Src Family Kinase Activity Is Required for Signal Tranducer and Activator of Transcription 3 and Focal Adhesion Kinase Phosphorylation and Vascular Endothelial Growth Factor Signaling *in Vivo* and for Anchorage-dependent and -independent Growth of Human Tumor Cells

A. Douglas Laird,[1] Guangmin Li, Katherine G. Moss,
Robert A. Blake, Martin A. Broome,
Julie M. Cherrington, and Dirk B. Mendel

Preclinical Research and Exploratory Development [A. D. L., G. L., K. G. M., J. M. C., D. B. M.], Discovery Technology [R. A. B.], and Research [M. A. B.], SUGEN, Inc., South San Francisco, California 94080

Abstract

The Src family kinases (SFKs) Src and Yes are believed to play critical roles in tumor growth, angiogenesis, invasion, and dissemination. Using a panel of highly selective and structurally diverse Src inhibitors, we found that phosphorylation of signal transducer and activator of transcription 3 [STAT3 (Y705)] and focal adhesion kinase [FAK (Y861)] was SFK dependent in cultured human colon, breast, lung, and ovarian tumor cells. These findings were reproduced *in vivo* in target modulation studies using tumors derived from fibroblasts overexpressing activated Src. Additionally, treatment of mice with multiple Src inhibitors resulted in inhibition of phosphorylation of FAK (Y861) and of a putative Src autophosphorylation epitope (Y419) in HT-29 human colon tumor xenografts. Next we pharmacologically examined the requirement for SFKs in asynchronous proliferation of human tumor cells. At concentrations sufficient to selectively inhibit Src, structurally diverse Src inhibitors inhibited growth of cultured human colon, breast, and lung cells on plastic under low serum conditions. In addition, these compounds inhibited anchorage-independent growth of HT-29 human colon tumor cells in soft agar. The role of SFK activity in vascular endothelial growth factor signaling was also evaluated. Inhibition of SFK signaling using structurally distinct Src inhibitors resulted in complete inhibition of vascular endothelial growth factor-dependent vascular permeability *in vivo*. These data demonstrate that STAT3 (Y705) and FAK (Y861) phosphoepitopes are SFK-dependent in tumor

cells and reveal a requirement for SFK function in tumor cell proliferation and vascular permeability.

Introduction

The SFKs[2] play a central role in the transduction of extracellular signals acting through activated RTKs, G-protein-coupled hormone receptors, and integrins to downstream cellular effectors (1). Three SFKs (Src, Yes, and Fyn) are widely expressed, whereas others (Lck, Lyn, Fgr, Hck, and Blk) have more restricted patterns of expression and are found primarily in hematopoietic cells (1–3). A series of elegant knockout studies has identified a limited number of functions uniquely dependent on the activity of single family members, most notably bone resorption (Src), T-cell development and signaling (Lck), and multiple B-cell signal pathways [Lyn (2–4)]. However, when coexpressed in less specialized cell types, a certain level of functional redundancy between family members is likely.

Src and Yes have been implicated in several human cancers (5). Although activating genetic mutations in SFKs are rare (they have been detected for Src in a small subset of advanced colon cancers and in a single endometrial cancer), high levels of Src activation secondary to the activation of oncogenic RTKs, such as EGF receptor and Met, are common (5–7). In colon cancer, Src activity increases with disease progression, and Src activity has recently been demonstrated to be an independent negative prognostic indicator for disease progression and patient survival (8, 9). Moreover, in patients with disseminated colon cancer, elevated Yes activity in metastases predicted poor survival (10). Overexpression/activation of Src has also been reported in other tumor types, including breast and pancreatic cancer (5).

Src activation has been reported to be associated with numerous facets of the transformed phenotype, including proliferation, survival, protease production, migration, and invasion (1, 5). STAT3 and FAK are implicated in cancer development, growth, and metastasis and have been reported to be substrates of SFKs (11, 12). In addition, SFKs have been implicated in VEGF-dependent endothelial cell

[2] The abbreviations used are: SFK, Src family kinase; STAT, signal transducer and activator of transcription; FAK, focal adhesion kinase; VEGF, vascular endothelial growth factor; RTK, receptor tyrosine kinase; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; FBS, fetal bovine serum; Erk, extracellular signal-regulated kinase.

Table 1   Selected biochemical IC$_{50}$ data (in μM) for the Src inhibitors used in these studies

Data for PP1 and PP2 are included for comparison. Enzymatic assays were performed as described previously (21).						
Kinase	SU11333	SU11336	SU12470	CGP77675	PP1	PP2
Src	0.1	0.07	0.01	0.02	0.2	<0.01
Yes	0.02	0.01	<0.009	0.2	0.01	0.03
Flk-1	1.1	0.5	2.0	1.2	0.1	0.8
PDGFRβ^a	3.3	4.0	2.0	0.01	1.3	0.6
Met	4.0	1.0	>20	>20	9.5	9.6

^{a} PDGFRβ, PDGF receptor β.

signaling, transducing signals leading to endothelial cell survival and VEGF-dependent vascular permeability (13).

A requirement for SFK function in G$_1$-S progression after treatment with PDGF and EGF has been demonstrated in fibroblasts using microinjection of mutant Src proteins and treatment with a selective small molecule Src kinase inhibitor, SU6656 (14–16). However, the evidence supporting a role for Src in maintaining the transformed phenotype in cancer cells, in phosphorylation of STAT3 and FAK, and in VEGF-dependent vascular permeability is less than decisive for several reasons. First, some studies depended on overexpression of activated Src mutants, which may exhibit qualitative as well as quantitative differences in signaling relative to native SFKs. Second, in other studies, putative inhibition of Src function was achieved using only a single inhibitor or single structural class of small molecule kinase inhibitor (17), leaving open the possibility that compound-dependent rather than target-dependent effects were being observed. In general, observed cellular responses were not tightly correlated with modulation of SFK activity, and in many cases the cellular readouts used were inadequate [*e.g.,* asynchronous proliferation in the presence of high levels of serum (17) or total phosphotyrosine levels of signaling proteins with complex phosphorylation patterns (18)]. Third, an otherwise outstanding study using genetic and pharmacological means to implicate Src in VEGF-dependent signaling (19) was flawed by reliance on an older tyrosine kinase inhibitor that is insufficiently selective to distinguish SFKs from many other kinases (Ref. 1; Table 1; data not shown).

One approach to address these concerns and to decisively elucidate the importance of SFKs in cancer would be to use several highly selective and structurally diverse small molecule inhibitors in parallel, looking for common biological responses to treatment. Using this strategy, we addressed the following three important questions: (*a*) Are STAT3 and FAK phosphorylation truly SFK dependent in cells representing multiple tumor types? (*b*) What are the consequences of pharmacological inhibition of SFK activity on anchorage-dependent and -independent proliferation of cultured tumor cells? and (*c*) Is there a requirement for SFK function in Flk-1/KDR signaling? We found that SFK activity is required for STAT3 and FAK phosphorylation in culture and *in vivo*, for tumor cell anchorage-dependent and -independent growth, and for VEGF-induced vascular permeability.

Materials and Methods
Inhibitors. The inhibitors used in these studies were adenine mimetics, which bind in the vicinity of the ATP-binding
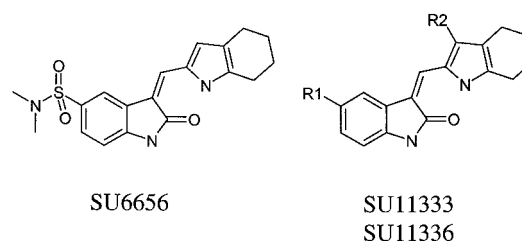

Fig. 1. SU11333 and SU11336 are derivatives of SU6656. *R1* and *R2* indicate sites of substitutions differentiating SU11333 and SU11336 from SU6656. See "Materials and Methods" for details.

pocket of their target kinases. CGP77675 is a selective inhibitor of SFKs, whose biochemical activities and structure have been reported previously (18). SU5416 is a selective inhibitor of the VEGF receptor Flk-1/KDR (20). SU6668 is a selective inhibitor of Flk-1/KDR, PDGF receptor β, and fibroblast growth factor receptor 1 (21). SU11333 and SU11336 are derivatives of SU6656 (Fig. 1), with a similar selectivity profile (16), but they exhibit enhanced potency relative to SU6656 in biochemical and cellular assays. SU12470 represents a third class of inhibitor, structurally distinct from both CGP77675 and SU11333/SU11336. In biochemical assays SU11333, SU11336, and SU12470 are highly selective for SFKs over a large panel of other tyrosine and serine/threonine kinases. Selected biochemical IC$_{50}$ data for the SFK inhibitors used in this study are summarized in Table 1. Biochemical IC$_{50}$ values for the previously published Src inhibitors PP1 and PP2 (22) are included for comparison.

Cell Culture. Cell culture reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD). All human tumor cell lines were obtained from the American Type Culture Collection (Manassas, VA), with the exception of NCI-HT-29 human colorectal adenocarcinoma cells and MDA-MB-468 human breast adenocarcinoma cells (National Cancer Institute, Bethesda, MD). Colo205 human colon carcinoma cells were grown in RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate, and 10 mM HEPES. MDA-MB-468 cells were grown in DMEM supplemented with 10% FBS. A549 human lung carcinoma cells were grown in F12K nutrient mixture supplemented with 10% FBS. SKOV3TP5 human ovarian carcinoma cells and NCI-HT-29 cells were grown in McCoy's 5A medium (modified) supplemented with 10% FBS. NIH 3T3 cells stably overexpressing a mutated, fully activated human Src (Y530F; Ref. 16) were maintained in DMEM supplemented with 10% FCS. Cells were propagated
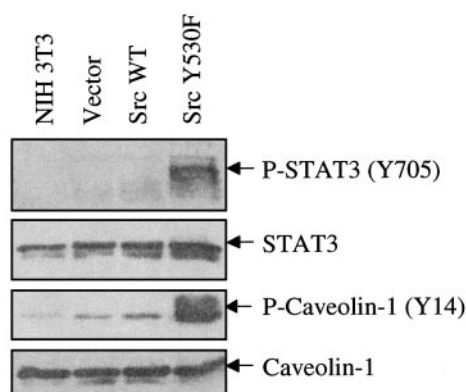
Fig. 2. Phosphorylation of STAT3 and Caveolin-1 is elevated in cells overexpressing activated Src. Untransfected NIH 3T3 cells or NIH 3T3 cells stably transfected with vector, wild-type Src, or activated Src (Y530F) were grown to 90% confluence in 10% FCS-containing growth medium. Cell lysates were prepared, and 30 μg of whole cell lysate were resolved by SDS-PAGE and immunoblotted as described in "Materials and Methods" using antibodies specific for putative SFK-dependent phosphoepitopes on STAT3 (Y705) and Caveolin-1 (Y14). Comparable loading was confirmed by immunoblotting of parallel blots for STAT3 and Caveolin-1.
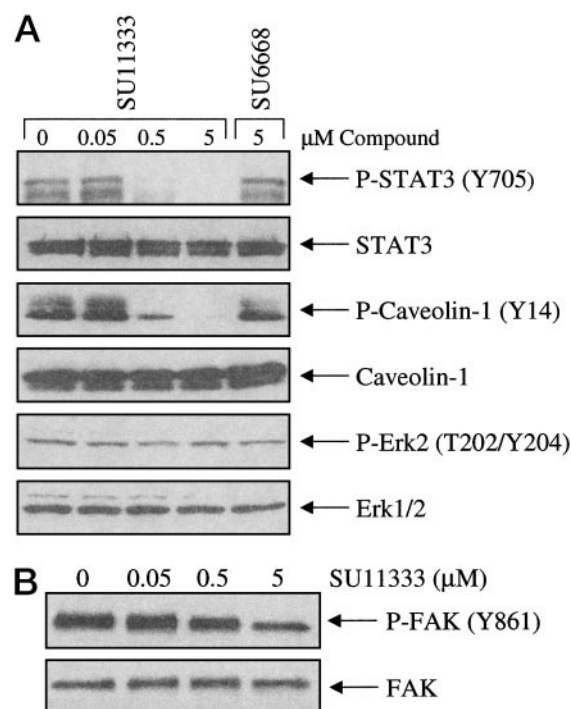

Fig. 3. SU11333 treatment inhibits Src-dependent phosphorylation of STAT3 (Y705), Caveolin-1 (Y14), and FAK (Y861) in NIH 3T3 cells stably overexpressing a mutated, fully activated Src Y530F. *A*, cells were grown to 80% confluence and treated with 0.05, 0.5, or 5 μM SU11333 or 5 μM SU6668 (as a negative control) for 24 h in complete growth medium. Cell lysates were prepared, and 30 μg of whole cell lysate were resolved by SDS-PAGE and immunoblotted as described in the Fig. 1 legend. *B*, an evaluation similar to that described in *A* was subsequently performed for FAK (Y861).

at 37°C in a humidified atmosphere of 5% CO_2 using standard cell culture techniques.

Antibodies. α-Phospho-STAT3 (Y705), α-total STAT3, α-phospho-SFK (Y419), α-phospho-p44/p42 Erk (T202/Y204), and α-total p44/p42 Erk antibodies were purchased from New England Biolabs/Cell Signaling Technology (Beverly, MA). α-phospho-FAK (Y861) and α-total Src antibodies were purchased from Biosource International (Camarillo, CA). α-total FAK, α-phospho-caveolin, and α-total caveolin antibodies were purchased from Transduction Laboratories (Lexington, KY).

Immunoblotting and Immunoprecipitation Analyses. Cells were lysed in an ice-cold buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1.0 mM EGTA, 10% glycerol, 1% Triton X-100, 10 mM $Na_4P_2O_4$, 1 mM Na_3VO_4, 50 mM NaF, 1 μM leupeptin, 0.3 μM aprotinin, 1 μM pepstatin A, 10 μM bestatin, and 1.4 μM E-64. Lysates were cleared by centrifugation at 4°C for 15 min at 14,000 rpm in a microcentrifuge, and protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). For immunoprecipitation, 1 mg of whole cell lysate was incubated with 3 μg of antibody and protein A-agarose-conjugated beads (Boehringer Mannheim) with continuous rocking at 4°C for 2 h. Beads were pelleted and washed three times with cold HNTG buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100] before being resuspended in an equal volume of 2× Laemmli sample buffer [100 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 200 mM DTT, and 0.2% bromphenol blue] and heated at 95°C for 5 min. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the indicated primary antibodies in Tris-buffered saline containing 0.1% Triton X-100 and 5% nonfat dry milk, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham) and visualization using enhanced chemiluminescence reagents (Amersham).

Growth Assays. Cells grown on plastic (anchorage-dependent growth assay) were plated at a density of 7,500 cells/well (HT-29) or 12,000 cells/well (MDA-MB-468 cells) in 96-well plates on day 0. Compounds were added on day 1, and the cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 2 days. Total cell protein was determined by staining cells with sulforhodamine B (Sigma, St. Louis, MO), and IC_{50} and LD_{50} values were determined for quadruplicate wells as described previously (23). For the soft agar assay (anchorage-independent growth assay), each well of a 24-well culture dish was coated with 0.5 ml of bottom agar mixture [McCoy's 5A medium (modified), 10% FBS or 0.5% FBS, 0.6% agar]. After the bottom layer had solidified, 0.5 ml of a top agar mixture [McCoy's 5A medium (modified), 10% FBS or 0.5% FBS, 0.3% agar] containing HT-29 cells (1,200 cells/well) along with the indicated inhibitors was added to each well. An additional 0.5-ml layer of medium [McCoy's 5A medium (modified), 10% FBS or 0.5% FBS] was added over the top of the solidified agar layer. Plates were incubated for 2 weeks before being stained overnight at 37°C in an atmosphere of 5% CO_2 in a solution of 1 μg/ml iodonitrotetrazolium salt (Sigma) in 50% ethanol. Colonies were counted at ×4 magnification, and the average number of colonies/triplicate wells was calculated.

***In Vivo* Target Modulation Studies.** NIH 3T3 cells stably overexpressing mutated, fully activated human Src (Y530F)
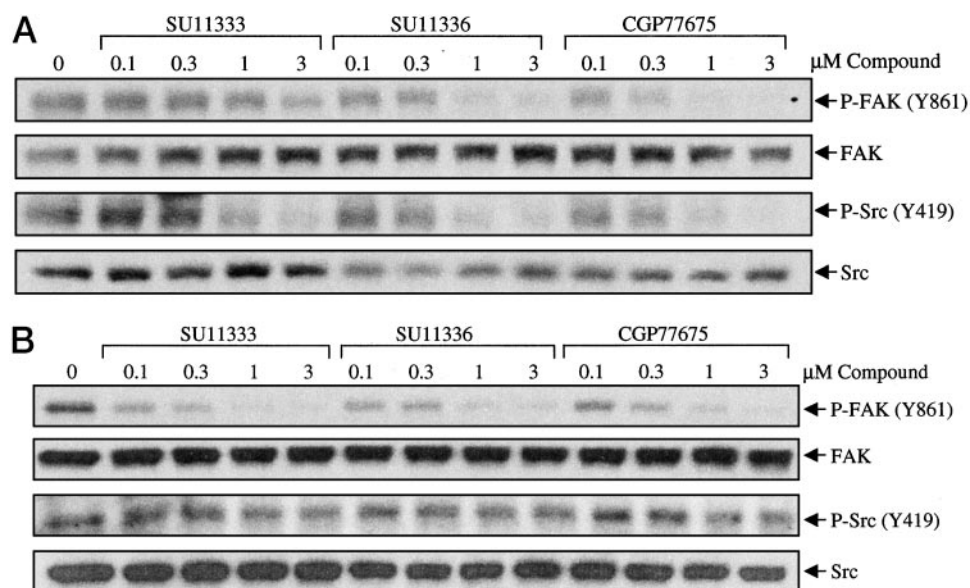
Fig. 4. Treatment with diverse Src inhibitors inhibits phosphorylation of FAK (Y861) and Src (Y419) in HT-29 cells in culture. HT-29 cells were grown to 90% confluence and treated with either 0.5 or 5 μM of each inhibitor for 24 h in medium containing low serum (0.5% FBS; *A*) or high serum (10% FBS; *B*). Cell lysates were prepared, and 30 μg of whole cell lysate were resolved by SDS-PAGE and immunoblotted as described in "Materials and Methods" using antibodies specific for putative SFK-dependent phosphoepitopes on Src (Y419) and FAK (Y861). Comparable loading was confirmed by immunoblotting of a parallel blot for total levels of Src and FAK.
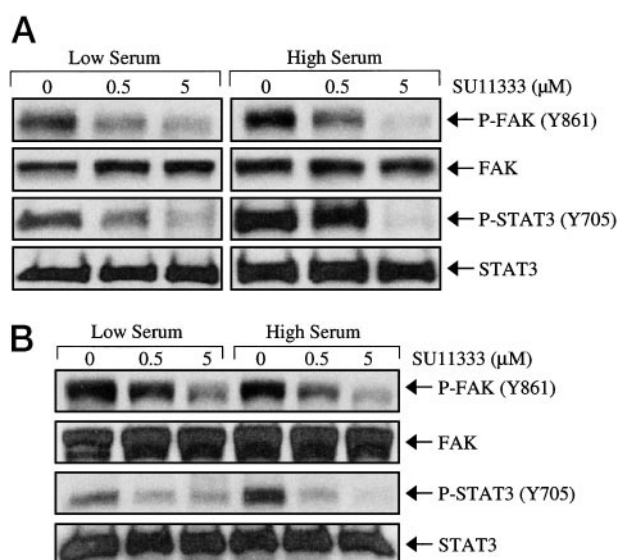

Fig. 5. SU11333 treatment inhibits phosphorylation of FAK (Y861) and STAT3 (Y705) in MDA-MB-468 (*A*) and A549 cells (*B*) in culture. Cells were grown to 90% confluence and treated with either 0.5 or 5 μM of each inhibitor for 24 h in medium containing low serum (0.5% FBS) or high serum (10% FBS). Cell lysates were prepared and analyzed for FAK (Y861) and STAT3 (Y705) as described in "Materials and Methods" (immunoblotting of 30 μg of whole cell lysate in the case of FAK; immunoblotting of anti-total STAT3 immunoprecipitate from 1 mg of lysate in the case of STAT3).

or HT-29 cells were implanted s.c. into athymic mice [5×10^5 Src (Y530F) cells/implant and 5×10^6 HT-29 cells/implant]. Tumors were established to approximately 500 mm^3 in volume. Mice were given a single i.p. injection of SU11333 dissolved in 50 μl of DMSO or 50 μl of DMSO alone. At the indicated time points, tumors were resected and subdivided; half of each tumor was fixed for subsequent histological and immunohistochemical evaluation (see below), and half was

snap-frozen, pulverized, homogenized in lysis buffer, and analyzed by Western blotting as described above.

Immunohistochemistry. Freshly resected tumor pieces to be evaluated by immunohistochemistry were fixed in 10% buffered formalin for 24 h and then transferred to 70% ethanol. These specimens were subsequently embedded in paraffin and sectioned. Phospho-FAK was detected using a 1:640 dilution of a polyclonal phospho-specific FAK (Y861) antibody (Biosource International) and visualized using a biotinylated antirabbit antibody (Vector Laboratories, Burlingame, CA). All immunostained sections were counterstained using hematoxylin.

Miles Assay for Vascular Permeability. The Miles assay for vascular permeability (13, 24) was adapted as follows. Mice were treated i.p. with the indicated inhibitors dissolved in 50 μl of DMSO or with 50 μl of DMSO alone. Three hours later, 100 μl of a 2.2% solution of FITC-dextran (M_r 150,000; Sigma) were administered i.v. via the tail vein. One h later, mice received intradermal injection (in duplicate sites on their backs) with 400 ng of VEGF (human recombinant VEGF$_{165}$; R&D Systems, Minneapolis, MN) dissolved in 20 μl of PBS and (in adjacent duplicate sites) with PBS alone. After an additional 30 min, the extent of VEGF-dependent dye leakage from the vasculature into skin was assessed visually and photographed using fluorescence microscopy.

Results

SU11333 Inhibits Putative SFK-dependent Phosphoepitopes in Fibroblasts Engineered to Overexpress an Activated Src Allele (Y530F). In published studies, STAT3 (Y705), Caveolin-1 (Y14), and FAK (Y861) have been proposed as potential SFK substrates (11, 25, 26). Consistent with this, we found that phosphorylation at these phosphoepitopes is elevated in NIH 3T3 cells stably overexpressing an activated Src allele (Y530F) relative to cells stably overexpressing wild-type Src, cells that were vector transfected, or untransfected NIH 3T3 cells (Fig. 2; data not shown).
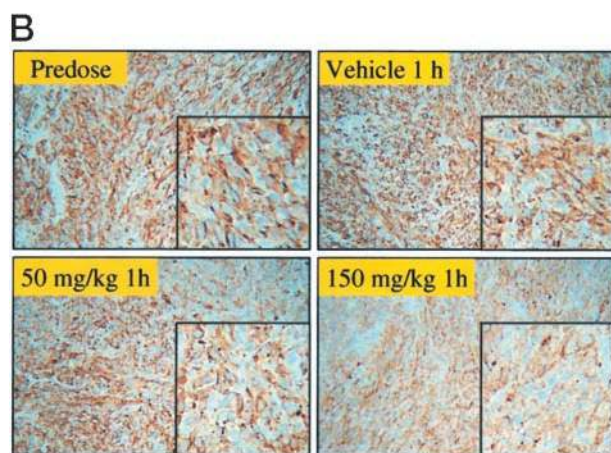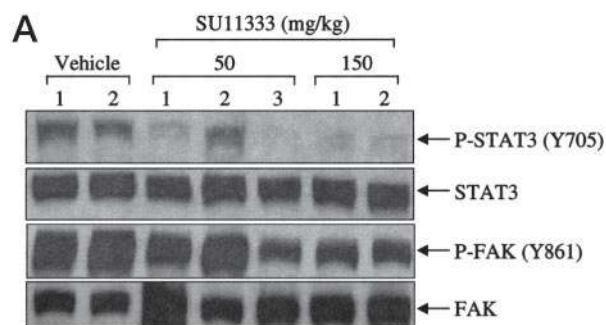
Fig. 6. SU11333 treatment inhibits phosphorylation of STAT3 (Y705) and FAK (Y861) phosphoepitopes in tumors derived from fibroblasts engineered to overexpress an activated Src allele. *A*, NIH 3T3 cells stably overexpressing a mutated, fully activated human Src (Y530F) were implanted s.c. into athymic mice, and tumors were established to approximately 500 mm^3 in volume. Mice were treated i.p. with vehicle (DMSO) alone or SU11333 at 50 or 150 mg/kg. Tumors were resected 1 h after treatment, frozen, pulverized, and homogenized in lysis buffer as described in "Materials and Methods." Fifty μg of whole cell protein lysate were analyzed by immunoblotting for FAK (Y861) and STAT3 (Y705) as described in "Materials and Methods." Protein levels of FAK and STAT3 were also determined by immunoblotting of duplicate blots using antibodies for total FAK and STAT3. Each lane represents data from a separate mouse. Predose (untreated) and vehicle-treated controls are included. *B*, tumor specimens were prepared, and phospho-FAK (Y861) was visualized immunohistochemically as described in "Materials and Methods." Slides were evaluated and photographed at ×400 magnification. Images from representative animals are shown, including *inset panels* at higher magnification (×800) showing more cellular detail.
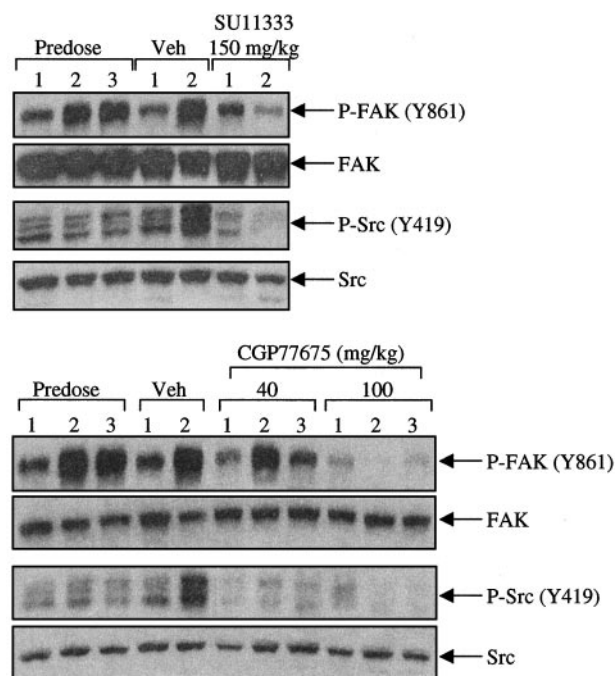
Fig. 7. SU11333 and CGP77675 inhibit phosphorylation of FAK (Y861) and Src (Y419) phosphoepitopes in HT-29 human colon tumor xenografts. HT-29 cells were implanted s.c. into athymic mice, and tumors were established to approximately 500 mm^3 in volume. Mice were treated i.p. with vehicle (DMSO) alone, SU11333 at 100 mg/kg, or CGP77675 at 40 and 100 mg/kg. Tumors were resected 4 h after treatment, frozen, pulverized, and homogenized in lysis buffer. Fifty μg of whole cell lysates were analyzed by immunoblotting for FAK (Y861) and the putative Src autophosphorylation epitope Y419 as described in "Materials and Methods." Protein levels of FAK and Src were also determined by immunoblotting of duplicate blots using antibodies for total FAK and Src. Each lane represents data from a separate mouse. Predose (untreated) and vehicle-treated (*Veh*) controls are included.

We set out to determine whether pharmacological inhibition of Src kinase activity, initially in the NIH 3T3 cells engineered to overexpress activated Src and subsequently in human tumor cells, would result in decreased phosphorylation at these phosphoepitopes. As indicated in Fig. 3*A*, SU11333 dose-dependently inhibited phosphorylation of STAT3 (Y705) and Caveolin-1 (Y14) at low submicromolar concentrations. Multiple FAK phosphoepitopes were shown to be elevated in the engineered NIH 3T3 cells, but only one (Y861) was potently inhibited by SU11333 (Fig. 3*B*; data not shown), suggesting that Y861 is the strongest candidate for a direct SFK phosphorylation site. In contrast, SU6668, a small molecule kinase inhibitor that does not inhibit SFKs (21), did not inhibit phosphorylation of STAT3 and Caveolin-1, and SU11333 did not inhibit phosphorylation of the Erk,

Erk2 (Fig. 3*A*), demonstrating that inhibition of STAT3, FAK, and Caveolin-1 phosphorylation was selective.

STAT3 and FAK Phosphorylation Is SFK dependent in Cultured Human Tumor Cells. Based on the above data, we set out to evaluate the effect of Src inhibition on FAK, STAT3, and Caveolin-1 phosphorylation in multiple human tumor cell types. However, because the Caveolin-1 (Y14) phosphoepitope was present at much lower levels in human tumor cells than in fibroblasts engineered to overexpress activated Src and because this phosphoepitope [unlike FAK (Y861) and STAT3 (Y705)] was not widely detectable in archival human tumors (data not shown), it was not investigated further. We evaluated the effects of multiple structurally distinct Src inhibitors on STAT3 and FAK phosphorylation in five diverse human tumor cell lines (HT-29 colon adenocarcinoma, Colo205 colon carcinoma, MDA-MB-468 breast adenocarcinoma, A549 lung carcinoma, and SKOV3TP5 ovarian carcinoma). The experiments were performed under both low (0.5% FBS) and high (10% FBS) serum conditions, and low serum conditions were thought to better mimic autocrine growth conditions *in vivo*. Treatment with SU11333, SU11336, and CGP77675 resulted in potent and dose-dependent submicromolar inhibition of tyrosine

Table 2   Structurally diverse selective Src inhibitors inhibit proliferation of human tumor cells grown under low serum conditions

Cells were plated at a density of 7,500 cells/well (HT-29) or 12,000 cells/well (MDA-MB-468 cells) in 96-well plates on day 0. Compounds were added on day 1, and the cells were incubated at 37°C for 2 days. Total cellular protein content was determined by staining cells with sulforhodamine B, and IC_{50} and LD_{50} values were calculated. IC_{50} indicates the inhibitor concentration required to reduce total protein accumulation by 50% relative to untreated cells; LD_{50} indicates the inhibitor concentration required to reduce total cellular protein by 50% relative to the starting amount (23). Similar values were obtained in two independent studies.

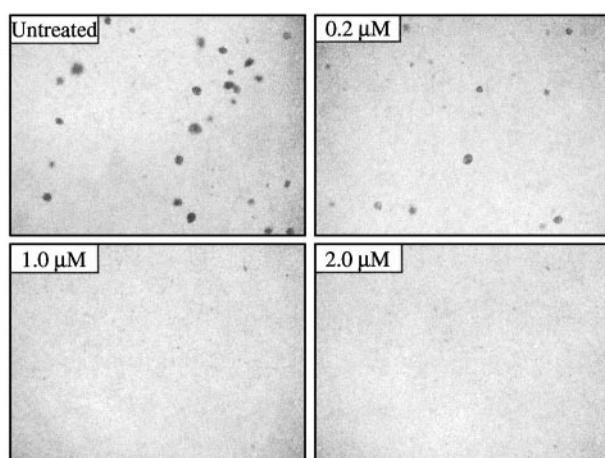|	HT-29				MDA-MB-468			
	Low serum		High serum		Low serum		High serum	
Compounds	IC_{50} (μM)	LD_{50} (μM)	IC_{50} (μM)	LD_{50} (μM)	IC_{50} (μM)	LD_{50} (μM)	IC_{50} (μM)	LD_{50} (μM)
SU11333	0.8	3.9	6.9	12.0	1.6	4.5	4.0	9.0
SU11336	1.2	2.7	7.2	17.0	1.2	7.2	6.5	10.5
SU12470	0.8	5.5	6.8	12.0	1.5	7.8	4.5	9.5
CGP77675	0.8	5.0	5.0	21.0	0.3	8.0	3.0	16.0

Fig. 8.   SU11333 inhibits anchorage-independent growth of HT-29 cells in soft agar. HT-29 cells (1200 cells/well) were seeded in soft agar along with the indicated inhibitors as described in "Materials and Methods." Plates were incubated for 2 weeks and stained overnight in a solution of 1 μg/ml iodonitrotetrazolium salt. Representative fields photographed at ×4 magnification are shown.

phosphorylation of FAK (Y861) under both low and high serum conditions in HT-29 cells. STAT3 was expressed at very low levels in this cell line, so potential changes in its phosphorylation status could not be accurately assessed. Src Y419 is located in the Src catalytic domain and is reported to be the major Src autophosphorylation site. We found that this phosphoepitope was inhibited by the compounds to an extent comparable with that seen with FAK (Y861) under low serum conditions (Fig. 4*A*) but was less susceptible in cells grown under high serum conditions (Fig. 4*B*).

SU11333 dose-dependently inhibited STAT3 (Y705) and FAK (Y861) phosphorylation in MDA-MB-468 (Fig. 5*A*) and A549 cells (Fig. 5*B*) in both low and high serum conditions with submicromolar potency. Similar results were obtained after treatment of Colo205 cells and SKOV3TP5 cells with SU11333 (data not shown). Hence, phosphorylation at STAT3 (Y705) and FAK (Y861) appears to be highly SFK dependent in multiple human tumor cell lines.

SU11333 Inhibits STAT3 and FAK Phosphorylation in Tumors Grown *in Vivo*. The studies described above, which demonstrated that STAT3 (Y705) and FAK (Y861) are SFK dependent, were performed in cultured cells. However, the signals driving phosphorylation of STAT3 and FAK in tumor cells *in vivo* could potentially be quite different, reflecting differences in cell adhesion, morphology, and signaling. To address this possibility, we evaluated the ability of SU11333 to inhibit phosphorylation of these targets *in vivo*. Tumors were established in athymic mice from the NIH 3T3 cells engineered to overexpress activated Src. As shown in Fig. 6*A*, a single dose of SU11333 was able to inhibit STAT3 and FAK phosphorylation in a dose-dependent manner relative to pretreatment and vehicle-treated controls, indicating that these phosphoepitopes are SFK dependent in these engineered tumors *in vivo*. Comparable levels of inhibition were evident 1 (Fig. 6*A*) and 5 h (data not shown) after treatment. Similar results were seen when Fak (Y861) phosphorylation changes were assessed by immunohistochemistry (Fig. 6*B*).

The effects of Src inhibition were also evaluated in mice bearing HT-29 human colon cancer xenografts (these tumors have naturally high levels of Src activity but are not engineered to overexpress an activated Src allele). We found that phosphorylation at FAK (Y861) was partially inhibited by both SU11333 and CGP77675 (Fig. 7). Moreover, as in cultured HT-29 cells (Fig. 4*A*), the putative Src autophosphorylation epitope Y419 was also inhibited with potency similar to that seen for FAK (Y861). These tumors express very low levels of STAT3, so the effects of Src inhibition on STAT3 signaling could not be explored in this model.

Src Inhibitors Suppress Anchorage-dependent Growth of Human Tumor Cells. To explore the potential requirement for SFKs in tumor cell proliferation, we evaluated the effect of four different SFK inhibitors in 2-day proliferation assays using HT-29, MDA-MB-468, and A549 cells. As shown in Table 2, we found that multiple Src compounds potently inhibited the growth of HT-29 and MDA-MB-468 cells under low serum (autocrine) conditions. These effects were seen at dosages consistent with those required to inhibit Src-dependent signaling (see the results above). In contrast, growth under high serum conditions was not inhibited at target-selective concentrations. These data suggest

Table 3 Structurally diverse selective Src inhibitors inhibit anchorage-independent growth of HT-29 cells in soft agar

HT-29 cells (1200 cells/well) were seeded in soft agar along with the indicated inhibitors as described in "*Materials and Methods.*" Plates were incubated for 2 weeks and stained overnight in a solution of 1 μg/ml iodonitrotetrazolium salt. Colonies were counted at $\times$4 magnification, and for each treatment group the total number of colonies in triplicate wells was calculated (variation was minimal between wells within groups).

Conditions	Compounds	Concentration (μM)	Total no. of colonies	% Inhibition
High Serum	Untreated	—	261	—
	SU11333	0.2	164	37
		1.0	3	99
		2.0	0	100
	SU11336	0.2	250	4
		1.0	141	46
		2.0	0	100
	CGP77675	0.2	187	28
		1.0	133	49
		2.0	47	82
Low Serum	Untreated	—	81	—
	SU11333	0.2	46	43
		1.0	2	97
		2.0	0	100
	SU11336	0.2	73	10
		1.0	58	28
		2.0	0	100
	CGP77675	0.2	54	33
		1.0	49	39
		2.0	0	100

that Src kinase activity is required for autocrine growth of HT-29 and MDA-MB-468 cells. A similar trend was also observed for A549 cells (data not shown).

Src Inhibitors Suppress Anchorage-independent Growth of HT-29 Human Colon Tumor Cells. Anchorage-independent growth is a hallmark of the transformed state (27). Therefore, to explore the contribution of SFKs to anchorage-independent growth, we evaluated the effect of three different SFK inhibitors on HT-29 human colon tumor cell colony formation in soft agar. Treatment with SU11333 resulted in dose-dependent suppression of the growth of HT-29 cells in soft agar under both low and high serum conditions (Fig. 8; Table 3). Again, the submicromolar potency of SU11333 in this assay is consistent with the concentrations of compound required to inhibit SFK signaling in HT-29 cells (Fig. 2). Similarly, SU11336 and CGP77675 were also potent inhibitors of colony formation (Table 3). In general, all three inhibitors appeared somewhat more potent in inhibiting anchorage-independent growth than in inhibiting anchorage-dependent growth (compare Table 3 with Table 2).

SU11333 Inhibits VEGF-dependent Vascular Permeability in Mice. To address the potential requirement for SFKs in signaling downstream of the VEGF receptor Flk-1/KDR, the ability of multiple Src inhibitors to suppress VEGF-dependent vascular permeability was assessed using the Miles assay. A single treatment with SU11333 or CGP77675 at 40 (Fig. 9) or 100 mg/kg (data not shown) inhibited VEGF-induced vascular permeability to an extent comparable with the Flk-1/KDR inhibitor SU5416 administered at 20 mg/kg. This effect of SU11333 and CGP77675, achieved at dosages

similar to those required to inhibit SFK-dependent phospho-epitopes *in vivo* (Figs. 6 and 7; data not shown), strongly supports the hypothesis that SFKs are effectors central to VEGFR signaling.

Discussion

Phosphorylation at tyrosine 705 results in dimerization and activation of STAT3, a transcription factor that is implicated in the expression of several genes important in cancer, including *myc* and *vegf* (15, 28). The ability of diverse SFK inhibitors to potently inhibit phosphorylation at STAT3 (Y705) in diverse cancer cells in culture demonstrates that SFKs are probably the major physiological effectors of phosphorylation at this phosphoepitope. These data are consistent with a previous report that STAT3 activation is Src dependent in unstimulated breast cancer cells (29). In contrast with solid tumors, STAT3 is likely to be predominantly activated by Janus kinase family members in leukemia (30).

The *fak* locus is frequently amplified in human cancers, and FAK expression is elevated in sarcomas and in colon, prostate, and breast tumors (12). FAK is activated after integrin engagement and mediates integrin-induced Src activation by relieving negative regulatory conformational constraints in Src. In turn, FAK is itself phosphorylated and activated by Src (12). FAK contains numerous phosphoepitopes, and the kinases responsible for phosphorylation of each phosphoepitope are not completely characterized. The ability of multiple independent SFK inhibitors to suppress phosphorylation of FAK (Y861) in diverse tumor cells in culture and *in vivo* indicates that this phosphoepitope is a site of regulation of FAK by SFKs. The observation that phosphorylation at STAT3 (Y705) and FAK (Y861) was readily pharmacologically reversible strongly, albeit indirectly, suggests that the enhanced phosphorylation seen at these phosphoepitopes in Src Y530F-transformed fibroblasts (Fig. 3) occurs via a direct mechanism *i.e.,* direct phosphorylation by Src, rather than being secondary to cell transformation *per se*. These data are consistent with the observation that, in biochemical phosphorylation assays using isolated c-Src and FAK proteins, Y861 is the predominant site of phosphorylation of FAK by Src (31).

A requirement for Src in G_1-S progression in fibroblasts after treatment with PDGF or EGF has been demonstrated (14). However, the role of Src kinase activity in proliferation of tumor cells has not been rigorously addressed. Previous studies using synthetic kinase inhibitors have demonstrated inhibition of cell growth, but the interpretation of these data is unclear because cells were grown in the presence of high levels of serum (17). We found that, when cultured under low serum conditions, the proliferation of colon, breast, and lung tumor cells was inhibited by diverse SFK inhibitors at compound concentrations similar to those required to inhibit SFK-dependent phosphorylation of STAT3 and FAK (Table 2; data not shown). In contrast, in cells cultured under high serum conditions, inhibition was not seen at compound concentrations considered to be highly selective. This divergence does not stem from differences in levels of soluble proteins (which might bind compounds) in the culture medium because all four compounds evaluated can inhibit Src-
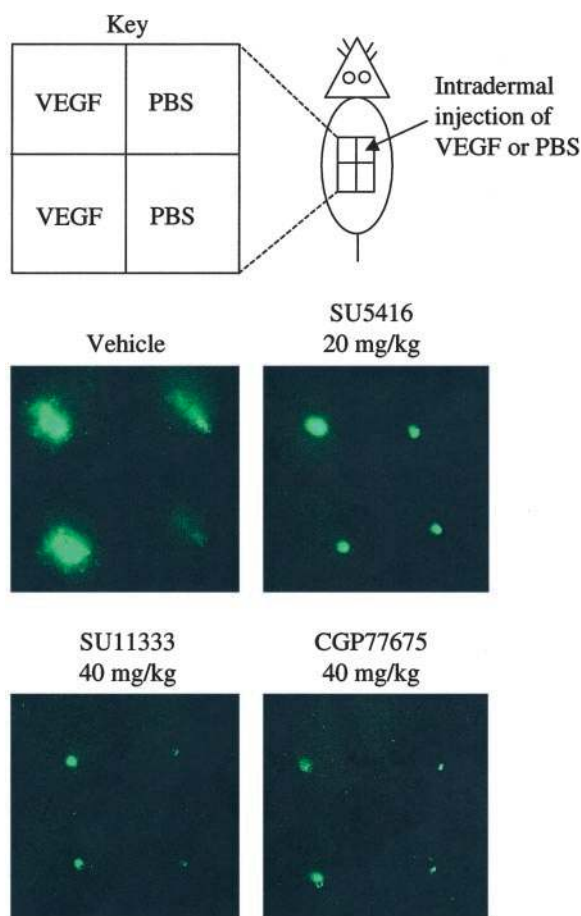
Fig. 9. Mice were given a single i.p. treatment with SU11333 at 40 and 100 mg/kg, CGP77675 at 40 and 100 mg/kg, SU5416 at 20 mg/kg, or vehicle (DMSO) alone (*n* = 3 mice/group). Three h later, 100 μl of a 2.2% solution of FITC-dextran were administered i.v. via the tail vein. One h later, mice received intradermal injections (in duplicate sites on their backs) with 400 ng of VEGF dissolved in 20 μl of PBS and (in adjacent duplicate sites) with PBS alone (location of injections are as indicated in key). After 30 min, the extent of VEGF-dependent dye leakage from the vasculature into skin was assessed visually and photographed using fluorescence *in vivo* microscopy. Images of representative mice are shown. SU11333 and CGP77675 were comparably efficacious at 40 (shown) and 100 mg/kg (data not shown).

dependent cellular morphological features in cells grown under high serum conditions at low submicromolar concentrations (data not shown). Hence, whereas growth under low serum conditions does not authentically simulate the complex mixture of signals driving tumor growth *in vivo* (including integrins, inflammatory cytokines, and so forth), it did allow elucidation of an underlying requirement for SFK function in tumor cell proliferation that had been previously masked by the unnatural stimulus of serum.

The proliferation of the tumor cell lines evaluated (HT-29 and MDA-MB-468) is probably mediated primarily by the production of autocrine growth factors, such as EGF, which activate their cognate RTKs, which in turn directly activate SFKs [predominantly Src and Yes in colon and breast tumors and cell lines (5)]. The Src inhibitors were cytostatic rather than cytotoxic in their effects on cultured cancer cells, sug-

gesting that SFKs are not major mediators of tumor cell survival, at least under the conditions evaluated.

Three different SFK inhibitors potently inhibited HT-29 colony formation in soft agar under both high and low serum conditions (Table 3). Consistent with the results for anchorage-dependent growth, greater potency was evident for colonies grown under low serum conditions, although strong inhibition was seen at selective concentrations of compound under high serum conditions as well. These effects on anchorage-independent growth are consistent with a previous report demonstrating that antisense RNA-mediated inhibition of Src expression in HT-29 cells inhibits their subsequent growth as s.c. tumor xenografts in nude mice (32). It has been hypothesized that abnormally activated SFKs can substitute for appropriate cell-matrix contacts, thereby permitting aberrant growth of cancer cells under anchorage-independent conditions (33).

Src has been implicated in VEGF-dependent angiogenesis at the level of VEGF production (34, 35). Interestingly, it has recently been demonstrated that v-Src-dependent induction of VEGF expression is mediated via STAT3 (28). It has also become apparent that SFKs may serve as important downstream effectors of signaling via activated Flk-1/KDR (13). Consistent with this hypothesis, administration of the semi-selective SFK inhibitor PP1 partially inhibited VEGF-induced vascular permeability in mice after stroke injury (19). However, interpretation of these data is complicated by the fact that PP1, unlike SU11333 and CGP77675, potently inhibits Flk-1/KDR in addition to SFKs (Table 1). Here we demonstrated that administration of either SU11333 or CGP77675 completely inhibited VEGF-dependent vascular permeability in mouse skin (Fig. 9). These data strongly support the hypothesis that SFKs are indispensable mediators of the VEGF-dependent vascular permeability signal cascade and are consistent with a published report demonstrating that mice deficient in Src or Yes exhibit reduced VEGF-dependent vascular permeability (13). Potential SFK effectors in this pathway include the gap junction protein connexin 43 (36).

We recognize that the small molecule inhibitors that we have used in these studies exhibit limited selectivity for SFKs over other kinases evaluated (Table 1). Therefore, we cannot exclude the possibility that the pharmacological effects we have documented may partially reflect effects on kinases other than SFKs. However, the fact that common biological effects were seen with several structurally diverse inhibitors administered at selective concentrations strongly supports the argument that the effects we have seen *in vitro* and *in vivo* are predominantly SFK dependent.

In conclusion, in this work, the use of multiple highly selective SFK inhibitors in culture and *in vivo* has decisively demonstrated the central involvement of SFK signaling in phosphorylation of signaling molecules important in cancer (STAT3 and FAK), in tumor cell proliferation, and in VEGF-dependent signaling.

Acknowledgments

References

1. Susva, M., Missbach, M., and Green, J. Src inhibitors: drugs for the treatment of osteoporosis, cancer or both? Trends Pharmacol. Sci., *21:* 489–495, 2000.

2. Kane, L. P., Lin, J., and Weiss, A. Signal transduction by the TCR for antigen. Curr. Opin. Immunol., *12:* 242–249, 2000.

3. Kurosaki, T. Genetic analysis of B cell antigen receptor signaling. Annu. Rev. Immunol., *17:* 555–592, 1999.

4. Soriano, P., Montgomery, C., Geske, R., and Bradley, A. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell, *64:* 693–702, 1991.

5. Irby, R. B., and Yeatman, T. J. Role of Src expression and activation in human cancer. Oncogene, *19:* 5636–5642, 2000.

6. Irby, R. B., Mao, W., Coppola, D., Kang, J., Loubeau, J. M., Trudeau, W., Karl, R., Fujita, D. J., Jove, R., and Yeatman, T. J. Activating SRC mutation in a subset of advanced human colon cancers. Nat. Genet., *21:* 187–190, 1999.

7. Sugimura, M., Kobayashi, K., Sagae, S., Nishioka, Y., Ishioka, S., Terasawa, K., Tokino, T., and Kudo, R. Mutation of the SRC gene in endometrial carcinoma. Jpn. J. Cancer Res., *91:* 395–398, 2000.

8. Talamonti, M. S., Roh, M. S., Curley, S. A., and Gallick, G. E. Increase in activity and level of pp60c-src in progressive stages of human colorectal cancer. J. Clin. Investig., *91:* 53–60, 1993.

9. Allgayer, H., Boyd, D. D., Heiss, M. M., Abdalla, E. K., Curley, S. A., and Gallick, G. E. Activation of Src kinase in primary colorectal carcinoma. An indicator of poor clinical prognosis. Cancer (Phila.), *94:* 344–351, 2002.

10. Han, N. M., Curley, S. A., and Gallick, G. E. Differential activation of pp60(c-Src) and pp62(c-Yes) in human colorectal carcinoma liver metastases. Clin. Cancer Res., *2:* 1397–1404, 1996.

11. Turkson, J., and Jove, R. STAT proteins: novel molecular targets for cancer drug discovery. Oncogene, *19:* 6613–6626, 2000.

12. Jones, R. J., Brunton, V. G., and Frame, M. C. Adhesion-linked kinases in cancer; emphasis on Src, focal adhesion kinases and PI 3-kinase. Eur. J. Cancer, *36:* 1595–1606, 2000.

13. Eliceiri, B. P., Paul, R., Schwartzberg, P. L., Hood, J. D., Leng, J., and Cheresh, D. A. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. Mol. Cell, *4:* 915–924, 1999.

14. Erpel, T., Alonso, G., Roche, S., and Courtneidge, S. A. The Src SH3 domain is required for DNA synthesis induced by platelet-derived growth factor and epidermal growth factor. J. Biol. Chem., *271:* 16807–16812, 1996.

15. Bowman, T., Broome, M. A., Sinibaldi, D., Wharton, W., Pledger, W. J., Sedivy, J. M., Irby, R., Yeatman, T., Courtneidge, S. A., and Jove, R. Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. Proc. Natl. Acad. Sci. USA, *98:* 7319–7324, 2001.

16. Blake, R. A., Broome, M. A., Liu, X., Wu, J., Gishizky, M., Sun, L., and Courtneidge, S. A. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. Mol. Cell. Biol., *20:* 9018–9027, 2000.

17. Kraker, A. J., Hartl, B. G., Amar, A. M., Barvian, M. R., Showalter, H. D. H., and Moore, C. W. Biochemical and cellular effects of c-Src kinase-selective pyrido [2,3-d]pyrimidine tyrosine kinase inhibitors. Biochem. Pharmacol., *60:* 885–898, 2000.

18. Missbach, M., Jeschke, M., Feyen, J., Muller, K., Glatt, M., Green, J., and Susa, M. A novel inhibitor of the tyrosine kinase Src suppresses phosphorylation of its major cellular substrates and reduces bone resorption *in vitro* and in rodent models *in vivo*. Bone, *24:* 437–449, 1999.

19. Paul, R., Zhang, Z. G., Eliceiri, B. P., Jiang, Q., Boccia, A. D., Zhang, R. L., Chopp, M., and Cheresh, D. A. Src deficiency or blockade of Src activity in mice provides cerebral protection following stroke. Nat. Med., *7:* 222–227, 2001.

20. Mendel, D. B., Laird, D. A., Smolich, B., Blake, R. A., Liang, C., Hannah, A. L., Shaheen, R. M., Ellis, L. M., Weitman, S., Shawver, L. K.,

and Cherrington, J. M. Development of SU5416, a selective small molecule inhibitor of VEGF receptor tyrosine kinase activity, as an anti-angiogenesis agent. Anticancer Drug Des., *15:* 29–41, 2000.

21. Laird, A. D., Vajkoczy, P., Shawver, L. K., Thurnher, A., Liang, C., Mohammadi, M., Schlessinger, J., Ullrich, A., Hubbard, S. R., Blake, R. A., Fong, T. A. T., Strawn, L. M., Sun, L., Tang, C., Hawtin, R., Tang, F., Shenoy, N., Hirth, K. P., McMahon, G., and Cherrington, J. M. SU6668, a broad spectrum angiogenesis inhibitor, exhibits potent anti-tumor activity in xenograft models, including regression of established tumors. Cancer Res., *60:* 4152–4160, 2000.

22. Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A., and Connelly, P. A. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. J. Biol. Chem., *271:* 695–701, 1996.

23. Rubinstein, L. V., Shoemaker, R. H., Paull, K. D., Simon, R. M., Tosini, S., Skehan, P., Scudiero, D. A., Monks, A., and Boyd, M. R. Comparison of *in vitro* anticancer-drug-screening data generated with a tetrazolium assay *versus* a protein assay against a diverse panel of human tumor cell lines. J. Natl. Cancer Inst. (Bethesda), *82:* 1113–1118, 1990.

24. Miles, A. A., and Miles, E. M. Vascular reactions to histamine, histamine-liberator and leukotaxine in the skin of guinea pigs. J. Physiol., *118:* 228–257, 1952.

25. Li, S., Seitz, R., and Lisanti, M. P. Phosphorylation of Caveolin by src tyrosine kinases. The α-isoform of Caveolin is selectively phosphorylated by v-Src *in vivo*. J. Biol. Chem., *271:* 3863–3868, 1996.

26. Eliceiri, B. P., Puente, X. S., Hood, J. D., Stupack, D. G., Schlaepfer, D. D., Huang, X. Z., Sheppard, D., and Cheresh, D. A. Src-mediated coupling of focal adhesion kinase to integrin $\alpha_v\beta_5$ in vascular endothelial growth factor signaling. J. Cell. Biol., *157:* 149–160, 2002.

27. Hanahan, D., and Weinberg, R. A. The hallmarks of cancer. Cell, *100:* 57–70, 2000.

28. Niu, G., Wright, K. L., Huang, M., Song, L., Haura, E., Turkson, J., Zhang, S., Wang, T., Sinibaldi, D., Coppola, D., Heller, R., Ellis, L. M., Karras, J., Bromberg, J., Pardoll, D., Jove, R., and Yu, H. Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis. Oncogene, *21:* 2000–2008, 2002.

29. Garcia, R., Bowman, T. L., Niu, G., Yu, H., Minton, S., Muro-Cacho, C. A., Cox, C. E., Falcone, R., Fairclough, R., Parsons, S., Laudano, A., Gazit, A., Levitzki, A., Kraker, A., and Jove, R. Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene, *20:* 2499–2513, 2001.

30. Lin, T. S., Mahajan, S., and Frank, D. A. STAT signaling in the pathogenesis and treatment of leukemias. Oncogene, *19:* 2496–2504, 2000.

31. Calalb, M. B., Zhang, X., Polte, T. R., and Hanks, S. K. Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. Biochem. Biophys. Res. Commun., *228:* 662–668, 1996.

32. Staley, C. A., Parikh, N. U., and Gallick, G. E. Decreased tumorigenicity of a human colon adenocarcinoma cell line by an antisense expression vector specific for c-Src. Cell Growth Differ., *8:* 269–274, 1997.

33. Guan, J. L., and Shalloway, D. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature (Lond.), *358:* 690–692, 1992.

34. Mukhopadhyay, D., Tsiokas, L., Zhou, X. M., Foster, D., Brugge, J. S., and Sukhatme, V. P. Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. Nature (Lond.), *375:* 577–581, 1995.

35. Ellis, L. M., Staley, C. A., Liu, W., Fleming, R. Y., Parikh, N. U., Bucana, C. D., and Gallick, G. E. Down-regulation of vascular endothelial growth factor in a human colon carcinoma cell line transfected with an antisense expression vector specific for c-src. J. Biol. Chem., *273:* 1052–1057, 1998.

36. Suarez, S., and Ballmer-Hofer, K. VEGF transiently disrupts gap junction communication in endothelial cells. J. Cell. Sci., *114:* 1229–1235, 2001.