THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 22, pp. 15736–15744, May 31, 2013

The Fer Tyrosine Kinase Is Important for Platelet-derived Growth Factor-BB-induced Signal Transducer and Activator of Transcription 3 (STAT3) Protein Phosphorylation, Colony Formation in Soft Agar, and Tumor Growth *in Vivo*[*]

Received for publication, April 10, 2013, and in revised form, April 12, 2013 Published, JBC Papers in Press, April 15, 2013, DOI 10.1074/jbc.M113.476424

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Background: Both PDGFR and STAT3 have been implicated in malignant disease, but the mechanisms by which PDGF regulates STAT3 are only partially understood.
Results: Fer is critical for PDGF-mediated STAT3 activation and formation of colonies in soft agar and tumor growth *in vivo*.
Conclusion: Fer has a critical role in PDGF-induced STAT3 activation and cell transformation.
Significance: Fer plays an important role in PDGF-driven tumorigenesis.

Fer is a cytoplasmic tyrosine kinase that is activated in response to platelet-derived growth factor (PDGF) stimulation. In the present report, we show that Fer associates with the activated PDGF β-receptor (PDGFRβ) through multiple autophosphorylation sites, *i.e.* Tyr-579, Tyr-581, Tyr-740, and Tyr-1021. Using low molecular weight inhibitors, we found that PDGF-BB-induced Fer activation is dependent on PDGFRβ kinase activity, but not on the enzymatic activity of Src or Jak kinases. In cells in which Fer was down-regulated using siRNA, PDGF-BB was unable to induce phosphorylation of STAT3, whereas phosphorylations of STAT5, ERK1/2, and Akt were unaffected. PDGF-BB-induced activation of STAT3 occurred also in cells expressing kinase-dead Fer, suggesting a kinase-independent adaptor role of Fer. Expression of Fer was dispensable for PDGF-BB-induced proliferation and migration but essential for colony formation in soft agar. Tumor growth *in vivo* was delayed in cells depleted of Fer expression. Our data suggest a critical role of Fer in PDGF-BB-induced STAT3 activation and cell transformation.

The platelet-derived growth factor (PDGF) family consists of five biologically active dimers of four polypeptide chains (PDGF-AA, -AB, -BB, -CC, and -DD) (1). PDGF isoforms exert their cellular effects via binding to two related tyrosine kinase receptors, *i.e.* PDGF receptor α (PDGFRα) that binds PDGF-A, -B, and -C chains, and PDGFRβ that binds PDGF-B and -D chains. Ligand binding induces dimerization and autophosphorylation of the receptors. Because of their binding specificities, the different PDGF isoforms induce characteristic dimeric receptor complexes (*i.e.* αα, αβ, ββ), which have overlapping, but also distinct, functional properties (2). Phosphorylated tyrosine residues in the intracellular regions of the PDGFRs[5] function as docking sites for signal transduction proteins with Src homology 2 domains (for review, see Ref. 3). Overactivity of PDGFRs is implicated in diseases involving excessive cell growth, including cancer, cardiovascular disease, and fibrosis (for review, see Ref. 4).

Fer is a ubiquitously expressed cytoplasmic tyrosine kinase that in addition to the kinase domain contains an Src homology 2 domain, coiled-coil domains, and an FCH (Fer/Fes/Fps/Cip4 homology) domain (5). Fer is closely related to Fes/Fps, which has a more restricted and primarily hematopoietic expression. Functionally, Fer or the related Fes/Fps has been proposed to be involved in cell adhesion, migration, and proliferation (6–11). A recent phosphoproteome profiling study identified phosphorylated Fer to be associated with invasion and metastasis of hepatocellular carcinoma cells, suggesting an important role for Fer in tumor progression (12).

Previous reports have shown that upon acute PDGF stimulation, Fer becomes tyrosine-phosphorylated and associated with the activated receptor (13). In addition, PDGF treatment also induces the formation of a complex between Fer and the p85 subunit of phosphatidylinositol (PI) 3-kinase, suggesting that Fer may bind PDGFR also indirectly via p85 (14).

STAT proteins make up a group of transcription factors (STAT1–6) that are activated through phosphorylation by growth factors and cytokines. Phosphorylated STAT proteins dimerize and translocate to the nucleus where they drive expression of specific genes. STAT3 is frequently found to be activated in human cancers (for review, see Ref. 15); hence, it is

* This work was supported by Swedish Cancer Society Grant 090715, Swedish Research Council Grant K2011-67X-21859-01-6, and the Ludwig Institute for Cancer Research.
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[5] The abbreviation used is: PDGFR, PDGF receptor.

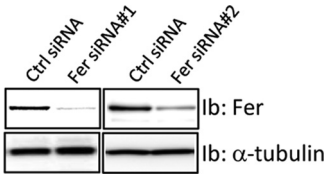

FIGURE 1. **Evaluation of siRNA targeting Fer.** NIH3T3 cells transfected with two distinct siRNA against Fer (siRNA-1 from Invitrogen and siRNA-2 from Dharmacon; for details, see "Experimental Procedures") or control siRNA. Efficiency of Fer silencing was measured by Fer immunoblotting (*Ib*), and α-tubulin served as a loading control. A representative experiment is shown.

important to understand the mechanisms controlling the function of this transcription factor.

The aim of the present investigation was to elucidate the mode of interaction and activation of Fer by PDGFRβ as well as the role the Fer protein in PDGF-BB-induced signal transduction and tumorigenicity.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant human PDGF-BB was generously provided by Amgen (Thousand Oaks, CA). The inhibitors SU6656 and AG490 were from Calbiochem. STI571 was from Novartis Pharma AG (Basel, Switzerland). Antibodies against Fer (sc-81272), Fps/Fes (sc-25415), phosphotyrosine (sc-7029), enolase (sc-15343), Akt (sc-8312), and PDGFRβ (sc-339) were from Santa Cruz Biotechnology. Rabbit antiserum recognizing ERK2 or Alix was raised against peptides corresponding to the sequences EETARFQPGYRS or CSYPFPQPPQQSYYPQQ conjugated to keyhole limpet hemocyanin, respectively. Antisera against phosphorylated ERK1/2 (9106), phosphorylated Akt (9271), phosphorylated Tyr-857-PDGFR (3170), phosphorylated STAT3 (9131), phosphorylated STAT5 (9351), and STAT5 (9352) were purchased from Cell Signaling Technology. STAT3 (610189) antibody was from BD Transduction Laboratories. α-Tubulin antibody was purchased from Sigma. [γ-^{32}P]ATP (BLU502A) was purchased from PerkinElmer Life Sciences.

Cell Culture—NIH3T3 or sis3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine supplemented with 10% bovine serum and 100 units/ml penicillin, 100 μg/ml streptomycin. For serum starvation, cells were washed once and incubated in DMEM containing 0.1% bovine serum.

siRNA Knockdown—Down-regulation of Fer was performed by using 100 nM specific siRNA (RNA sequence: GGUGAAGUAUAUAAGGGCACAUUAAdTdT) purchased from Invitrogen or Fer-specific siGENOME from Dharmacon (siGENOME, D-045318-02). For every experiment performed, luciferase-targeting siRNA (RNA sequence CGUACGCGGAAUACUUCGAdTdT) was used as a control. Transfection of siRNA was done for 24 h with SilentFect from Bio-Rad. Levels of knockdown were tested after an additional 48 h by measuring protein levels by immunoblotting. All experiments were performed with Fer targeting siRNA-1 and then confirmed with siRNA-2. Both Fer siRNAs efficiently silenced Fer expression (Fig. 1).

Lentivirus Transfection—Sis3T3 cells were seeded to 30% confluence. The next day, 8 μg/ml hexadimethrine was added to the cells in fresh medium. An equal number of five different Fer shRNA lentivirus particles and one control lentivirus construct were added. After a gentle swirl the plate was incubated 18–20 h at 37 °C. Next, the medium containing the lentivirus particles was removed, and fresh selection medium containing 3 μg/ml puromycin was added to select for stable Fer shRNA and ctr shRNA silencing clones. The selection medium was exchanged every 3–4 days until resistant clones could be identified. To ensure a high efficiency in the silencing, we performed another round of lentiviral infection.

Western Blotting—Subconfluent cells, transfected as indicated, were starved and stimulated with 20 ng/ml PDGF-BB for the indicated periods of time. Cells were washed with ice-cold phosphate-buffered saline and lysed in 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM Pefabloc, 1% aprotinin, and 1 mM sodium orthovanadate. Extracts were clarified by centrifugation, and equal amounts of lysates were boiled with sodium dodecyl sulfate (SDS) sample buffer containing dithiothreitol. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and then electrotransferred to polyvinylidene difluoride membranes (Immobilon P), which were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline solution containing 0.1% Tween 20. Primary antibodies were diluted according to the manufacturer's instructions and membranes incubated overnight at 4 °C. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (both from Amersham Biosciences), and proteins were visualized using enhanced chemoluminescence Western blotting detection systems from Roche Applied Science on a cooled charge-coupled device camera (Fuji, Minami-Ashigata, Japan).

Peptide Pulldown Assay—Cells were washed in ice-cold PBS and lysed in radioimmune precipitation assay buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM Tris, 150 mM NaCl, 1 mM EDTA, and 10% glycerol), supplemented with 1 mM Pefabloc and 1 mM Na_3VO_4. Peptides of 15–20 amino acid residues in length derived from the sequence surrounding Tyr-579, Tyr-581, Tyr-716, Tyr-740, Tyr-751, Tyr-763, Tyr-771, Tyr-775, Tyr-778, Tyr-857, Tyr-1009, and Tyr-1021 in PDGFRβ, either phosphorylated or not, were synthesized with the addition of a N-terminal cysteine residue to enable coupling to Sulfolink (Pierce)-beads according to the manufacturer's instructions. Coupled peptides were incubated with cell lysates for 2 h rotating at 4 °C. Subsequently, beads were washed three times with radioimmune precipitation assay buffer, and attached proteins were eluted by boiling for 5 min in reducing sample buffer followed by SDS-PAGE and immunoblotting against Fer.

[^{3}H]Thymidine Incorporation Assay—Cells were transfected with Fer or control siRNA, as described above, and seeded into 12-well plates. Cells were serum-starved and incubated with 0.2 μCi/ml [^{3}H]thymidine and increasing concentrations of PDGF-BB or 10% FBS overnight. Macromolecules including DNA were then precipitated using 5% trichloroacetic acid followed by washing in water and then dissolved in 1 M NaOH. The solutions were then neutralized with HCl, and radioactivity was measured with a liquid scintillation counter.

In Vitro Kinase Assay—Subconfluent cells were starved overnight in serum-free medium and stimulated with PDGF-BB for 5 min, followed by lysis and immunoprecipitation with Fer antibodies and *in vitro* kinase assay using acid-denatured enolase as a substrate, as described (16).

Cell Migration Assay—A polycarbonate membrane (polyvinylpyrrolidone-free, pore size, 8.0 μm; NeuroProbe, Gaithersburg, MD) was coated with 50 μg/ml fibronectin (BD Biosciences). Different concentrations of PDGF-BB were added to the lower chambers, and cells were added to the upper chambers at 3×10^4 cells/well. After 4 h of incubation, cells remaining on the upper membrane surface were removed, and the cells that had traversed and spread on the lower surface of the filter were fixed with ethanol and stained with Giemsa. The migrated cells were quantified with a cooled CCD camera (Fuji).

Soft Agar Colony Formation Assay—Colony formation and cell growth rate in soft agar were tested by plating 2.5×10^4 sis3T3 cells transfected with control or Fer siRNA in 0.4 ml of DMEM, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml amphotericin B, 3% FBS, and 0.3% low melting temperature agarose (Seaplaque) in 12-well plates (6 wells for each) coated with 0.8 ml of 0.6% low melting temperature agarose. PDGF-BB (50 ng/ml) was added to half of the wells of each type of siRNA-transfected cells. Colony formation of each type of siRNA-transfected cells was monitored for 5 days in 37 °C incubator, and colony number and size was recorded for comparison and then microphotographed by Axiovert 40 CFL microscope.

Animal Experiments—Animal experiments were approved by the local ethical committee and performed according to the UKCCCR guidelines. All manipulations were performed under isoflurane (Abbott Scandinavia, Solna, Sweden) gas anesthesia. BALB/c nude mice (5–6 weeks old) received subcutaneous inoculation in the dorsal skinfold of 2×10^6 control and Fer-shRNA-transfected sis3T3 cells suspended in 100 μl of cold PBS. The cells were growing at 40–50% confluence before collection. Tumor length and width were measured using calipers, and tumor volume was calculated as $\pi/6$ x length $\times$ width $\times$ width.

Statistics—Data are presented as mean ± S.D. Student's *t* test was used to determine *p* values, and $p < 0.05$ was considered statistically significant.

RESULTS

PDGF-BB Promotes Fer Tyrosine Phosphorylation and Association with PDGFRβ—Previous reports have shown that Fer can interact with the PDGF receptor (13, 14). To confirm this finding, we treated NIH3T3 cells with PDGF-BB and performed immunoprecipitation with Fer-specific antibodies and immunoblotting against phosphotyrosine, PDGFRβ, and Fer. In concurrence with earlier reports, we found that PDGF-BB stimulation caused tyrosine phosphorylation of Fer and co-immunoprecipitation with the activated PDGFRβ (Fig. 2*A*).

To characterize further the interaction between Fer and PDGFRβ, we investigated which tyrosine residues in PDGFRβ mediate the interaction with Fer, by *in vitro* peptide pulldown assays using peptides corresponding to the autophosphorylation sites in the receptor, either tyrosine-phosphorylated or not. We observed robust interactions with peptides containing tyrosine residues 579 and 581 in the juxtamembrane region of the receptor, as well as with peptides containing tyrosine residues 740 and 1021 (Fig. 2*B*). All interactions were dependent on tyrosine phosphorylation of the peptides. A alignment of the amino acid sequences from the identified binding sites (Fig. 2*C*) revealed, with the exception of Tyr-1021, a negatively charged amino acid in position +2 from the phosphotyrosine binding sites (because Tyr-581 is phosphorylated in the Tyr-579 sequence) and a hydrophobic amino acid in position +1. It should be noted that we cannot conclude from the peptide pulldown assay whether the binding is direct or indirect, and this may influence the binding sequence requirements.

Next, we investigated whether phosphorylation of Fer is performed directly by the PDGFR or whether other downstream kinases are involved. Therefore, we determined the effect of low molecular mass inhibitors against PDGFRβ (STI571), Src family kinases (SU6656), and Jak kinases (AG490) on Fer phosphorylation. Whereas inhibition of the kinase activity of the PDGFRβ blocked Fer phosphorylation, inhibition of Src or Jak activity did not affect the PDGF-BB-induced Fer phosphorylation (Fig. 2*D*).

To conclude, Fer binds to several autophosphorylation sites in PDGFRβ. Following Fer binding to the receptor complex, it becomes tyrosine-phosphorylated in a manner sensitive to PDGF receptor kinase inhibition, but not to inhibition of Src and Jak kinases, suggesting that Fer may be phosphorylated directly by the receptor.

PDGF-BB Stimulates Sustained Phosphorylation and Activation of Fer—Many signaling proteins become activated by PDGF stimulation in a transient manner. To elucidate the kinetics of Fer activation, we stimulated cells for different periods of time with PDGF-BB and analyzed the tyrosine phosphorylation of Fer. We found that Fer was rapidly phosphorylated by PDGF-BB and that this phosphorylation was maintained for at least 8 h of continuous stimulation (Fig. 3*A*). In contrast, autophosphorylation of PDGFRβ was maximal at 5–15 min of PDGF-BB stimulation and then declined upon longer stimulation (Fig. 3*A*).

We next investigated whether the observed Fer phosphorylation was correlated with its kinase activity. Fer was immunoprecipitated from cells stimulated with PDGF-BB for different time periods and washed with high salt lysis buffer to remove potential co-immunoprecipitating proteins. An *in vitro* kinase reaction was performed using acid-denatured enolase as an exogenous substrate; robust enolase and Fer phosphorylations throughout the time course of stimulation were observed (Fig. 3*B*), suggesting that Fer tyrosine phosphorylation correlated with increased enzymatic activity. Furthermore, no significant effect of STI571 on Fer kinase activity was observed if STI571 was added to the lysates from PDGF-BB-stimulated cells (Fig. 4), arguing against a direct effect of STI571 on Fer kinase activity in the immunoblot experiment shown in Fig. 2*D*. However, it should be noted that STI571 also inhibits other kinases, such as c-Kit and Abl, whose contribution cannot be excluded in our experiment.

Fer Is Critical for PDGF-BB-induced STAT3 Phosphorylation—To assess the involvement of Fer in PDGF-BB-induced signal

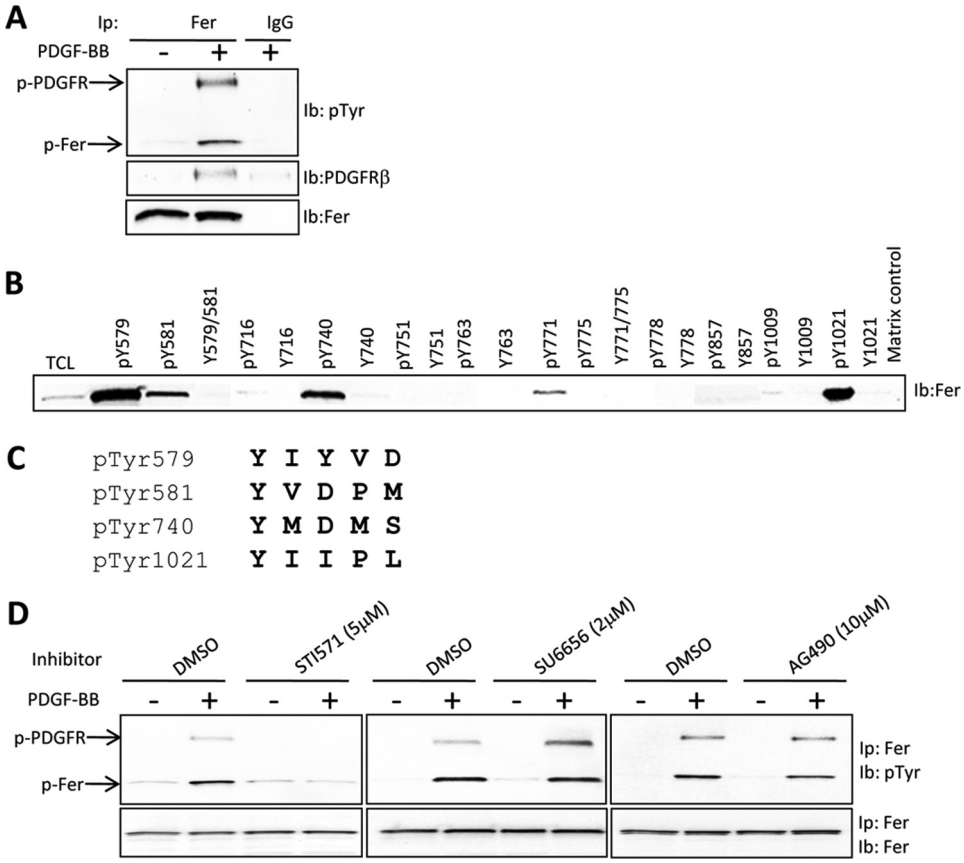

FIGURE 2. **PDGF-BB-induced Fer phosphorylation and association with PDGFRβ.** *A* and *D*, NIH3T3 cells were serum-starved overnight and pretreated for 1 h with inhibitors, as indicated, followed by stimulation with 20 ng/ml PDGF-BB. Fer was immunoprecipitated (*Ip*) followed by SDS-PAGE and immunoblotting (*Ib*) against phosphotyrosine (*pTyr*), PDGFRβ, and Fer, as shown. *DMSO*, dimethyl sulfoxide. *B*, to control for unspecific interactions, we performed precipitation with control IgG from PDGF-BB-stimulated cells. Synthetic peptides corresponding to autophosphorylation sites in the PDGFRβ were incubated with cell lysate, and Fer interaction was determined by immunoblotting. Total cell lysates (*TCL*) were used as a positive control and sulfo-link beads without coupled peptide as a negative control. One representative experiment is shown of three performed. *C*, the amino acid sequences surrounding the identified Fer binding sites were aligned.

transduction, we down-regulated Fer with siRNA and analyzed the activation of major pathways downstream of the PDGF receptor, including ERK1/2, Akt, STAT3, and STAT5. We found that after Fer down-regulation, PDGF-BB was not able to induce STAT3 phosphorylation (Fig. 5*A*). Other pathways analyzed, *i.e.* STAT5, ERK1/2, and Akt, were not dramatically affected by Fer silencing. Furthermore, we observed a decrease in PDGFRβ autophosphorylation, suggesting that Fer phosphorylates the receptor (Fig. 5*A*). The effect of Fer knockdown on STAT3 and PDGFRβ phosphorylation was confirmed using a second siRNA which gave similar results (data not shown). Notably, the strong effect of Fer silencing on STAT3 phosphorylation occurred predominantly in sparse cell cultures and required efficient reduction in Fer protein expression (>90%). The reason for the cell density dependence is not clear, but may relate to up- or down-regulation of proteins or miRNAs affecting STAT3 phosphorylation in a cell density-dependent manner.

Despite our finding that down-regulation of Fer abolished the PDGF-BB-induced STAT3 phosphorylation, a study on fibroblasts from mice into which kinase-deficient Fer had been knocked in, did not report any effect of PDGF-induced STAT3 phosphorylation (17). We therefore addressed the possibility that Fer has a kinase-independent role in PDGF-BB-induced STAT3 phosphorylation, by down-regulating the kinase-deficient Fer in the knock-in cell line. As can be seen in Fig. 5*B*, cells in which the wild-type Fer was replaced with kinase-dead Fer transfected with control siRNA were capable of inducing STAT3 phosphorylation in response to PDGF-BB treatment, as reported previously (17); however, when the expression of kinase-deficient Fer was down-regulated with siRNA, PDGF-BB did not activate STAT3. Activation of STAT5 or PDGFRβ was not appreciably affected by down-regulation of Fer expression or loss of its kinase activity (Fig. 5*B*). Thus, Fer may have a kinase-independent function in PDGF-BB-induced STAT3 activation.

Src Is Involved in PDGF-induced STAT3 Phosphorylation, and Fer Influences PDGF-BB-induced Src Activation—It has been suggested that Src family kinases are involved in growth factor-induced STAT activation (for review, see Ref. 18). Additionally, Jak kinases are established activators of STAT signaling downstream of cytokines receptors. Therefore, we investigated STAT3 and STAT5 activation in response to PDGF-BB in the presence of the Src kinase inhibitor SU6656 or the Jak inhibitor AG490. Suppression of Src activity resulted in a reduction of STAT3 phosphorylation, but no effect was observed on STAT5 or PDGFRβ phosphorylation (Fig. 6*A*). In contrast, treatment with the Jak inhibitor AG490 did not

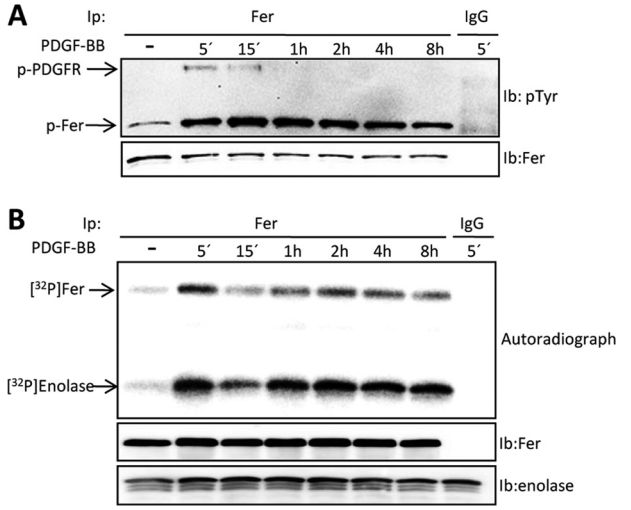

FIGURE 3. **Kinetics of Fer phosphorylation and enzymatic activity.** *A*, NIH3T3 cells were serum-starved overnight and treated with 20 ng/ml PDGF-BB for the indicated periods of time. Cells were lysed, and Fer was immunoprecipitated (*Ip*), followed by SDS-PAGE and immunoblotting (*Ib*) for phosphotyrosine (*pTyr*) or Fer, as indicated. *B*, alternatively, the Fer immunoprecipitates were incubated with [γ-^{32}P]ATP and acid-denatured enolase for 10 min. Proteins were separated by SDS-PAGE, and incorporated radioactivity was visualized by autoradiography, and Fer and enolase by immunoblotting. To control for unspecific interactions we performed precipitation with control IgG from PDGF-BB-stimulated cells. One representative experiment is shown of three performed.

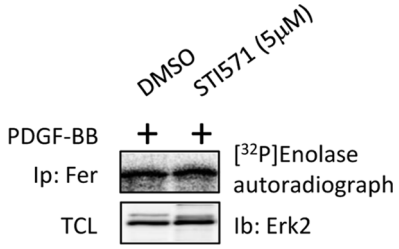

FIGURE 4. **STI571 does not directly inhibit Fer *in vitro*.** NIH3T3 cells were starved overnight treated with STI571 as indicated and then stimulated with 20 ng/ml PDGF-BB. Fer was immunoprecipitated (*Ip*) and incubated with [γ-^{32}P]ATP and acid-denatured enolase for 10 min. Proteins were separated by SDS-PAGE, and incorporated radioactivity was visualized by autoradiography. Equal loading was verified by immunoblotting (*Ib*) for ERK2. *DMSO*, dimethyl sulfoxide; *TCL*, total cell lysate. A representative experiment is shown.

have any effect on PDGF-BB-induced STAT3 or STAT5 activation (Fig. 6*A*).

Because we observed inhibition of STAT3 phosphorylation when Fer was down-regulated as well as after Src inhibition, we tested whether Fer down-regulation affected the PDGF-BB-induced Src activation. We found that reduction of Fer expression in NIH3T3 cells suppressed PDGF-BB-induced Src activation, although it should be noted that the basal Src kinase activity increased (Fig. 6*B*), indicating a role for Fer in PDGF-BB-induced Src activation.

Our results suggest that Fer, but not its kinase activity, is required for efficient STAT3 phosphorylation (Fig. 5). In addition, Src kinases are also involved because SU6656 inhibits STAT3 phosphorylation (Fig. 6*A*). Furthermore, Fer may be involved in Src activation (Fig. 6*B*). Therefore, we investigated whether Fer and Src could form a complex. Indeed, by co-immunoprecipitation experiments we could observe a rapid interaction between Src and Fer (Fig. 6*C*).

Fer Down-regulation Is Necessary for Soft Agar Colony Formation, but Does Not Affect PDGF-BB-induced DNA Synthesis or Cell Migration—To elucidate whether Fer silencing affects the ability of PDGF-BB to promote cell proliferation, we performed [^{3}H]thymidine incorporation assay to measure DNA synthesis. We found no significant difference in PDGF-BB-induced DNA synthesis between control and Fer-silenced cells (Fig. 7*A*).

PDGFs are known to regulate cell migration, and previous reports have suggested a function of Fer or Fes/Fps in focal contacts (6, 8, 11, 19). Therefore, we investigated whether silencing of Fer expression affected chemotaxis of NIH3T3 cells toward PDGF-BB. However, we were not able to observe any significant effects of Fer down-regulation on cell migration toward different concentrations of PDGF-BB (Fig. 7*B*).

Because the Fer-related Fes/Fps kinase was initially identified as an oncogene product of a transforming retrovirus (20), we investigated whether silencing of Fer expression affected the *in vitro* transforming ability of sis3T3 cells, which are NIH3T3 cells expressing the PDGF-B-like v-Sis oncoprotein; these cells form colonies in soft agar due to a PDGF-BB-driven autocrine loop. As can be seen in Fig. 7*C*, sis3T3 cells formed colonies in soft agar, and PDGF-BB stimulation enhanced colony growth. Down-regulation of Fer caused a marked reduction in the number of colonies formed (containing >50 cells) with or without PDGF-BB stimulation; in fact, we were unable to detect any colonies larger than 100 cells in Fer siRNA-transfected conditions, whereas such colonies could be readily seen under control conditions.

To explore the role of Fer in tumorigenesis further, we inoculated BALB/c nude mice with sis3T3 cells depleted of Fer expression by shRNA. We found that Fer-depleted tumor cells had a reduced ability to initiate tumor growth (Fig. 7*D*).

In summary, Fer has no or a redundant role in PDGF-BB-induced cell proliferation and migration; however, it exerts a major influence on colony formation in soft agar of sis3T3 cells and on the initiation of tumor growth *in vivo*.

DISCUSSION

In the present study, we have confirmed and extended the previous reports that Fer interacts with the PDGFR in a Src homology 2 domain-dependent manner (13, 14). The strongest interaction was detected with phosphorylated Tyr-579 and Tyr-581, which also are major Src kinase binding sites (21), as well as with Tyr-1021 in the C-terminal tail of the receptor that also binds phospholipase Cγ (22). Fer also bound phosphorylated Tyr-740, although this may be indirect because PI3-kinase binds to Tyr(P)-740, and an interaction between Fer and the p85 subunit of PI3-kinase has been reported (14). Our studies on Fer activation in response to PDGF-BB stimulation showed that Fer is phosphorylated independently of Src and Jak kinase activities, suggesting that phosphorylation and activation occur directly by the receptor kinase. A kinetic analysis revealed that Fer is rapidly activated by PDGF-BB and remains active up to 8 h, *i.e.* long after the autophosphorylation of the receptor has declined. The mechanism for the extended Fer activation is not known; it is possible that the PDGFRβ-induced phosphoryla-

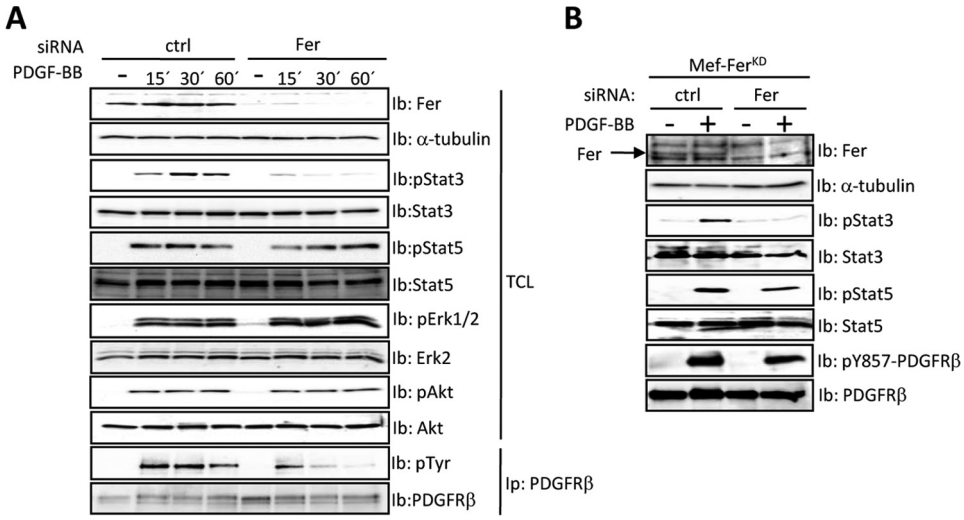

FIGURE 5. **Effect of Fer down-regulation on PDGF-BB-induced STAT3, STAT5, ERK1/2, Akt, and PDGFRβ activation.** NIH3T3 cells (*A*) or mouse embryonic fibroblasts (*Mef*) derived from a knock-in mouse of kinase-deficient Fer, Mef-Fer^{KD} (*B*) were transfected with siRNA targeting Fer or control siRNA and serum-starved overnight. Cells were then stimulated with 20 ng/ml PDGF-BB for the indicated periods of time, followed by cell lysis and immunoblotting (*Ib*) for total and phosphorylated STAT3, STAT5, ERK1/2, and Akt as well as pY857-PDGFRβ and PDGFRβ. *Ip*, immunoprecipitation; *TCL*, total cell lysate. One representative experiment is shown of three performed.

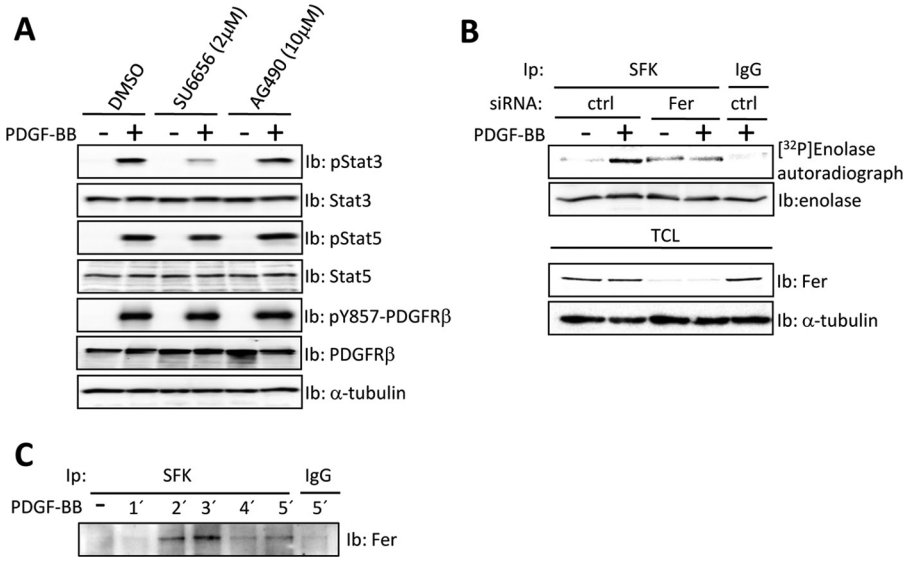

FIGURE 6. **Src kinases contribute to PDGF-induced STAT3 phosphorylation, and Fer is involved in Src activation.** *A*, serum-starved NIH3T3 cells were treated for 1 h with Src (SU6656) or Jak (AG490) inhibitors prior to PDGF-BB stimulation. Cells were lysed, and phosphorylation and total amount of STAT3, STAT5, and PDGFRβ were analyzed by immunoblotting (*Ib*). *B*, Src kinases (Src, Yes, and Fyn) were immunoprecipitated (*Ip*) using the cst-1 antiserum from cells transfected with Fer or control siRNA. Immunoprecipitated proteins were incubated with [γ-^{32}P]ATP and acid-denatured enolase for 10 min. Proteins were separated by SDS-PAGE, and incorporated radioactivity was visualized by autoradiography and enolase by immunoblotting. *TCL*, total cell lysate. *C*, to control for unspecific interactions, we used in parallel control IgG. Efficiency of Fer silencing was measured by Fer immunoblotting, and α-tubulin served as a loading control. One representative experiment is shown of three performed.

tion is sustained by subsequent Fer autophosphorylation within the trimeric complex where it has been shown to reside (23).

Fer, or the related Fes/Fps kinase, has been implicated in various signal transduction pathways. For example, expression of an activated form of Fes/Fps causes a potentiation of GM-CSF-, Epo-, and SCF-induced phosphorylation of STAT3, STAT5, and ERK1/2 (24, 25). Overexpression of Fer in COS cells, or stimulation of myogenic or human prostate cancer cells with insulin or IL6, respectively, resulted in complex formation between Fer and STAT3 as well as STAT3 phosphorylation (10, 26, 27). Fer has also been shown to be important for sustained ERK1/2 activation and VEGF expression under hypoxic conditions (28). In our experiments, we investigated the PDGF-BB-induced activation of STAT3, STAT5, ERK1/2, Akt, and PDGFRβ in NIH3T3 cells under conditions with siRNA-mediated silencing of Fer expression. We found that STAT3 tyrosine phosphorylation was dependent on the presence of Fer, whereas other pathways were not much affected. In contrast to previous studies where a complex between Fer and STAT3 could be observed after cytokine stimulation (10, 27), we were unable to detect such a complex after PDGF-BB treatment (data not shown). In addition to abolished STAT3 phosphorylation, the phosphorylation of PDGFRβ was also reduced in the absence of Fer; however, the importance of

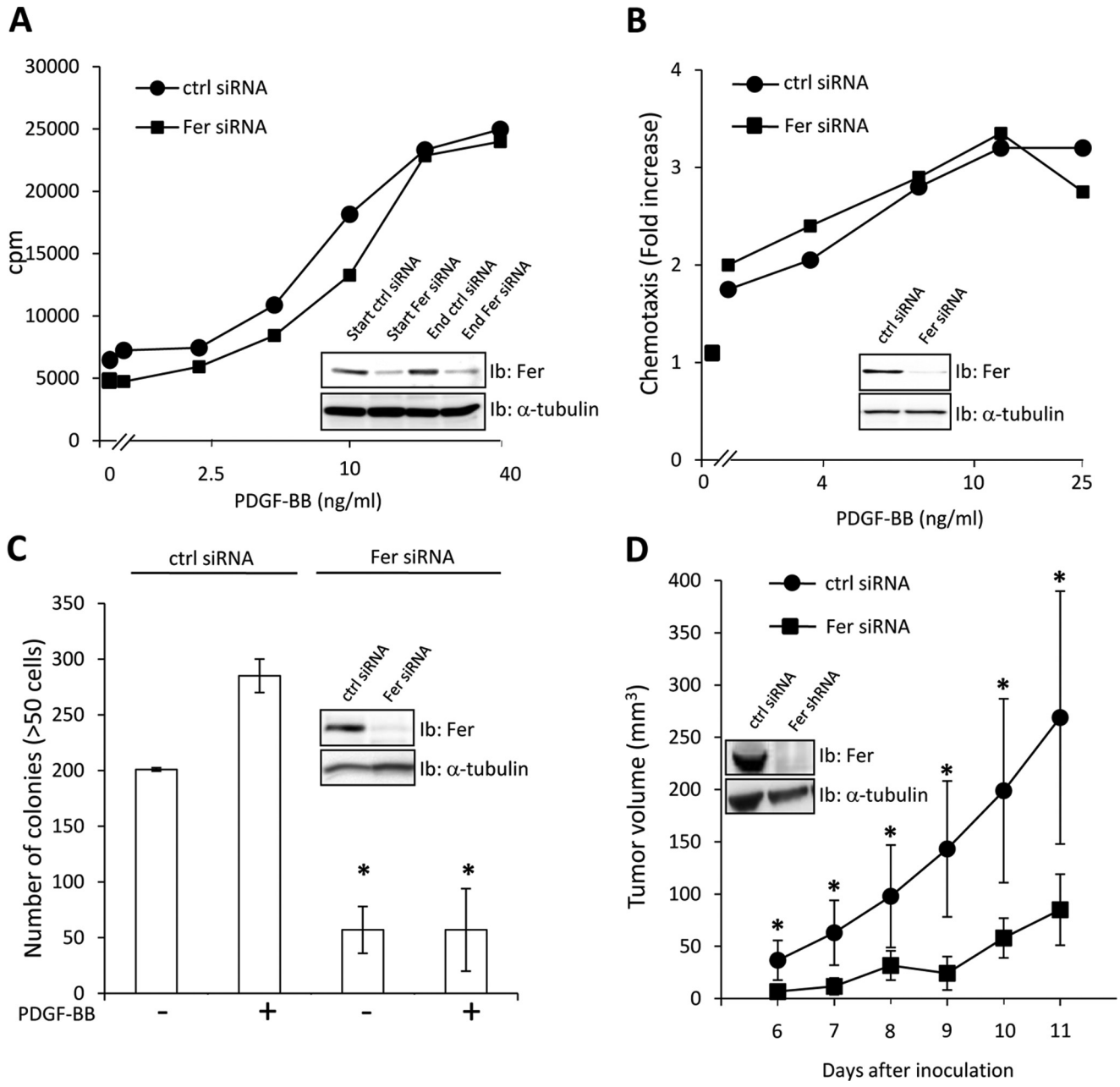

FIGURE 7. **Fer is critical for colony formation in soft agar and initiation of *in vivo* tumor growth but not for cell proliferation or migration.** *A*, NIH3T3 cells transfected with siRNA against Fer or control (*ctrl*) siRNA were starved overnight and then stimulated with different concentrations PDGF-BB for 24 h in the presence of [^{3}H]thymidine. The amount of [^{3}H]thymidine incorporated into DNA was determined using a scintillation counter. *Ib*, immunoblotting. *B*, NIH3T3 cells were plated on a fibronectin-coated polycarbonate membrane (pore size, 8.0 μm) in a 96-well microchemotaxis chamber. The cells were then allowed to migrate for 4 h toward increasing concentrations PDGF-BB placed in the lower chamber. The membrane was then fixed in ethanol, and cells that had migrated through the membrane were stained with Giemsa. The intensity of the staining was measured using a CCD camera. Data are plotted as mean of quadruplicate samples. *C*, sis3T3 cells transfected with control or Fer siRNA were plated into soft agar in the absence or presence of 50 ng/ml PDGF-BB for 5 days. Colonies containing >50 cells were counted. Values are means of three independent experiments. *D*, sis3T3 cells infected with lentivirus containing Fer or control shRNA were inoculated into nude BALB/c mice (five mice in each group), and the establishment of tumors was monitored daily by determining tumor volume. *, significant change between corresponding condition using control and Fer si/shRNA, $p < 0.05$. *Error bars*, S.D.

this finding is unclear because all pathways investigated, except STAT3, as well as cell proliferation and chemotaxis, were activated to a more or less normal extent. Mice have been generated expressing a mutant Fer lacking tyrosine kinase activity, and they are viable and fertile (17). Interestingly, whereas PDGF stimulation of fibroblasts derived from these mice displayed normal STAT3 phosphorylation, knockdown of the kinase-deficient Fer inhibited STAT3 phosphorylation. These observations suggest that Fer may have a kinase-independent role in STAT3 activation. In fact, a kinase-independent role for the Fer ortholog FRK-1 in *Caenorhabditis elegans* has been demonstrated in embryonic closure and morphogenesis (29). Hence, it is possible that Fer functions as an adaptor protein in the process of PDGF-BB-induced STAT3 activation, although further investigations are needed to clarify this issue.

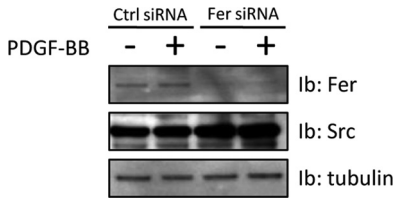

FIGURE 8. **Fer depletion does not affect Src kinase levels.** NIH3T3 cells transfected with control or Fer siRNA were starved overnight and then stimulated with 20 ng/ml PDGF-BB. Lysates were separated by SDS-PAGE and immunoblotted (*Ib*) against Fer or Src kinases. Equal loading was verified by immunoblotting for tubulin. A representative experiment is shown.

Src and Jak kinases have been implicated in activation of STATs in different receptor systems. Therefore, we investigated the involvement of Src and Jak in PDGF-BB-induced STAT3 and STAT5 activation using low molecular mass inhibitors. Our data showed that PDGF-BB-induced STAT3 phosphorylation was sensitive to Src kinase inhibition but not to Jak inhibition; however, consistent with a previous report (30), STAT5 phosphorylation requires neither Src nor Jak activity. We showed that Src activity is not essential for PDGF-BB-induced Fer activation. Importantly, silencing of Fer did not influence Src kinase expression (Fig. 8). In contrast, under conditions of reduced Fer expression, PDGF-BB was not able to increase Src kinase activity. However, the basal Src kinase activity was increased, which may relate to the fact that both Fer and Src interact with Tyr-579 and Tyr-581 in the juxtamembrane region of the receptor. Thus, it is possible that Src and Fer compete for binding, and when Fer is absent Src may interact more efficiently. Collectively, our data on STAT3 phosphorylation are compatible with a scenario where Fer is a component of a scaffolding complex that contributes to Src activation and thus facilitates STAT3 phosphorylation. Consistent with this possibility we could detect a rapid and transient complex formation between Fer and Src kinases in response to PDGF-BB.

Fer, Fes/Fps, and STAT3 have been implicated in cell biological processes such as proliferation and cell migration. However, our results showed that there was no significant effect on PDGF-BB-induced DNA synthesis or chemotaxis in cells where Fer was silenced. This result contrasts with what has been reported for IL6-induced proliferation, where Fer-mediated activation of STAT3 promotes PC-3 cell proliferation (10) and suggests that Fer may have a redundant role in PDGF-BB-induced proliferation and migration in our experimental model. Notably, we were unable to detect expression of the related Fes/Fps protein in NIH3T3 cells (Fig. 9), indicating that other pathways are involved.

STAT3 has been shown to promote expression of a large number of genes involved in cell survival and proliferation (for review, see Ref. 31). Therefore, it is not surprising that aberrant activation of STAT3 has been observed frequently in various cancers (for review, see Ref. 32). Because the major signaling defect we were able to identify in cells with reduced Fer expression was abolished PDGF-BB-induced STAT3 activation, we investigated whether this could affect tumorigenesis as determined by *in vitro* colony formation in soft agar. We found that NIH3T3 cells with an autocrine activation of PDGF receptors were not able to form colonies in soft agar when the Fer expression was silenced using siRNA, consistent with the notion that

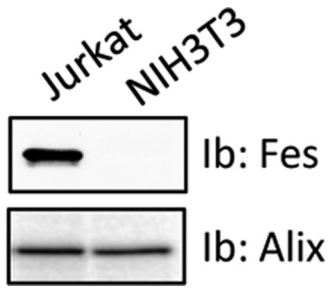

FIGURE 9. **NIH3T3 cells does not express detectable levels of Fes protein.** Lysates from NIH3T3 cells, or Jurkat cells as a positive control, were separated by SDS-PAGE and immunoblotted (*Ib*) against Fes or Alix as a loading control. A representative experiment is shown.

STAT3 is important for tumorigenicity. In fact, expression of a constitutively activated mutant form of STAT3 has been shown to promote NIH3T3 and 3Y1 fibroblasts to form colonies in soft agar and for the 3Y1 cells also tumors in mice (33). When we inoculated nude BALB/c mice with sis3T3 cells stably depleted of Fer, we observed a strong reduction in tumor formation. This suggests that Fer may play a crucial role in PDGF-induced tumorigenesis.

To conclude, we have demonstrated that Fer binds to the activated PDGFRβ through multiple tyrosine residues and becomes activated in a sustained manner. Reducing the expression of Fer resulted in a decreased phosphorylation of STAT3, whereas other pathways were not dramatically affected. Functionally, we found that Fer expression was critical for the ability of an autocrine PDGF-BB-driven loop to promote *in vitro* transformation and in the initiation of tumor growth *in vivo*. Our results suggest that Fer kinase may play an important role in PDGF-driven tumorigenesis.

Acknowledgments—We thank Dr. Peter Greer (Queen's University, Queen's Cancer Research Institute, Kingston, ON, Canada) for the mouse embryonic fibroblasts expressing kinase-deficient Fer and Linda Sooman for technical assistance.

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