Tumor and Stem Cell Biology

Signal Transducers and Activators of Transcription-3 Binding to the Fibroblast Growth Factor Receptor Is Activated by Receptor Amplification

Anna A. Dudka, Steve M.M. Sweet, and John K. Heath

Abstract

Fibroblast growth factor receptors (FGFR) are cell surface tyrosine kinases that function in cell proliferation and differentiation. Aberrant FGFR signaling occurs in diverse cancers due to gene amplification, but the associated oncogenic mechanisms are poorly understood. Using a proteomics approach, we identified signal transducers and activators of transcription-3 (STAT3) as a receptor-binding partner that is mediated by Tyr[677] phosphorylation on FGFR. Binding to activated FGFR was essential for subsequent tyrosine phosphorylation and nuclear translocation of STAT3, along with activation of its downstream target genes. Tyrosine phosphorylation of STAT3 was also dependent on concomitant FGFR-dependent activity of SRC and JAK kinases. Lastly, tyrosine (but not serine) phosphorylation of STAT3 required amplified FGFR protein expression, generated either by enforced overexpression or as associated with gene amplification in cancer cells. Our findings show that amplified FGFR expression engages the STAT3 pathway, and they suggest therapeutic strategies to attack FGFR-overexpressing cancers. *Cancer Res; 70(8); 3391–401. ©2010 AACR.*

Introduction

Fibroblast growth factor receptors (FGFR) of the receptor tyrosine kinase family mediate a diversity of biological processes, including regulation of embryogenesis, proliferation, differentiation, migration, cell survival, and angiogenesis (1). These functions are executed by transmitting signals that initiate gene expression. Thus, upon ligand binding, FGFRs dimerize, triggering tyrosine kinase activity, leading to autophosphorylation of the intracellular domains of both receptors and receptor-associated adaptors and subsequent recruitment of signaling partners. Formation of these multiprotein complexes results in the activation of several well-characterized downstream pathways, including mitogen-activated protein kinase and phosphoinositide-3-kinase/Akt (1).

Abnormal expression of FGFRs is often linked with the development and progression of a variety of human cancers. FGFR gene amplifications and protein expression have been described in several tumor types, including breast (2–4), gastric (5, 6), and prostate (7) tumors. As a result of this, and

other oncogenic manifestations of FGFR function, the FGFR pathway has elicited significant interest as a target for the development of therapeutic interventions (8). However, despite rapidly advancing knowledge of the prevalence of FGFR amplicons in tumors, the exact effect of enhanced FGFR expression on downstream signaling processes is unknown. Here, we implicate the transcription factor STAT3 as a mediator of amplified FGFR signaling.

The signal transducers and activators of transcription (STAT) were identified as transcription factors mediating signaling via the cytokine family of signaling receptors (9). Activation of STATs mediates cell migration, differentiation, proliferation, apoptosis, and wound healing and is classically induced via the cytokine receptor-associated JAK family of non–receptor tyrosine kinases. JAKs phosphorylate tyrosine residues within the intracellular domain of cytokine receptors, providing SH2 domain-mediated docking sites for STAT3. STAT3 is then phosphorylated by JAKs on Tyr[705] within the COOH terminus yielding STAT dimerization (10). Dimeric STAT3 translocates to the nucleus where it induces downstream transcriptional activation of specific target genes (9). The dynamic status of the STAT3/JAK pathway is regulated by two key determinants: STAT3 dephosphorylation and nuclear export (11), as STAT3 constitutively shuttles between the cytoplasm and the nucleus, and tyrosine phosphorylation inactivates nuclear export (12).

STAT3 is often expressed at a high level and is constitutively activated in many human tumor specimens (13–15); its role in cancer development is firmly established (16). The first observation of oncogenic STAT3 activation was made in cells transformed by v-Src (17). In addition, a constitutively active form of STAT3 was shown to be able to transform fibroblasts and induce tumorigenicity (18).

Authors' Affiliation: CRUK Growth Factor Group, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom

Corresponding Author: John K. Heath, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom. Phone: 44-12141-47529; Fax: 44-12141-44534; E-mail: J.K.Heath@bham.ac.uk.

American Association for *Cancer Research*

In this study, we show a connection between overexpression of FGFRs and STAT3 activation. We report that STAT3 is a binding partner for phosphorylated Tyr[677] of FGFR1 and this tyrosine is shown to be critical for STAT3-FGFR1 association. Importantly, tyrosine activation of STAT3 requires overexpression of FGFR1 or FGFR2; either in tumor cells, SUM-52PE, expressing high level of endogenous FGFR2 or experimentally by transient transfection of FGFR1. Moreover, our results imply that JAK2 and Src also form a complex with active FGFR and TyrSTAT3 phosphorylation by FGF is JAK2 and Src-dependent. These findings suggest that oncogenic FGFR amplification, and hyperexpression of receptor, results in ectopic activation of the STAT3 transcriptional response.

Materials and Methods

Cell culture. HEK293T, MCF7, and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum, 0.2 units/mL of penicillin, 0.1 mg/mL of streptomycin, and 2 mmol/L of L-glutamine. SUM-52PE cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 0.2 units/mL of penicillin, 0.1 mg/mL of streptomycin, and 2 mmol/L of L-glutamine.

Antibodies and reagents. Rabbit anti-Bek, rabbit anti-Flg, rabbit anti-ERK1, mouse anti-phosphorylated ERK1, rabbit anti-JunB, and rabbit c-fos antibodies were obtained from Santa Cruz Biotechnology, Inc. Rabbit anti-phosphorylated Jak2 (Tyr[1007/1008]), rabbit anti-Jak2, mouse anti-Src, rabbit anti-phosphorylated Src family (Tyr[416]), mouse anti-STAT3, rabbit anti-phosphorylated STAT3 (Tyr[705]), rabbit anti-phosphorylated STAT3 (Ser[727]), rabbit anti-phosphorylated STAT5 (Tyr[694]), mouse anti-phosphorylated FGFR (Tyr[653/654]), rabbit anti–c-myc, and mouse myc-tag antibodies were obtained from Cell Signaling Technology. Mouse anti–α-tubulin (Sigma), goat anti-human IgG-Fc-HRP conjugate (Pierce), goat anti-mouse IRDye-conjugated, goat anti-rabbit IRDye-conjugated (Li-cor), goat anti-mouse IgG Texas red, goat anti-rabbit Alexa Fluor 594, and goat anti-rabbit IgG FITC (Molecular Probes). Inhibitors SU5402, SU6656, Jak inhibitor I, SP600125, SB203580, calphostin C, and cucurbitacin I (all from Calbiochem), U0126 (Cell Signaling Technology), and PD173074 (Sigma). Lactate dehydrogenase toxicology kit and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) were used according to the instructions of the manufacturer.

Peptide pull-down experiment. Dynabeads MyOne Streptavidin T1 (Invitrogen) were washed and resuspended in PBST. Desthiobiotinylated FGFR1 peptide (1 μmol/L) was added to Dynabeads, incubated for 1 h and washed with PBST before the addition of HEK293T whole cell lysate. After 1 h, whole cell lysate was removed and beads were extensively washed with PBST. Following the addition of 2× reduced SDS sample buffer, proteins were resolved by SDS-PAGE and analyzed by Western blotting.

Transient transfection and cDNA constructs. FcFGFR1 pEF-BOS constructs have been previously described (19). Human full-length FGFR1WT pcDNA3.1 was provided by Pamela Maher (Department of Cell Biology, The Scripps Research Institute, La Jolla, CA). Mouse STAT3 pZeoSV2 was provided by Valeria Poli (Molecular Biotechnology Center, University of Turin, Turin, Italy). Point mutations in FGFR1 Y677F, STAT3, and R609L constructs were obtained by QuickChange site-directed mutagenesis (Stratagene) and were confirmed by DNA sequencing. NH₂-terminal myc-tagged-JAK2 constructs were made by Gateway cloning (Invitrogen) according to the instructions of the manufacturer. Short interfering RNA (siRNA) ON-TARGET oligonucleotides against FGFR2 were purchased from Thermo Fisher Scientific. C-Src and Jak2 siRNA were purchased from Santa Cruz Biotechnology. Nontargeting control siRNA were purchased from Qiagen. Gene Juice reagent (Novagen, MERCK Bioscience) and DharmaFECT3 (Dharmacon) were used according to the instructions of the manufacturer.

Immunoblot and immunoprecipitation. For all experiments, cells were serum-starved, stimulated with 20 ng/mL of FGF1 and 10 μg/mL of heparin for 20 min (or as indicated). Cell lysis and immunoprecipitation were essentially performed as described previously (20). Proteins were separated on NuPAGE 4% to 12% Bis-Tris gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore). Primary antibodies were incubated with the membrane overnight at 4°C. Membranes were washed and probed with InfraRedDye-conjugated secondary antibody, and then proteins were visualized using fluorescence detection on the Odyssey Infrared Imaging System (Li-cor).

Immunofluorescence. Cells growing on coverslips were fixed with 4% paraformaldehyde, permeabilized for 5 min with methanol at −20°C, and incubated with 4% bovine serum albumin-PBS blocking buffer for 1 h. Cells were incubated with primary antibody followed by incubation with fluorescence-conjugated secondary antibody. Coverslips were mounted with Mowiol solution on the slide and observed under a laser scanning confocal microscope (Leica TCS SP2). All further image processing was carried out with Adobe Photoshop 7.0.

Results

STAT3-FGFR1 interaction is phosphorylation-dependent. To identify phosphorylation-dependent protein partners for FGFR1, we performed peptide pull-down experiments from HEK293T cells using phosphorylated and nonphosphorylated pairs of FGFR1 peptides. Using mass spectrometry, STAT3 was identified as a novel binding partner for the FGFR1 peptide containing phosphorylated Tyr[677] (Supplementary Table S1). Tyr[677] is part of a pYxxQ motif on FGFR1, which is a consensus binding motif for the STAT3 SH2 domain (21), and conserved in human FGFR2, FGFR3, and FGFR4 and other vertebrate FGFRs. To confirm the interaction, whole cell lysates from HEK293T cells expressing either STAT3WT (wild-type) or STAT3 R609L, a mutation that specifically disrupts the SH2-binding pocket of STAT3, were incubated with Tyr[677] FGFR1 peptides. Figure 1A shows an increased association of STAT3WT with phosphorylated Tyr[677] FGFR1 peptide compared with the nonphosphorylated peptide. R609L mutation in STAT3 abolished its ability to bind the FGFR1
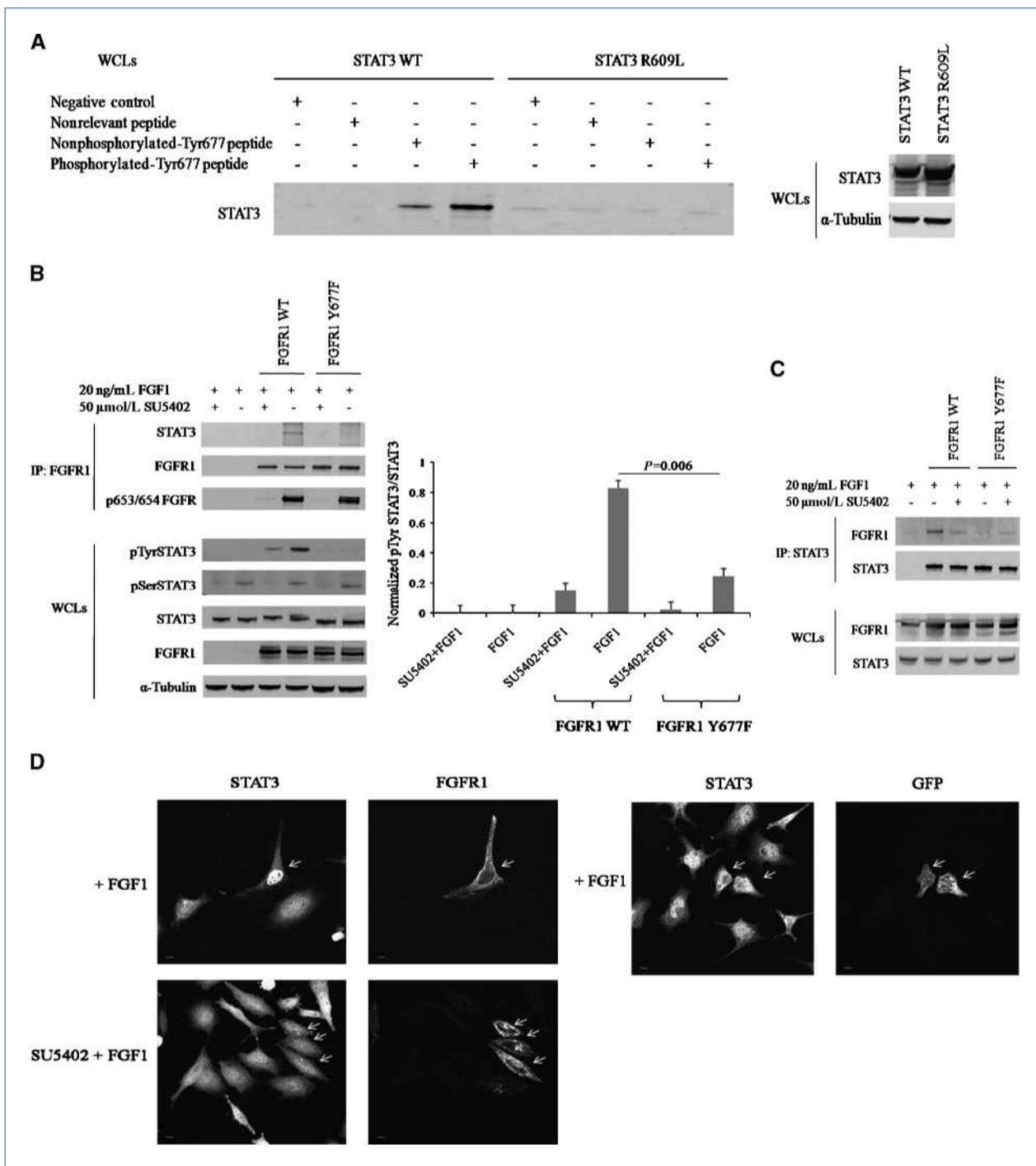
Figure 1. STAT3-FGFR1 interaction is phosphorylation-dependent. A, peptide pull-down was performed using Tyr[677], phosphorylated Tyr[677] FGFR1 peptides, or nonrelevant peptide. Peptides were incubated with Dynabeads followed by whole cell lysates from HEK293T cells overexpressing STAT3WT or STAT3R609L. Negative controls comprised sample without any peptide. Samples from peptide pull-down experiments and whole cell lysates were analyzed by Western blotting. B, HEK293T cells were transiently transfected with FGFR1WT or FGFR1 Y677F. Cells were serum-starved, treated with SU5402 or DMSO, and stimulated with FGF1 and heparin. Immunoprecipitation samples and cell lysates were analyzed by Western blotting. Densitometric analysis of Western blots for pTyrSTAT3 normalized to STAT3 was performed from three separate experiments. Error bars, SD. Statistical significance was determined by t test. C, HEK293T cells were transiently transfected with FGFR1WT or FGFR1 Y677F. Cells were serum-starved, treated with SU5402 or DMSO, and stimulated with FGF1 and heparin. Immunoprecipitation samples and cell lysates were analyzed by Western blotting. D, HeLa cells growing on coverslips were transiently transfected with FGFR1WT or green fluorescent protein construct as negative control. Cells were serum-starved, treated with SU5402 or DMSO, stimulated with FGF1 and heparin, fixed, permeabilized, and stained using STAT3 and FGFR1 antibodies and Texas red or FITC-conjugated secondary antibody, respectively. Bars, 10 μm. Arrows, cells overexpressing FGFR1WT or green fluorescent protein constructs.

Dudka et al.

peptide, demonstrating the importance of the STAT3 SH2 domain (Fig. 1A). These results reveal the interaction between the phosphorylated Tyr[677] FGFR1 peptide and STAT3, and show that the interaction is dependent on the SH2 domain of STAT3.

The STAT3-FGFR1 interaction was further verified by coimmunoprecipitation. A FGFR1 construct was created in which Tyr[677] was substituted with phenylalanine. FGFR1WT and FGFR1 Y677F were expressed in HEK293T cells (Fig. 1B and C). Overexpression of FGFR1WT caused constitutive activation of the receptor, therefore, to compare conditions in which FGFR1 was phosphorylated and dephosphorylated, we used SU5402 as a FGFR inhibitor. The interaction between activated FGFR1WT and STAT3 was confirmed by independent immunoprecipitation experiments in which overexpressed FGFR1 or endogenous STAT3 were pulled-down (Fig. 1B and C). In both experiments, the association between FGFR1 Y677F and STAT3 was decreased, confirming the importance of Tyr[677] of FGFR1 in this interaction (Fig. 1B and C). Additionally, tyrosine phosphorylation of STAT3 was significantly enhanced when FGFR1WT was overexpressed and phosphorylated (Fig. 1B). Increased STAT3 tyrosine phosphorylation was not observed when FGFR1 Y677F was expressed, however, the Y677F point mutation in FGFR1 did not affect serine phosphorylation of STAT3 (Fig. 1B). Serine phosphorylation of STAT3 was also detected in cells expressing endogenous FGFR1, in the presence of FGF1, suggesting that SerSTAT3 was activated as a downstream effect of FGFR1 and was independent of Tyr[677]. Furthermore, STAT3 recruitment to

phosphorylated FGFR1 was shown to be independent of FRS2 (Supplementary Fig. S1). Taken together, our results indicate that STAT3 and FGFR1 specifically interact with each other in a phosphorylation-dependent manner and that Tyr[677] of FGFR1 plays a critical role in STAT3 recruitment and Tyr-STAT3 phosphorylation. Given the crucial roles of the receptor YxxQ motif and the STAT3 SH2 domain, it is most likely that this interaction is direct. Nevertheless, the formal possibility that it is mediated via another protein cannot be excluded.

In the cytokine receptor pathway, activated STAT3 is accumulated inside the nucleus where it exerts its biological effects. Immunofluorescence studies on wild-type HeLa cells showed no STAT3 nuclear accumulation after FGF1 stimulation (data not shown); therefore, we examined localization of STAT3 in HeLa cells expressing high levels of FGFR1. Nuclear accumulation of STAT3 was observed in HeLa cells overexpressing FGFR1WT (Fig. 1D). Moreover, treatment with SU5402 suppressed STAT3 nuclear accumulation proving that the observed effect was due to FGFR1WT activation (Fig. 1D). Transfection with control vector did not induce any effect on STAT3 location (Fig. 1D). Additionally, immunofluorescence analysis for phosphorylated TyrSTAT3 was performed. HeLa cells transfected with FGFR1WT showed nuclear accumulation of tyrosine-phosphorylated STAT3 which was effectively decreased with SU5402 (Supplementary Fig. S2). These results confirm that TyrSTAT3 is phosphorylated only in cells expressing FGFR1WT to higher levels than those required to elicit Tyr[677]-independent serine phosphorylation of STAT3.
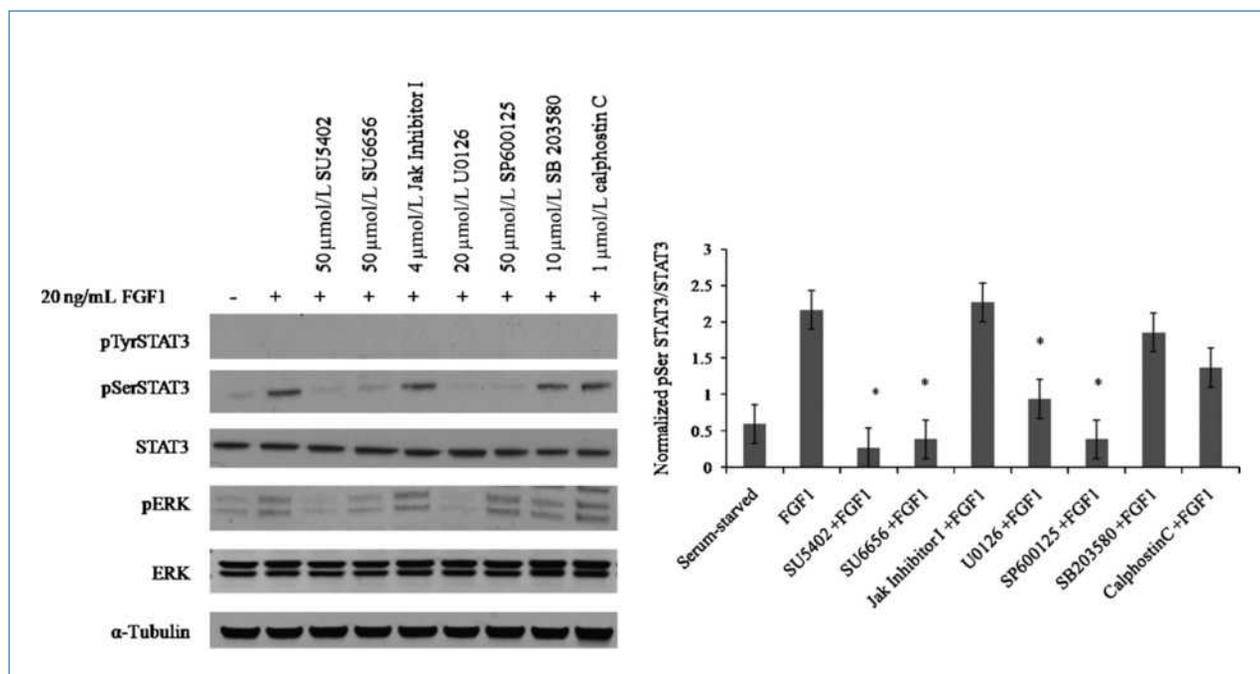

Figure 2. Serine STAT3 activation is a downstream effect of FGFR signaling. MCF7 cells were treated with inhibitors or DMSO for 30 min and stimulated with FGF1 and heparin. Whole cell lysates were analyzed by Western blotting. Densitometric analysis of Western blots for pSerSTAT3 normalized to STAT3 was performed from three separate experiments. Error bars, SD. Statistical significance was determined by t test comparison between each sample treated with inhibitor and sample stimulated with FGF1 (*, $P < 0.05$).
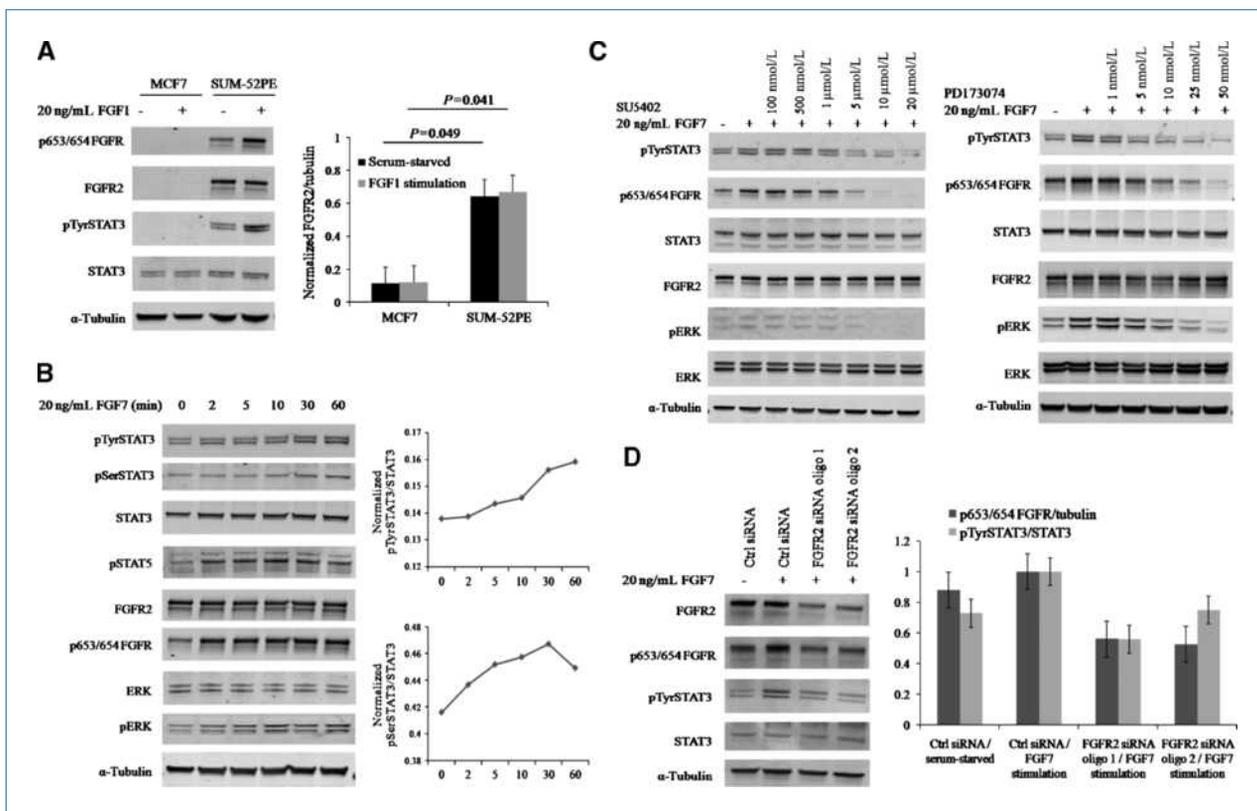
Figure 3. Tyrosine STAT3 phosphorylation depends on high levels of FGFR. A, MCF7 and SUM-52PE cells were serum-starved and stimulated with FGF1 and heparin. Whole cell lysates were analyzed by Western blotting. Densitometric analysis of Western blots for FGFR2 normalized to α-tubulin was performed from three separate experiments. Error bars, SD. Statistical significance between samples was determined by t test. B, SUM-52PE cells were serum-starved and stimulated with FGF7 and heparin as indicated. Whole cell lysates were analyzed by Western blotting. Densitometric analysis of Western blots for pTyr- and pSerSTAT3 normalized to STAT3 was performed from one experiment. C, SUM-52PE cells were serum-starved, treated with inhibitors as indicated or DMSO for 30 min, and stimulated with FGF7 and heparin. Whole cell lysates were analyzed by Western blotting. D, SUM-52PE cells were transfected with FGFR2 siRNA oligonucleotides. Cells were serum-starved, stimulated with FGF7 and heparin. Whole cell lysates were analyzed by Western blotting. Densitometric analysis of Western blots for p653/654FGFR normalized to α-tubulin and pTyrSTAT3 normalized to STAT3 was performed from three separate experiments. Error bars, SD.

Tyrosine, but not serine, STAT3 phosphorylation depends on high expression levels of FGFRs. We examined the effect of FGF1 stimulation on tyrosine and serine phosphorylation of STAT3 in several FGF-responsive cell lines to probe this point further. No tyrosine phosphorylation of STAT3 was observed upon FGF1 stimulation in MCF7, HEK293T, HeLa, and NIH3T3 cells although STAT3 protein was clearly detectable (data not shown). This suggested that stimulation of endogenous FGFR1 in these cells had no detectable effect on STAT3 tyrosine activation. On the other hand, FGF-induced SerSTAT3 phosphorylation did not rely on high level of FGFRs (Fig. 1B). FGF-induced STAT3 serine activation in MCF7 cells was also shown (Fig. 2). To examine which Ser/Thr kinases phosphorylate SerSTAT3, we used specific inhibitors. Decreased SerSTAT3 phosphorylation with U0126 (MEK inhibitor) and SP600125 (JNK inhibitor), but not Jak inhibitor I, SB203580 (p38 inhibitor) and calphostin C (PKC inhibitor), was observed (Fig. 2). These data suggest that SerSTAT3 is phosphorylated downstream of JNK and Ras/mitogen-activated protein kinase pathways, which

are both activated upon FGF1 stimulation. When FGFR1 and Src were inhibited by treatment with SU5402 and SU6656, respectively, the level of phosphorylated SerSTAT3 was also significantly reduced (Fig. 2), presumably due to the lack of JNK and MEK activation. Thus, JNK and MEK kinases were responsible for the phosphorylation of SerSTAT3, which is a downstream effect of FGF1-mediated signaling.

As phosphorylation of TyrSTAT3 required overexpression of FGFR1, we hypothesized that this pathway functions only in cells that express high levels of FGFRs. Therefore, we examined several tumor cells in which the FGFR gene had been amplified. Surprisingly, from this set, only SUM-52PE exhibited elevated FGFR protein expression. SUM-52PE is a breast cancer cell line reported to have a 12-fold amplification of the FGFR2 gene and a 40-fold overexpression of FGFR2 isoform IIIb (2). The expression levels of FGFR2 in two cell lines, MCF7 and SUM-52PE, were compared. Figure 3A shows that FGFR2 in MCF7 cells is barely detectable, whereas SUM-52PE cells express the receptor at high levels. Importantly, the phosphorylation of TyrSTAT3 was only observed in SUM-52PE cells, but

not in MCF7, which supports the notion that TyrSTAT3 activation by FGF requires high levels of FGFR expression.

To specifically activate FGFR2 isoform IIIb, we used FGF7 (1). FGF7 stimulation induced STAT3 tyrosine and serine phosphorylation (Fig. 3B). Moreover, tyrosine STAT5 (and to a lesser extent, TyrSTAT1; data not shown) phosphorylation was also induced (Fig. 3B), which implicates the possibility of interaction between FGFRs and other members of the STAT family. To further verify that this effect was due to FGFR2 activity, we used increasing concentrations of two FGFR inhibitors, SU5402 and PD173074, following stimulation with FGF7 (Fig. 3C). Phosphorylation of TyrSTAT3 gradually decreased together with the inhibition of FGFR2 (Fig. 3C), suggesting that receptor activity was essential.

To validate the data showing that elevated levels of FGFR2 caused tyrosine STAT3 activation, the effect of receptor depletion was studied. Even though the knockdown of FGFR2 was not complete, a reduction of tyrosine STAT3 phosphorylation was observed (Fig. 3D). To exclude the possibility of an off-target effect, two siRNA oligonucleotides against FGFR2 were used and the same effect was detected (Fig. 3D). This reveals a significant role for FGFR2 kinase activity in FGF-induced tyrosine STAT3 phosphorylation in SUM-52PE cells.

STAT3 interacts with FGFR2. We further verified whether STAT3 interacts with FGFR2. Immunoprecipitation experiments were performed in which endogenous FGFR2 or endogenous STAT3 were pulled down from whole cell lysates (Fig. 4A and B). As expected, the association between STAT3 and FGFR2 was enhanced by FGF1 stimulation, although treatment with SU5402 efficiently reduced the interaction, confirming that STAT3 binds preferentially to activated receptor (Fig. 4A and B).

FGFR2-STAT3 pathway activation leads to early response gene expression and cell proliferation. STAT3 is a transcription factor that, when tyrosine phosphorylated, translocates to the nucleus where it regulates the transcription of gene targets. The increased expression of c-fos, c-myc, and JunB was observed after FGF1 stimulation in SUM-52PE cells (Fig. 5A). Because expression of these genes could also be induced by other pathways, including Ras/mitogen-activated protein kinase cascade, SUM-52PE cells were treated with cucurbitacin I to confirm that the observed effect was due to STAT3 activity. STAT3 inhibition significantly reduced the expression of c-fos, c-myc, and JunB (Fig. 5A), indicating that STAT3 contributes to the regulation of the expression of early response transcription factors via its activation with FGFR2. Furthermore, tyrosine STAT3 phosphorylation and its subsequent nuclear accumulation in SUM-52PE cells were confirmed by immunofluorescence (Supplementary Fig. S3). Phosphorylation of TyrSTAT3 was effectively reduced with SU5402 treatment, indicating the importance of FGFR2 activity (Supplementary Fig. S3).

We next examined the effect of blocking FGFR and STAT3 activity on proliferation and viability of SUM-52PE cells. PD173074 treatment led to growth inhibition and cell death as assessed by either cell number–dependent catabolism of the dye MTT or lactate dehydrogenase leakage to the medium (Fig. 5B and C). Cucurbitacin I treatment resulted in the suppression of cell proliferation but did not induce cell death (Fig. 5B and C). The combined effects of both inhibitors gave similar results with PD173074 alone. These data suggest that whereas STAT3-independent FGFR2 activity in SUM-52PE cells is essential for their survival, FGF-activated STAT3 plays a role in the regulation of cell proliferation. Similar dependence of receptor activity in breast and lung cancer cells overexpressing
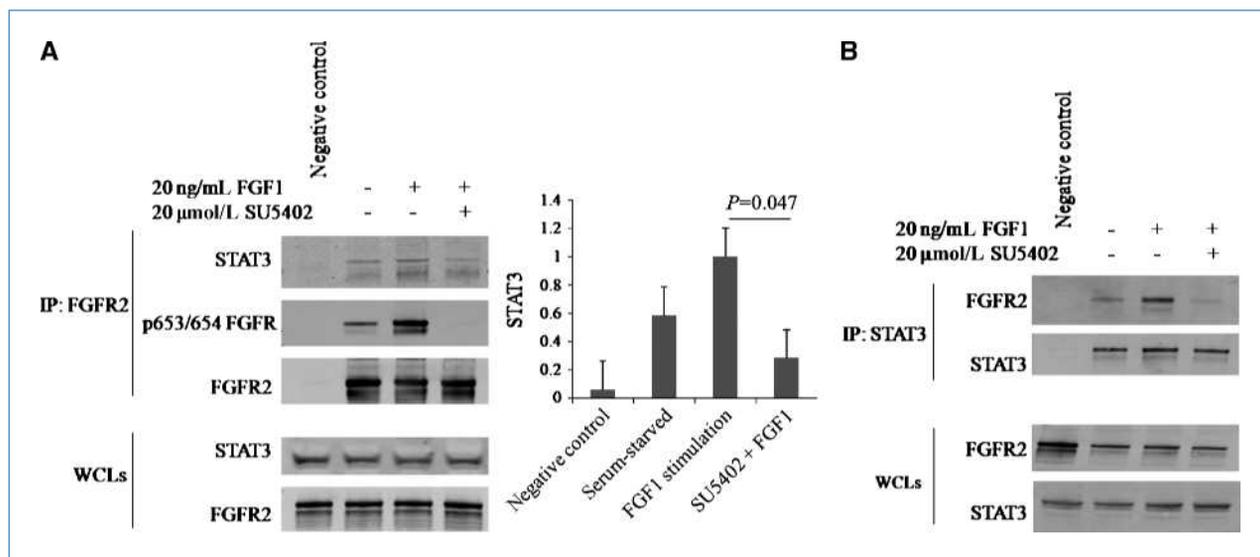

Figure 4. STAT3 interacts with FGFR2. SUM-52PE cells were serum-starved, treated with SU5402 or DMSO for 30 min, and stimulated with FGF1 and heparin. Equal amounts of protein were immunoprecipitated using FGFR2 antibody (A) or STAT3 antibody (B) and Sepharose protein-G. Immunoprecipitation samples and cell lysates were analyzed by Western blotting. A, densitometric analysis performed from three separate immunoprecipitation experiments for STAT3 blots. Statistical significance was determined by t test.
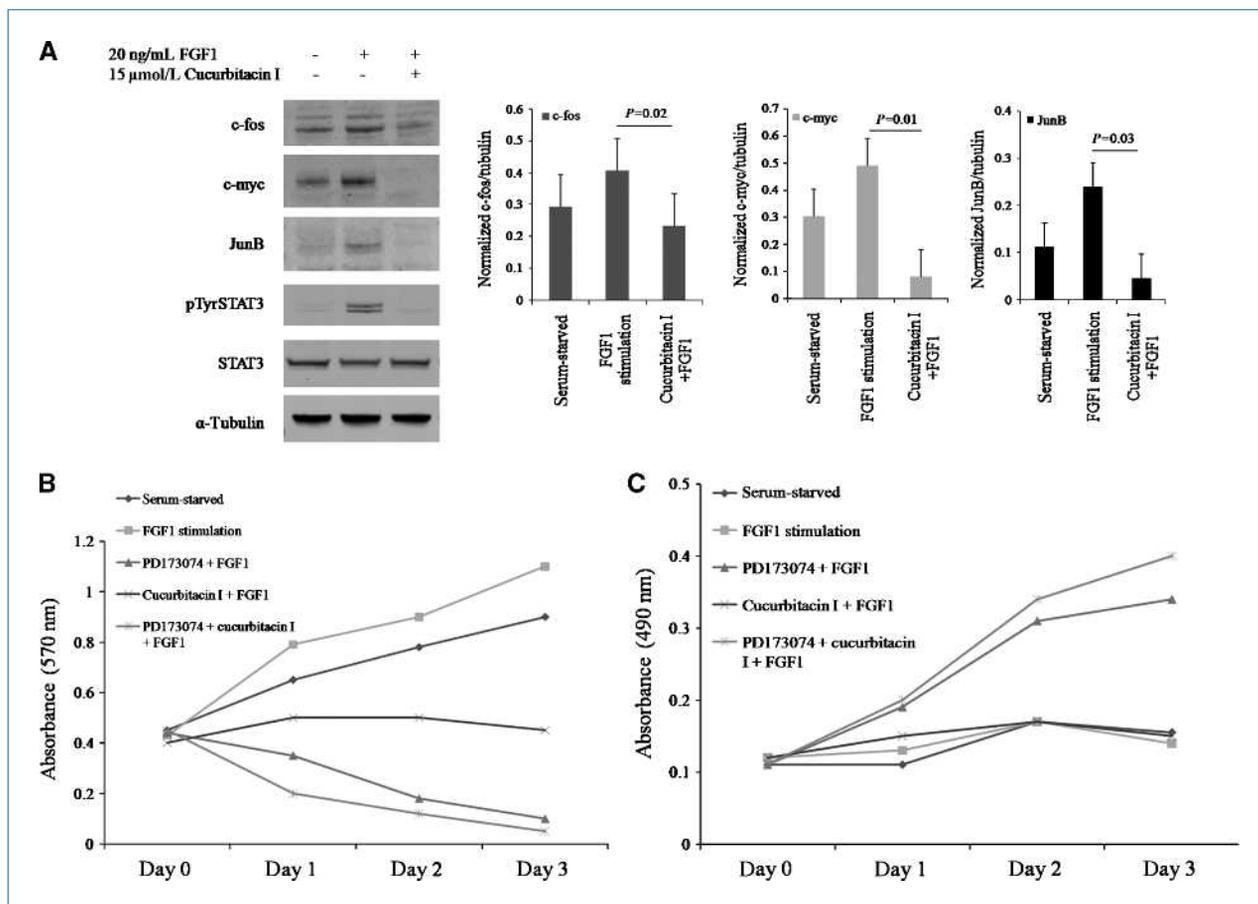
Figure 5. FGFR2-STAT3 pathway activation leads to early response gene expression and cell proliferation. A, SUM-52PE cells were serum-starved, treated with cucurbitacin I or DMSO for 30 min, and stimulated with FGF1 and heparin for 4 h. Whole cell lysates were analyzed by Western blotting. Densitometric analysis of Western blots for c-fos, c-myc, and JunB normalized for α-tubulin was performed from three separate experiments. Error bars, SD. Statistical significance was determined by t test. B and C, SUM-52PE cells were plated in 96-well plates. After 24 h, medium was changed with serum-free medium, cells were treated with 50 nmol/L of PD173074, cucurbitacin I (1 μmol/L), both or DMSO, and stimulated with FGF1 and heparin. After 0, 1, 2, and 3 d, cell proliferation and viability was measured by MTT assay (B) or cell death was evaluated by lactate dehydrogenase leakage into the medium (C). Results are mean from three separate experiments plotted on histogram as units of absorbance. Greater absorbance indicates greater proliferation (B) or greater apoptosis (C).

FGFR has been previously shown (4, 22, 23). However, we cannot eliminate the formal possibility that STAT3 activation in SUM-52PE cells occurs by an FGR kinase–dependent mechanism that does not involve Tyr[677] and is not active in HEK293T cells.

Src and JAK involvement in FGF-induced TyrSTAT3 phosphorylation. To identify the role of Src and JAK in Tyr-STAT3 activation, inhibitors for Src and JAK family members were preincubated with SUM-52PE cells prior to FGF1 stimulation (Fig. 6A). Both inhibitors significantly reduced Tyr-STAT3 activation indicating that Src and JAK contribute to TyrSTAT3 phosphorylation in FGF1-stimulated SUM-52PE cells (Fig. 6A). Furthermore, the same effect was observed using other pharmacologic inhibitors, dasatinib and AG490 (Supplementary Fig. S4A). Additionally, we verified the observed effect by Src and JAK2 depletion using siRNA oligonucleotides. Knockdown experiments confirmed that both kinases, Src and JAK2, take part in FGF-induced TyrSTAT3 phosphorylation (Fig. 6B).

To further investigate the involvement of Src and JAK2 in the STAT3-FGFR pathway, we performed an immunoprecipitation experiment to establish if they associate with the receptor. Src kinase was identified as a binding partner for Tyr[730] of FGFR1 in our initial peptide pull-down (Supplementary Table S1) and the interaction between Src and active FGFR1 was confirmed by immunoprecipitation (Fig. 6C). We transiently transfected four FcFGFR1 constructs into HEK293T cells: the FcFGFR1 kinase active, the kinase dead construct, the truncated version lacking the COOH-terminal intracellular domain, and the FcFGFR1 VT– construct that was kinase active but was unable to bind FRS2 (19). We found that Src interacted with kinase active and VT– FGFR1 (Fig. 6C). Increased phosphorylation of Src was shown with kinase active and VT– FGFR1 expression (Fig. 6C). The interaction between FGFR1 and myc-JAK2 was also shown. Similar to Src, JAK2-FGFR1 association was phosphorylation-dependent and FRS2-independent (Fig. 6D). Phosphorylation of JAK2
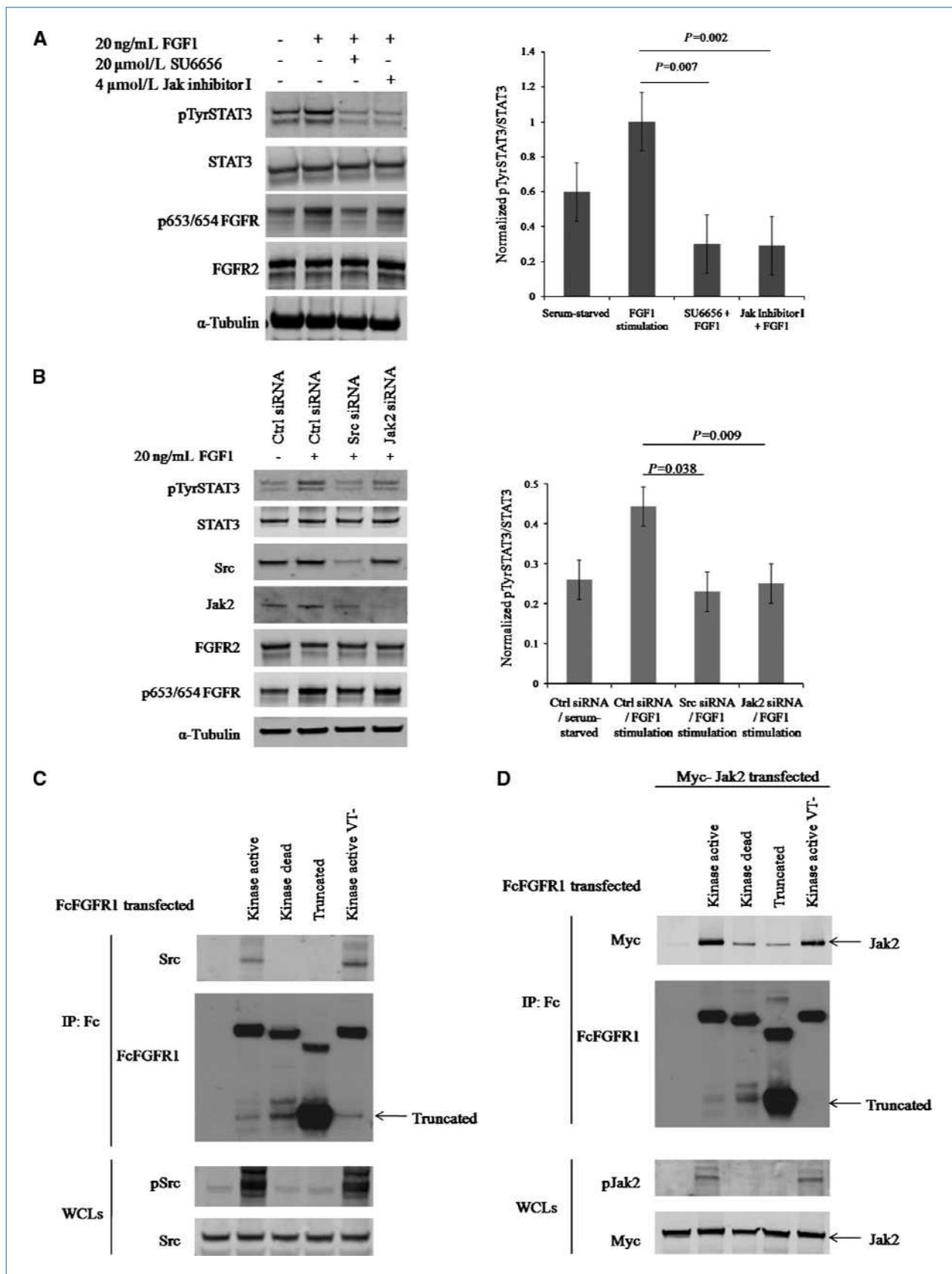
was detected with kinase active FGFR1 and VT–, suggesting that JAK2 acted downstream of FGFR1 and FGFR1 kinase activity was necessary for its activation (Fig. 6D). The reverse immunoprecipitation experiments for Src and JAK2 are presented in Supplementary Fig. S4B. Altogether, our results show that FGFR kinase activity is crucial for the formation of a complex with Src and JAK2, which are activated downstream of FGFR. The results of pharmacologic inhibition studies, as well as Src and JAK2 knockdown, reveal that both non–receptor kinases are required for tyrosine phosphorylation of STAT3.

Discussion

In this study, a connection between overexpression of FGFRs and STAT3 phosphorylation in cancer cells was demonstrated. We show that FGF-induced STAT3 activation via tyrosine phosphorylation requires high expression levels of FGFRs and is Src- and JAK2-dependent. This was shown in breast cancer cell line SUM-52PE, which has amplified FGFR2 gene and elevated levels of receptor expression. STAT3 phosphorylation induced by FGF stimulation in these cells leads to nuclear accumulation and regulation of gene expression. Moreover, we show a phosphorylation-dependent interaction between FGFRs and STAT3. The association between FGFR1WT and STAT3 is mediated by Tyr[677] of FGFR1 that is part of a highly conserved YxxQ SH2-domain binding motif for STAT3 (21). We show that mutation of this tyrosine residue significantly reduced the binding of STAT3 to FGFR1WT. These data are in agreement with our initial peptide pull-down experiment followed by mass spectrometry in which STAT3 was identified as a binding partner for Tyr[677] of FGFR1. Furthermore, a mutation in the SH2 domain of STAT3 abolished the association with a Tyr[677] FGFR1 peptide, suggesting that SH2 domain is necessary for the interaction with the receptor. Thus, the physical interaction between phosphorylated Tyr[677] of FGFR1 and SH2 domain of STAT3 is mandatory for STAT3 recruitment and its subsequent phosphorylation.

The interaction between STATs and growth factor receptors and subsequent STATs activation was previously shown (24–28). FGF-mediated phosphorylation of STAT3 was described in cells harboring FGFR mutations, however, the mechanism of the interaction between them remained elusive. The ability to activate STAT1 and STAT3 seems to be restricted to FGFR3 with K650E mutation, which leads to constitutive activation of the receptor (29–32). The same mutation generated in other FGFR isoforms results in a similar activation of both STATs (33). STAT1 was also described as a

downstream substrate for FGF signaling that negatively regulates the proliferation of chondrocytes (34). However, Krejci and colleagues were unable to detect STAT1 tyrosine activation by FGF in RCS chondrocytes (35). Recently, FGF-induced activation of STAT5 via JAK2 and Src was described as necessary for angiogenesis (36). Here, we tested the activation of STAT3 following FGF1 stimulation: SerSTAT3 phosphorylation was observed at endogenous levels in FGFRs in several cell lines, whereas TyrSTAT3 phosphorylation required the overexpression of FGFRs. Transient transfection of FGFR1WT in HEK293T or HeLa cells induced TyrSTAT3 phosphorylation and nuclear accumulation in contrast to cells with endogenous level of FGFRs. Additionally, FGF stimulation of SUM-52PE resulted in enhanced TyrSTAT3 phosphorylation, which was significantly decreased by deactivation of the receptor using chemical inhibitors, as well as by FGFR2 knockdown. Thus, it is possible that oncogenic overexpression of FGFRs induces STAT3 activation as an additional signaling pathway amplified in cancer cells. Moreover, FGFR autophosphorylation is an ordered and sequential event that when disturbed, for example by activating mutations, might contribute to aberrant assembly of FGFR-binding partners (37). Thus, possible changes in the order of FGFR phosphorylation, including Tyr[677], due to overexpression could lead to modification of adaptor protein recruitment.

Serine STAT3 phosphorylation was believed to enhance STAT3 transcription activity (38), however, other aspects of SerSTAT3 activity have recently emerged (39) and the role of SerSTAT3 in FGFR signaling remains unclear. Udayakumar and colleagues showed the induction of promatrilysin expression in prostate carcinoma cell line by FGF1-induced SerSTAT3 (40). Here, we show that STAT3 serine phosphorylation is induced by ERK and JNK as a downstream effect of FGFR signaling and it does not require the overexpression of FGFRs. However, the physiologic function of SerSTAT3 activation remains to be determined because the involvement of serine STAT3 in oncogenesis was suggested and its activity in cancer cells might be as important as tyrosine phosphorylated STAT3 (41).

We also investigated the potential role of the non–receptor tyrosine kinases, Src and JAK2. Both kinases act downstream of FGFR and their activity is also essential for TyrSTAT3 phosphorylation. Src has been described as an important kinase in STAT phosphorylation in many cell types under various conditions (24, 25, 28, 42), but its activity is usually dependent on upstream receptor tyrosine kinase activation (15). Src activation upon FGF stimulation has been previously described (43), and both direct and indirect interaction

Figure 6. Src and JAK involvement in FGF-induced TyrSTAT3 activation. A, SUM-52PE cells were serum-starved, treated with inhibitors or DMSO for 30 min, and stimulated with FGF1 and heparin. Whole cell lysates were analyzed by Western blotting. Densitometric analysis of Western blots for pTyrSTAT3 normalized for STAT3 was performed from three separate experiments. Error bars, SD. Statistical significance was determined by t test. B, SUM-52PE cells were transfected with Src and JAK2 siRNA oligonucleotides. Cells were serum-starved and stimulated with FGF1 and heparin. Whole cell lysates were analyzed by Western blotting. Densitometric analysis of Western blots for pTyrSTAT3 normalized to STAT3 was performed from three separate experiments. Error bars, SD. Statistical significance was determined by t test. FcFGFR1 constructs (C) or FcFGFR1 and myc-JAK2 (D) constructs were transiently transfected into HEK293T cells. Samples were immunoprecipitated by Sepharose protein-G. Immunoprecipitation samples and cell lysates were analyzed by Western blotting.

between Src family members and FGFR were presented (20, 44). We show that complex formation between active FGFR1 and Src is FRS2-independent. We also show that Src potentially plays a direct role in FGF-induced STAT3 phosphorylation. On the other hand, Src can be indirectly involved in STAT3 activation by maintaining the activity and dynamics of FGFR as activation of Src and FGFR is interdependent (43).

STAT3 activation by various receptor tyrosine kinases might be JAK-dependent or JAK-independent, and both models of STAT3 phosphorylation are presented in the literature (25–28, 36, 45). In our study, we found that JAK2 was necessary for TyrSTAT3 phosphorylation in SUM-52PE cells but its activity depended on the concurrent tyrosine kinase activity of FGFR. Furthermore, immunoprecipitation results showed an association between JAK2 and the kinase-active FGFR1. Phosphorylation-dependent JAK2 association with FGFR1 suggests that JAK2 is recruited only to the activated receptor. The exact mechanism for JAK recruitment to FGFR remains to be further determined.

Finally, we report that STAT3 could be a mediator of amplified FGFR signaling in cancer cells, SUM-52PE, which is a Src- and JAK2-dependent process. We propose a model in which overexpression of FGFR leads to the recruitment of STAT3 to the receptor. At the same time, Src and JAK2 form a complex with active receptor and both kinases are subsequently phosphorylated. Then, Src and JAK2 facilitate tyrosine STAT3 phosphorylation, which leads to STAT3 dimerization, translocation in the nucleus, and regulates cell proliferation. Emerging evidence indicates that aberrant FGFR signaling leads to cancer development (8),; however, the mechanism is thus far unclear. Meanwhile, STAT3 has been found to be active in various cancer cells and its role in cancer development is firmly established (16). This study supports a potential contribution of STAT3 in the process of tumorigenesis in cells overexpressing FGFRs, in which case, the FGFR-STAT3 pathway might be an attractive therapeutic target.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. Cytokine Growth Factor Rev 2005; 16:139–49.
2. Tannheimer SL, Rehemtulla A, Ethier SP. Characterization of fibroblast growth factor receptor 2 overexpression in the human breast cancer cell line SUM-52PE. Breast Cancer Res 2000;2:311–20.
3. Xian W, Pappas L, Pandya D, et al. Fibroblast growth factor receptor 1-transformed mammary epithelial cells are dependent on RSK activity for growth and survival. Cancer Res 2009;69:2244–51.
4. Reis-Filho JS, Simpson PT, Turner NC, et al. FGFR1 emerges as a potential therapeutic target for lobular breast carcinomas. Clin Cancer Res 2006;12:6652–62.
5. Takeda M, Arao T, Yokote H, et al. AZD2171 shows potent antitumor activity against gastric cancer over-expressing fibroblast growth factor receptor 2/keratinocyte growth factor receptor. Clin Cancer Res 2007;13:3051–7.
6. Kunii K, Davis L, Gorenstein J, et al. FGFR2-amplified gastric cancer cell lines require FGFR2 and Erbb3 signaling for growth and survival. Cancer Res 2008;68:2340–8.
7. Sahadevan K, Darby S, Leung HY, Mathers ME, Robson CN, Gnanapragasam VJ. Selective over-expression of fibroblast growth factor receptors 1 and 4 in clinical prostate cancer. J Pathol 2007; 213:82–90.
8. Beenken A, Mohammadi M. The FGF family: biology, pathophysiology and therapy. Nat Rev Drug Discov 2009;8:235–53.
9. Lim CP, Cao X. Structure, function, and regulation of STAT proteins. Mol Biosyst 2006;2:536–50.
10. Schindler C, Levy DE, Decker T. JAK-STAT signaling: from interferons to cytokines. J Biol Chem 2007;282:20059–63.
11. Guerriero ML, Dudka A, Underhill-Day N, Heath JK, Priami C. Narrative-based computational modelling of the Gp130/JAK/STAT signalling pathway. BMC Syst Biol 2009;3:40.
12. Pranada AL, Metz S, Herrmann A, Heinrich PC, Muller-Newen G.

13. Real time analysis of STAT3 nucleocytoplasmic shuttling. J Biol Chem 2004;279:15114–23.
14. Coffer PJ, Koenderman L, de Groot RP. The role of STATs in myeloid differentiation and leukemia. Oncogene 2000;19:2511–22.
15. Mora LB, Buettner R, Seigne J, et al. Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Res 2002; 62:6659–66.
16. Garcia R, Bowman TL, Niu G, et al. Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells. Oncogene 2001;20:2499–513.
17. Yu H, Pardoll D, Jove R. STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 2009;9:798–809.
18. Yu CL, Meyer DJ, Campbell GS, et al. Enhanced DNA-binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. Science 1995;269:81–3.
19. Bromberg JF, Wrzeszczynska MH, Devgan G, et al. Stat3 as an oncogene. Cell 1999;98:295–303.
20. Burgar HR, Burns HD, Elsden JL, Lalioti MD, Heath JK. Association of the signaling adaptor FRS2 with fibroblast growth factor receptor 1 (Fgfr1) is mediated by alternative splicing of the juxtamembrane domain. J Biol Chem 2002;277:4018–23.
21. Li X, Brunton VG, Burgar HR, Wheldon LM, Heath JK. FRS2-dependent SRC activation is required for fibroblast growth factor receptor-induced phosphorylation of Sprouty and suppression of ERK activity. J Cell Sci 2004;117:6007–17.
22. Shao H, Xu X, Mastrangelo MA, et al. Structural requirements for signal transducer and activator of transcription 3 binding to phosphotyrosine ligands containing the YXXQ motif. J Biol Chem 2004;279: 18967–73.
23. Koziczak M, Holbro T, Hynes NE. Blocking of FGFR signaling inhibits breast cancer cell proliferation through downregulation of D-type cyclins. Oncogene 2004;23:3501–8.

23. Pardo OE, Latigo J, Jeffery RE, et al. The fibroblast growth factor receptor inhibitor PD173074 blocks small cell lung cancer growth *in vitro* and *in vivo*. Cancer Res 2009;69:8645–51.

24. Wang YZ, Wharton W, Garcia R, Kraker A, Jove R, Pledger WJ. Activation of Stat3 preassembled with platelet-derived growth factor β receptors requires Src kinase activity. Oncogene 2000;19:2075–85.

25. Olayioye MA, Beuvink I, Horsch K, Daly JM, Hynes NE. ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. J Biol Chem 1999;274:17209–18.

26. Paukku K, Valgeirsdottir S, Saharinen P, Bergman M, Heldin CH, Silvennoinen O. Platelet-derived growth factor (PDGF)-induced activation of signal transducer and activator of transcription (Stat) 5 is mediated by PDGF β-receptor and is not dependent on c-src, fyn, jak1 or jak2 kinases. Biochem J 2000;345:759–66.

27. Bong YS, Lee HS, Carim-Todd L, et al. ephrinB1 signals from the cell surface to the nucleus by recruitment of STAT3. Proc Natl Acad Sci U S A 2007;104:17305–10.

28. Ren Z, Schaefer TS. ErbB-2 activates Stat3 α in a Src- and JAK2-dependent manner. J Biol Chem 2002;277:38486–93.

29. Su WC, Kitagawa M, Xue N, et al. Activation of Stat1 by mutant fibroblast growth-factor receptor in thanatophoric dysplasia type II dwarfism. Nature 1997;386:288–92.

30. Legeai-Mallet L, Benoist-Lasselin C, Munnich A, Bonaventure J. Overexpression of FGFR3, Stat1, Stat5 and p21Cip1 correlates with phenotypic severity and defective chondrocyte differentiation in FGFR3-related chondrodysplasias. Bone 2004;34:26–36.

31. Li C, Chen L, Iwata T, Kitagawa M, Fu XY, Deng CX. A Lys644Glu substitution in fibroblast growth factor receptor 3 (FGFR3) causes dwarfism in mice by activation of STATs and ink4 cell cycle inhibitors. Hum Mol Genet 1999;8:35–44.

32. Krejci P, Salazar L, Kashiwada TA, et al. Analysis of STAT1 activation by six FGFR3 mutants associated with skeletal dysplasia undermines dominant role of STAT1 in FGFR3 signaling in cartilage. PLoS One 2008;3:e3961.

33. Hart KC, Robertson SC, Kanemitsu MY, Meyer AN, Tynan JA, Donoghue DJ. Transformation and Stat activation by derivatives of FGFR1, FGFR3, and FGFR4. Oncogene 2000;19:3309–20.

34. Sahni M, Raz R, Coffin JD, Levy D, Basilico C. STAT1 mediates the increased apoptosis and reduced chondrocyte proliferation in mice overexpressing FGF2. Development 2001;128:2119–29.

35. Krejci P, Salazar L, Goodridge HS, et al. STAT1 and STAT3 do not participate in FGF-mediated growth arrest in chondrocytes. J Cell Sci 2008;121:272–81.

36. Yang X, Qiao D, Meyer K, Friedl A. Signal transducers and activators of transcription mediate fibroblast growth factor-induced vascular endothelial morphogenesis. Cancer Res 2009;69:1668–77.

37. Lew ED, Furdui CM, Anderson KS, Schlessinger J. The precise sequence of FGF receptor autophosphorylation is kinetically driven and is disrupted by oncogenic mutations. Sci Signal 2009; 2:ra6.

38. Wen Z, Zhong Z, Darnell JE, Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell 1995;82:241–50.

39. Decker T, Kovarik P. Serine phosphorylation of STATs. Oncogene 2000;19:2628–37.

40. Udayakumar TS, Stratton MS, Nagle RB, Bowden GT. Fibroblast growth factor-1 induced promatrilysin expression through the activation of extracellular-regulated kinases and STAT3. Neoplasia 2002;4: 60–7.

41. Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. Oncogene 2000;19:2474–88.

42. Kloth MT, Laughlin KK, Biscardi JS, Boerner JL, Parsons SJ, Silva CM. STAT5b, a mediator of synergism between c-Src and the epidermal growth factor receptor. J Biol Chem 2003;278:1671–9.

43. Sandilands E, Akbarzadeh S, Vecchione A, McEwan DG, Frame MC, Heath JK. Src kinase modulates the activation, transport and signalling dynamics of fibroblast growth factor receptors. EMBO Rep 2007; 8:1162–9.

44. Zhan X, Plourde C, Hu X, Friesel R, Maciag T. Association of fibroblast growth factor receptor-1 with c-Src correlates with association between c-Src and cortactin. J Biol Chem 1994;269:20221–4.

45. Vignais ML, Gilman M. Distinct mechanisms of activation of Stat1 and Stat3 by platelet-derived growth factor receptor in a cell-free system. Mol Cell Biol 1999;19:3727–35.

Cancer Research

AACR American Association for Cancer Research

Signal Transducers and Activators of Transcription-3 Binding to the Fibroblast Growth Factor Receptor Is Activated by Receptor Amplification

Anna A. Dudka, Steve M.M. Sweet and John K. Heath

Updated version	Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-09-3033
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2010/04/05/0008-5472.CAN-09-3033.DC1

Cited articles	This article cites 45 articles, 23 of which you can access for free at: http://cancerres.aacrjournals.org/content/70/8/3391.full#ref-list-1
Citing articles	This article has been cited by 7 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/70/8/3391.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/70/8/3391. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.