabilize tubes, SFKs may also protect from regression by increasing PtdIns-4,5-P₂. There are reports that SFKs modulate the amount of PtdIns-4,5-P₂ (36, 37) and elevating the level of PtdIns-4,5-P₂ in cells prevents regression (13). If SFKs protect from regression by elevating the level of PtdIns-4,5-P₂, then the chain of events that mediate this SFK-dependent phenomenon may include activation of enzymes that produce PtdIns-4,5-P₂ (PtdIns 4-phosphate 5-kinase and PtdIns 5-phosphate 4-kinase) or antagonize those that reduce the level of PtdIns-4,5-P₂ (PLC, PI3K, and PtdIns-4,5-P₂ 5-phosphatases) (38).

The observation that SFKs act through the ERK pathway to prevent regression suggests that activating the ERK pathway would be sufficient to stabilize tubes. However, this does not appear to be the case. The α/β PI3K receptor activated ERK at least as well as the α/β SFK receptor (Fig. 4B), yet α/β PI3K + tubes regressed (Fig. 2A). Furthermore, although α/β SFK chimaera suppressed ROCK activity, α/β PI3K + promoted it (Fig. 3D). Thus it appears that although ERK activity was required for inhibiting ROCK and regression, it was not sufficient. Alternatively, SFKs and PI3K may activate ERK in different ways, and these differences may constitute the basis for subsequent ROCK inhibition and protection from regression.

Acknowledgments—We thank Dr. Philip Cole (The Johns Hopkins University, Baltimore) for providing the anti-phospho-MLC2 and anti-MLC2 antibodies. We also thank Dr. Alexey M. Belkin (University of Maryland, Baltimore) for providing the SrcR388A and SrcD386N cDNAs. We also thank Dr. Philip Cole (The Johns Hopkins University, Baltimore) for providing the anti-phospho-MLC2 and anti-MLC2 antibodies.

REFERENCES

1. Carmeliet, P. (2005) Nature 438, 932–936
2. Giriano, R. F., and Gardner, T. W. (2005) Nature 438, 960–966
3. Gragoudas, E. S., Adamis, A. P., Cunningham, E. T. J., Feinsod, M., Geyer, D. R., and VEGF Inhibition Study in Ocular Neovascularization Clinical Trial Group (2004) N. Engl. J. Med. 351, 2805–2816
4. Eter, N., Krohne, T. U., and Holz, F. G. (2006) BioDrugs 20, 167–179
5. Carmeliet, P., Ferreira, V., Breier, G., Polleto, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenheuvel, A., Harpal, K., Eberhardt, C., and Declercq, C. (1996) Nature 380, 435–439
6. Nishijima, K., Ng, Y. S., Zhong, L., Bradley, J., Schubert, W., Jo, N., Akita, J., Samuelsson, S. J., Robinson, G. S., Adams, A. P., and Shima, D. T. (2007) Am. J. Pathol. 171, 53–67
7. Bayless, K. J., and Davis, G. E. (2004) J. Biol. Chem. 279, 11686–11695
8. Mavria, G., Vergouwen, Y., Yeo, M., Paterson, H., Carasarides, M., Marais,
R., Bird, D., and Marshall, C. J. (2006) Cancer Cell 9, 33–44
9. Benjamin, L. E., Golijanin, D., Itin, A., Pode, D., and Keshet, E. (1999) J. Clin. Investig. 103, 159–165
10. Im, E., and Kazlauskas, A. (2006) Cell Cycle 5, 2057–2059
11. Lang, R., Lustig, M., Francois, F., Sellinger, M., and Plesken, H. (1994) Development (Camb.) 120, 3395–3403
12. Oshima, Y., Oshima, S., Nambu, H., Kachi, S., Takahashi, K., Umeda, N., Shen, J., Dong, A., Apte, R. S., Duh, E., Hackett, S. F., Okoye, G., Ishibashi, K., Handa, J., Melia, M., Wiegand, S., Yancopoulos, G., Zack, D. J., and Campochiaro, P. A. (2005) FASEB J. 19, 963–965
13. Im, E., and Kazlauskas, A. (2006) EMBO J. 25, 2075–2082
14. Alavi, A., Hood, J. D., Frausto, R., Stupack, D. G., and Cheresh, D. A. (2003) Science 301, 94–96
15. Eliceiri, B. P., Paul, R., Schwartzberg, P. L., Hood, J. D., Leng, J., and Cheresh, D. A. (1999) Mol. Cell 4, 915–924
16. Lipshitz, L. A., Lewis, A. J., and Brugge, J. S. (1983) J. Virol. 48, 352–360
17. Valius, M., Bazenet, C., and Kazlauskas, A. (1993) Mol. Cell. Biol. 13, 133–143
18. Gitlin, J. D., and D’Amore, P. A. (1983) Microvasc. Res. 26, 74–80
19. Im, E., Venkatakrishnan, A., and Kazlauskas, A. (2005) Mol. Biol. Cell 16, 3488–3500
20. Baxter, R. M., Secrist, J. P., Vaillancourt, R. R., and Kazlauskas, A. (1998) J. Biol. Chem. 273, 17050–17055
21. Mori, S., Ronnstrand, L., Yokote, K., Engstrom, A., Courtemeide, S. A., Claesson-Welsh, L., and Heldin, C. H. (1993) EMBO J. 12, 2257–2264
22. Gelderloos, J. A., Rosenkrantz, S., Bazenet, C., and Kazlauskas, A. (1998) J. Biol. Chem. 273, 5908–5915
23. DeMali, K. A., and Kazlauskas, A. (1998) Mol. Cell. Biol. 18, 2014–2022
24. DeMali, K. A., Whiteford, C. C., Ulug, E. T., and Kazlauskas, A. (1997) J. Biol. Chem. 272, 9011–9018
25. Ory, D. S., Neugeboren, B. A., and Mulligan, R. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11400–11406
26. Qiao, Y., Molina, H., Pandey, A., Zhang, J., and Cole, P. A. (2006) Science 311, 1293–1297
27. Knott, R. M., Robertson, M., Muckersie, E., Folefac, V. A., Fairhurst, F. E., Wileman, S. M., and Forrester, J. V. (1999) Diabetologia 42, 870–877
28. Werdich, X. Q., and Penn, J. S. (2005) Angiogenesis 8, 315–326
29. Arcaro, A., Aubert, M., Espinosa del Hierro, M. E., Khanzada, U., Angelidou, S., Tetley, T. D., Bittermann, A. G., Frame, M. C., and Seckl, M. J. (2007) Cell. Signal. 19, 1081–1092
30. Jin, W., Yun, C., Hobbie, A., Martin, M. J., Sorensen, P. H., and Kim, S. J. (2007) Cancer Res. 67, 3192–3200
31. Thamilselvan, V., Craig, D. H., and Basson, M. D. (2007) FASEB J. 21, 1730–1741
32. Janiak, A., Zemskov, E. A., and Belkin, A. M. (2006) Mol. Biol. Cell 17, 1606–1619
33. Chen, X., Wang, R., Li, Q., Li, L., Kaibuchi, K., and Clark, R. A. (2004) J. Cell Biol. 167, 3511–3518
34. Eliceiri, B. P., Klemke, R., Stromblad, S., and Cheresh, D. A. (1998) J. Cell Biol. 140, 1255–1263
35. Li, Z., Dong, X., Wang, Z., Liu, W., Deng, N., Ding, Y., Tang, L., Hla, T., Zeng, R., Li, L., and Wu, D. (2005) Nat. Cell Biol. 7, 399–404
36. Tolloczko, B., Turkewitsch, P., Choudry, S., Bisotto, S., Fixman, E. D., and Martin, J. G. (2002) Am. J. Physiol. 282, L1305–L1313
37. Halstead, J. R., van Rheenen, J., Snel, M. H., Meeuws, S., Mohammed, S., D’Santos, C. S., Heck, A. J., Jalink, K., and Divecha, N. (2006) Curr. Biol. 16, 1850–1856
38. Halstead, J. R., Jalink, K., and Divecha, N. (2005) Trends Pharmacol. Sci. 26, 654–660