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Differential effect of the focal adhesion kinase Y397F mutant on v-Src-stimulated cell invasion and tumor growth

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Summary

Upon cell adhesion to extracellular matrix proteins, focal adhesion kinase (FAK) rapidly undergoes autophosphorylation on its Tyr-397 which consequently serves as a binding site for the Src homology 2 domains of the Src family protein kinases and several other intracellular signaling molecules. In this study, we have attempted to examine the effect of the FAK Y397F mutant on v-Src-stimulated cell transformation by establishing an inducible expression of the Y397F mutant in v-Src-transformed FAK-null ($FAK^{-/-}$) mouse embryo fibroblasts. We found that the FAK Y397F mutant had both positive and negative effects on v-Src-stimulated cell transformation; it promoted v-Src-stimulated invasion, but on the other hand it inhibited the v-Src-stimulated anchorage-independent cell growth *in vitro* and tumor formation *in vivo* . The positive effect of the Y397F mutant on v-Src-stimulated invasion was correlated with an increased expression of matrix metalloproteinase-2, both of which were inhibited by the specific phosphatidylinositol 3-kinase inhibitor wortmannin or a dominant negative mutant of AKT, suggesting a critical role for the phosphatidylinositol 3-kinase/AKT pathway in both events. However, the expression of the Y397F mutant rendered v-Src-transformed $FAK^{-/-}$ cells susceptible to anoikis, correlated with suppression on v-Src-stimulated activation of ERK and AKT. In addition, under anoikis stress, the induction of the Y397F mutant in v-Src-transformed $FAK^{-/-}$ cells selectively led to a decrease in the level of $p130^{Cas}$, but not other focal adhesion proteins such as talin, vinculin, and paxillin. These results suggest that FAK may increase the susceptibility of v-Src-transformed cells to anoikis by modulating the level of $p130^{Cas}$.

Abbreviations: Cas – Crk-associated substrate; ERK – extracellular signal-regulated kinase; FAK – focal adhesion kianse; HA – hemagglutinin; IB – immunoblotting; IP – immunoprecipitation; JNK – c-Jun N-terminal kinase; MMP – matrix metalloproteinase; PI3K – phosphatidylinositol 3-kinase; SH – Src homology; Src – sarcoma; STAT3 – signal transducers and activators of transcription 3; Tet – tetracycline; Tyr – tyrosine; v-Scr – viral Scr; Wt – wild type

Introduction

The v-*src* oncogene, which was first identified from the transforming gene of Rous sarcoma virus, encodes a constitutively activated form of c-Src nonreceptor tyrosine kinase [1–3]. Cell transformation by v-Src results in a wide variety of phenotypic changes, including morphological transformation, increase in cell migration or invasiveness, and acquisition of anchorage and

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growth-factor independence, and tumorigenicity [4]. Activation of several intracellular signal transduction pathways has shown to be associated with transforming potential of activated Src, such as the Ras/ERK [5], the PI3K/AKT [6], and the STAT3/Myc [7] pathways. Considerable evidence now implicates that elevated expression and/or activity of Src is associated with human cancers [8].

FAK, a 125-kDa cytoplasmic protein tyrosine kinase localized in focal contacts, was originally identified as a highly tyrosine-phosphorylated protein in v-Src-transformed chicken fibroblasts [9]. The full-length cDNA of mouse FAK was independently cloned on the basis of homology cloning [10]. FAK is now known to play a crucial role in the control of integrin-mediated cellular functions including cell spreading [11], cell migration [12, 13], cell cycle progression [14, 15], and cell survival [16–19]. In addition to cell adhesion, the tyrosine phosphorylation of FAK is also stimulated by growth factors [20–22] as a result of Src activation by growth factor receptor [22] and/or FAK association with growth factor receptor [23]. Increased level of FAK has been found in a variety of invasive human tumors and has been implicated to play a role in tumor progression to an invasive phenotype [24–27].

The tyrosine residue 397 has been identified as the major site of FAK autophosphorylation [28] and the binding site for the Src homology (SH) 2 domains of the Src family kinases [28, 29], PI3K [30, 31], phospholipase C-γ1 [32], and Grb7 [33]. After binding to FAK, the Src family kinases (Src or Fyn) phosphorylates FAK on particular tyrosine residues, leading to activation of the FAK catalytic activity [34] and creating a binding site for the Grb2/Sos complex that relays signals to the Ras/ERK pathway [35, 36]. In addition, Src can also phosphorylate FAK-associated proteins, such as paxillin [37] and $p130^{Cas}$ [38] to transmit signals. Through these mechanisms, Src intimately cooperates with FAK to regulate various aspects of cell functions.

Crk-associated substrate (Cas or $p130^{Cas)}$ was originally identified and cloned as a highly tyrosine phosphorylated protein in cells transformed by v-Src [39] or v-Crk [40]. It contains an NH_2-terminal SH3 domain, a substrate domain that consists of 15 YXXP motifs, and a COOH-terminal Src-binding domain [41, 42]. The SH3 domain of $p130^{Cas}$ could bind proline-rich sequences of various signaling molecules such as FAK [43], guanine nucleotide exchange factor C3G [44], tyrosine phosphatase PTP-PEST [45]. $p130^{Cas}$-deficient mouse embryonic fibroblasts showed impaired actin bundling and cell migration and were refractory to be transformed by activated Src [46]. The phosphorylated $p130^{Cas}$ has been demonstrated to be essential for FAK to promote cell migration [47], cell cycle progression [15] and cell survival [18].

Since the Tyr-397 of FAK is the binding site for Src and other signaling molecules, mutation at this site was found to impair the function of FAK [13, 48] and could serve as a dominant negative version of FAK capable of inhibiting cell cycle progress [14] and cell migration [49]. In this study, we set out to examine the effect of the FAK Y397F mutant on the v-Src-stimulated cell transformation. Our results revealed that the expression of the Y397F mutant in v-Src-transformed $FAK^{-/-}$ cells promotes their invasiveness, but inhibits their anchorage independent growth *in vitro* and tumor formation *in vivo*.

Materials and methods

Materials

The 24-well transwell chamber for invasion assay was purchased from Costar. Methylcellulose (Methocel® 65HG) was purchased from Fluka. Hygromycin B, SP600125, and PD98059 were purchased from Calbiochem. The monoclonal anti-talin, anti-vinculin, and wortmannin were purchased from Sigma-Aldrich. The monoclonal anti-FAK (clone 77), anti-phosphotyrosine (PY20), and anti-STAT3 were purchased from BD Transduction Laboratories. The rabbit polyclonal anti-$p130^{Cas}$ (C-20), anti-Src (N-16), anti-ERK (K-23), anti-JNK (C-17), anti-MMP2 (C-19) were purchased from Santa Cruz Biotechnology. The rabbit polyclonal anti-phosphoERK (Thr202/Tyr204), anti-phosphoJNK (Thr183/Tyr185), anti-phosphoSTAT3 (Tyr705), anti-AKT, and anti-phosphoAKT (Ser473) were purchased from Cell Signaling Technology. The rabbit polyclonal anti-phosphoSrc (Tyr418) and anti-phosphoFAK (respectively for Tyr397, Tyr407, Tyr576, Tyr861, and Tyr925) were purchased from Biosource. The monoclonal anti-hemagglutinin (HA) epitope was

purchased from Roche. The mouse ascites containing monoclonal anti-Src (peptide 2–17) produced by the hybridoma (ATCC, CRL-2651) was collected in our laboratory.

Cell lines

Tet-FAK-Y397F cells harboring a tetracycline repression system for inducible FAK Y397F expression were generated from $FAK^{-/-}$ fibroblasts and kindly provided by Dr. Steven Hanks at Vanderbilt University, as described [49]. To generate v-Src-transformed Tet-FAK-Y397F cells (designated as Tet-FAK-Y397F/v-Src), Tet-FAK-Y397F cells were grown on 60-mm dishes and co-transfected with 4 μg of pM-vSrc and 0.2 μg of pREP3 using lipofectamine following the manufacturer's instructions. To generate hygromycin-resistant control cells, Tet-FAK-Y397F cells were transfected with 0.2 μg of pREP3 alone. The cells were selected in medium containing 100 U/ml hygromycin and 2 μg/ml tetracycline. 10 days later, the drug-resistant colonies were pooled, expanded, and subjected to analysis for their morphology, the expression level of Src, and inducible expression of the FAK Y397F mutant. The expression of FAK Y397F was induced by incubating the cells in the medium without tetracycline.

Soft agar-colony formation assay

5×10^3 cells were suspended in 2 ml of Dulbecco's modified Eagle's medium containing 0.3% agar and 10% serum $\pm$ 2 μg/ml tetracycline and added onto a layer of medium containing 0.5% agar and 10% serum in a 60-mm dish. 2 ml of medium containing 0.3% agar and 10% serum ± 2 μg/ml tetracycline was added to the dish every other day. Each experiment was performed in duplicate. After 21 days, the number of colonies (> 1.5 mm) was measured.

Matrigel invasion assay

10^4 cells in 250 μl of serum-free medium were added to an inner cup of the 24-well transwell chamber that had been coated with 150 μl of Matrigel (1:10 dilution in serum-free medium). Seven-hundred and fifty microlitre of medium supplemented with 10% serum was added to the outer cup. After 12 h, cells that had migrated through Matrigel and filter membrane with 8-μm pores were fixed, stained, and counted under a light microscope. Each experiment was performed in triplicate.

Gelatin zymography

5×10^6 cells were plated on 10-cm dishes in growth medium with or without tetracycline for 24 h and then incubated in 5 ml of serum-free medium with or without tetracycline for another 12 h. The conditioned media was collected, concentrated by centrifugation with Ultra-4 centrifugal filter (Millipore-Amicon), and separated in a 7.5% SDS-polyacrylamide gel electrophoresis containing 0.1% gelatin. The gel was washed several times in Tris buffer (50 mM Tris, pH 7.4) containing 2.5% (v/v) Triton X-100 at room temperature, followed by further incubation in substrate buffer (50 mM Tris, pH 7.4, 10 mM $CaCl_2$, 150 mM NaCl) at 37 °C for 24 h. The gel was fixed in 10% methanol and 10% acetic acid for 10 min and gelatinolytic activity was visualized following Coomassie blue staining.

Anoikis assay

5×10^6 cells were suspended in 5 ml of 2X Dulbecco's modified Eagle's medium containing 20% serum and then mixed with 5 ml of 1% methylcellulose. The mixture was plated onto a 10-cm culture dish for 10 h. The cells were harvested by centrifugation and the cell survival rate was determined by trypan blue exclusion, as described previously [50]. Each experiment was performed in duplicate.

Tumorigenicity in nude mice

Five to six-week-old female nude mice were subcutaneously injected with 5×10^5 Tet-FAK-Y397F/Hygro cells on the left flank and 5×10^5 Tet-FAK-Y397F/v-Src cells on the right flank. To control the expression of the FAK Y397F mutant *in vivo* , nude mice were fed with water containing with or without tetracycline (150 μg/ml). At 14 or 30 days after injection, the size (length $\times$ width $\times$ height) of tumor was measured by a caliper. Mice were euthanasized and tumors were surgically removed. The tumors were homogenized and

analyzed for the expression of the FAK Y397F mutant.

Immuoprecipitation and immunoblotting

Cells were solubilized in 1% NP-40 lysis buffer [22] containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.2 trypsin inhibitory units/ml aprotinin, and 20 μg/ml leupeptin). The lysates were centrifuged for 10 min at 4 °C to remove debris, and the protein concentrations were determined using the Bio-Rad Protein Assay. Immunoprecipitation and immunoblotting were performed as described previously [51].

Immunofluorescent staining

Cells were grown on glass coverslips for 48 h prior to fixation in phosphate-buffered saline containing 4% paraformadehyde at room temperature for 30 min. Samples were permeabilized with phosphate-buffered saline containing 0.3% Triton X-100 for 30 min. Coverslips were stained with primary antibody for 60 min and followed by goat anti-rabbit Cy5-conjugated or anti-mouse tetramethyl rhodamine isothiocyanate-conjugated secondary antibodies (Jackson ImmunoReseach Laboratories) at 4 μg/ml for 60 min. Polyclonal anti-FAK and monoclonal anti-paxillin were diluted 1:200 before use. Fluorescein isothiocyanate-conjugated phalloidin (Sigma-Aldrich) at 2 μM was used to stain actin filaments. Coverslips were mounted in anti-fading solution and viewed using a Zeiss LSM laser-scanning confocal microscope.

Statistics

Statistical analyses were performed with Student's *t*-test. Differences were considered to be statistically significant at $p < 0.05$.

Results

v-Src phosphorylates FAK independently of the Tyr-397

To examine the effect of the FAK Y397F mutant on v-Src-stimulated cell transformation, a tetracycline repression system for inducible expression of the Y397F mutant was established in MEF $FAK^{-/-}$ cells (*Tet-FAK-Y397F/Hygro cells*) and those stably expressing v-Src (*Tet-FAK-Y397F/v-Src cells*). When those cells were grown in the medium containing tetracycline, the expression of the Y397F mutant was suppressed. In contrast, the Y397F mutant was induced to express by tetracycline withdrawal (Figure 1a). The induced level of the Y397F mutant in $FAK^{-/-}$ cells was comparable to that of endogenous FAK in $FAK^{+/+}$ cells

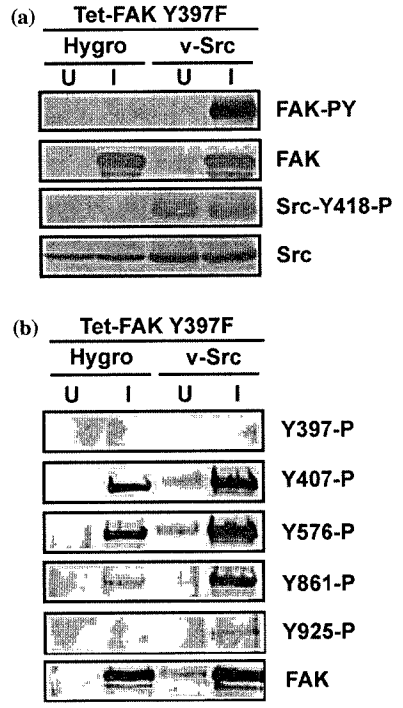


Figure 1. v-Src phosphorylates FAK independently of the Tyr-397 of FAK. (a) A tetracycline repression system for inducible expression of the FAK Y397F mutant was established in $FAK^{-/-}$ cells (*Tet-FAK-Y397F/Hygro*) or those stably expressing v-Src (*Tet-FAK-Y397F/v-Src*). When those cells were grown in the medium with tetracycline (2 μg/ml), the expression of the Y397F mutant remained un-induced (*U*). In contrast, when those cells were grown in the medium without tetracycline, the expression of the Y397F mutant was induced (*I*). 48 h after induction, the cells were lyzed and subjected to analysis for the expression and phosphorylation of FAK and Src. FAK was immunoprecipitated by anti-FAK and analyzed by immunoblotting with anti-phosphotyrosine or anti-FAK. For the expression and phosphorylation of Src, an equal amount of whole cell lysates was analyzed by immunoblotting with anti-phosphoSrcY418 and anti-Src. (b) FAK was immunoprecipitated by anti-FAK and analyzed by immunoblotting with antibodies specifically recognizing phosphorylated FAK at Y397, Y407, Y576, Y861, or Y925. These residues, except Y397, were known to be phosphorylated by c-Src.

(data not shown). Although a similar level of the Y397F mutant was induced in both Tet-FAK-Y397F/v-Src cells and Tet-FAK-Y397F/Hygro cells, the tyrosine phosphorylation level of the Y397F mutant was much higher in Tet-FAK-Y397F/v-Src cells than in Tet-FAK-Y397F/Hygro cells (Figure 1a). In addition, the phosphorylation of the Y397F mutant on particular tyrosine residues known to be phosphorylated by c-Src was all increased in the cells expressing v-Src (Figure 1b), indicating that the Tyr-397 of FAK is dispensable for v-Src to phosphorylate FAK.

Induction of the FAK Y397F expression in v-Src-transformed FAK $^{-/-}$ cells induces a more spread morphology, accompanied by a more organized actin cytoskeleton and an increased formation of focal adhesions

Our results showed that v-Src was able to transform the morphology of the FAK$^{-/-}$ cells into long spindle-like phenotype (Figure 2a). The induction of the FAK Y397F expression caused Tet-FAK-Y397F/v-Src cells to spread (Figure 2a), accompanied by more organized actin filaments (Figure 2d). By time-lapse video-microscopy, we found that Tet-FAK-Y397F/v-Src cells did not spread until 9 h after induction (Figure 2b), correlated with the expression of the Y397F mutant in those cells (Figure 2c). The induced Y397F mutant was localized in focal adhesions, revealed by co-staining a focal adhesion protein, paxillin (Figure 2d). The FAK Y397F-induced cell spreading was associated with an increased formation of focal adhesions (Figure 2d).

Induction of the FAK Y397F expression potentiates v-Src-stimulated invasion, but suppresses v-Src-stimulated anchorage-independent cell growth in vitro and tumor growth in vivo

The induction of the Y397F mutant potentiated the ability of v-Src to stimulate invasion (Figure 3a), correlated with increased gelatinase activity in the conditioned medium (Figure 3b). Based on the position of the major clearing zone on the zymograph, MMP-2, which has a molecular mass about 66-kDa, was speculated. Our result (Figure 3c) showed that the amount of MMP-2 in the conditioned medium from Tet-FAK-Y397F/v-Src cells was higher than that from Tet-FAK-Y397F/Hygro cells. Importantly, the induction of the Y397F mutant further increased (twofold) the amount of secreted MMP-2 from Tet-FAK-Y397F/v-Src cells. These results suggest that the promotion of v-Src-stimulated invasion by the FAK Y397F mutant may be at least through its stimulatory effect on the MMP-2 expression.

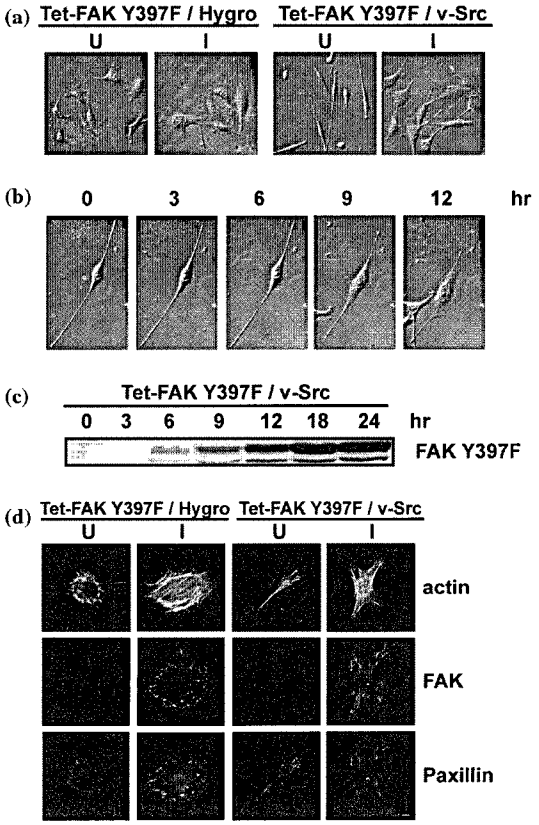


Figure 2. FAK Y397F mutant induces a more spread cell morphology, accompanied by a more organized actin cytoskeleton and an increased formation of focal adhesions. (a) The expression of the FAK Y397 mutant was induced (*I*) or un-induced (*U*) in Tet-FAK-Y397F/Hygro cells and Tet-FAK-Y397F/v-Src cells. Forty-eight hours after induction, micrographs were taken under a differential interference contrast microscope at magnification 400×. (b) Tet-FAK-Y397F/v-Src cells were allowed to express the Y397F mutant. The time-lapse micrographs were taken every 5 min for 12 h to record the morphological changes of the cells. The representative micrographs at 0, 3, 6, 9, and 12 h are shown (c). Tet-FAK-Y397F/v-Src cells were allowed to express the Y397F mutant for various times and lyzed. An equal amount of cell lysates was analyzed by immunoblotting with anti-FAK. (d). The cells were maintained in the medium with or without tetracycline. Forty-eight hours later, the cells were fixed and co-stained for the actin filaments, FAK, and paxillin.

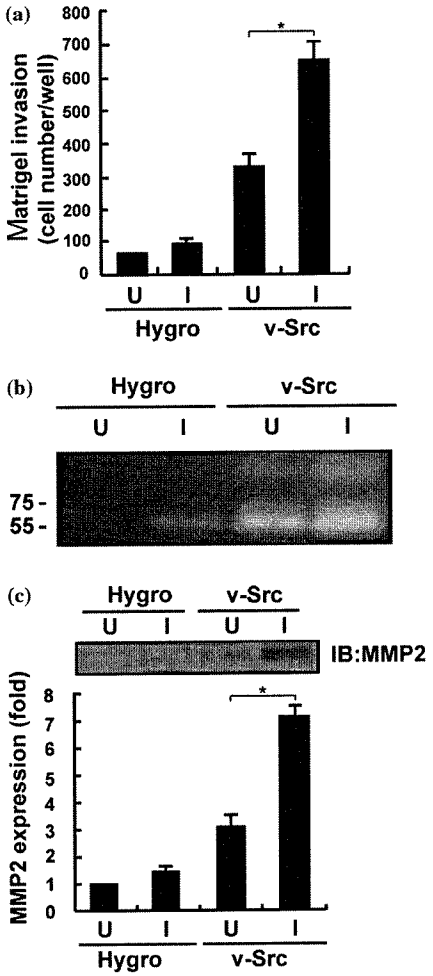


Figure 3. FAK Y397F mutant potentiates v-Src-stimulated invasion and MMP2 expression. (a) The expression of the FAK Y397F mutant was induced (*I*) or un-induced (*U*) in Tet-FAK-Y397F/Hygro cells and Tet-FAK-Y397F/v-Src cells. Forty-eight hours after induction, the cells (10^4) were subjected to a Matrigel invasion assay in the presence or absence of tetracycline. Twelve hours later, the cells invading through Matrigel to the lower side of the membrane were fixed, stained, and counted. The experiments were performed in triplicate. The value (mean ± SE) was from nine data points from three separate experiments. (b) The cells were allowed to express the Y397F mutant for 24 h and then the medium was replaced by serum-free medium with or without tetracycline. Twelve hours later, the conditioned medium was collected, concentrated, and analyzed by gelatin zymography. The clear zones represent the activity of gelatinases. (c) An equal volume of the conditioned medium, as described in the panel B, was analyzed by immunoblotting with anti-MMP2. The level of MMP2 was measured and expressed as – fold relative to that of Tet-FAK-Y397F/Hygro cells without induction of the Y397F mutant. The value (mean ± SE) was from three experiments.

Next, we examined whether induction of the FAK Y397F mutant has an effect on v-Src-stimulated anchorage-independent cell growth. To our surprise, induction of the Y397F mutant completely inhibited Tet-FAK-Y397F/v-Src cells to grow in soft agar (Figure 4a). To further examine the effect of the FAK Y397F mutant on v-Src-stimulated tumor growth *in vivo*, equal numbers of Tet-FAK-Y397F/v-Src cells and Tet-FAK-Y397F/Hygro cells were subcutaneously injected into nude mice. The expression of the FAK Y397F mutant *in vivo* was controlled by addition with or without tetracycline in drinking water for nude mice. Two weeks after injection, Tet-FAK-Y397F/v-Src cells formed measurable tumors only in the nude mice fed with tetracycline-containing water, but not in the nude mice fed with tetracycline-free water (Figure 4b). One month after injection, the tumors in the mice drinking tetracycline-containing water were threefold bigger than those in the mice drinking tetracycline-free water (Figure 4b). To verify whether the induction of FAK Y397F mutant was tightly controlled *in vivo*, the tumors were excised from the mice and subjected to analysis for the expression of the Y397F mutant. The result (Figure 4c) clearly showed that addition of tetracycline in drinking water tightly suppressed the expression of the Y397F mutant in Tet-FAK-Y397F/v-Src cells *in vivo*. Together, our results indicate that the FAK Y397F mutant suppresses v-Src-stimulated anchorage-independent cell growth *in vitro* and tumor formation *in vivo*.

Activation of the PI3K pathway is important for the FAK Y397F mutant to promote v-Src-stimulated Matrigel invasion

To examine the pathway(s) selectively promoted by the FAK Y397F mutant, the phosphorylation (activation) status of several signaling molecules was examined. Among the molecules examined in this study, we found that the induction of the Y397F mutant selectively increased the activation of AKT, ERK, and JNK, but not STAT3, in Tet-FAK-Y397F/v-Src cells (Figure 5a). Next, we employed a pharmacological approach to examine which of these molecules may be involved in the FAK Y397F promotion of v-Src-stimulated invasion. Our results showed that the specific PI3K inhibitor wortmannin, but not the specific inhibitor for MEK or JNK, efficiently blocked the ability of the Y397F mutant to

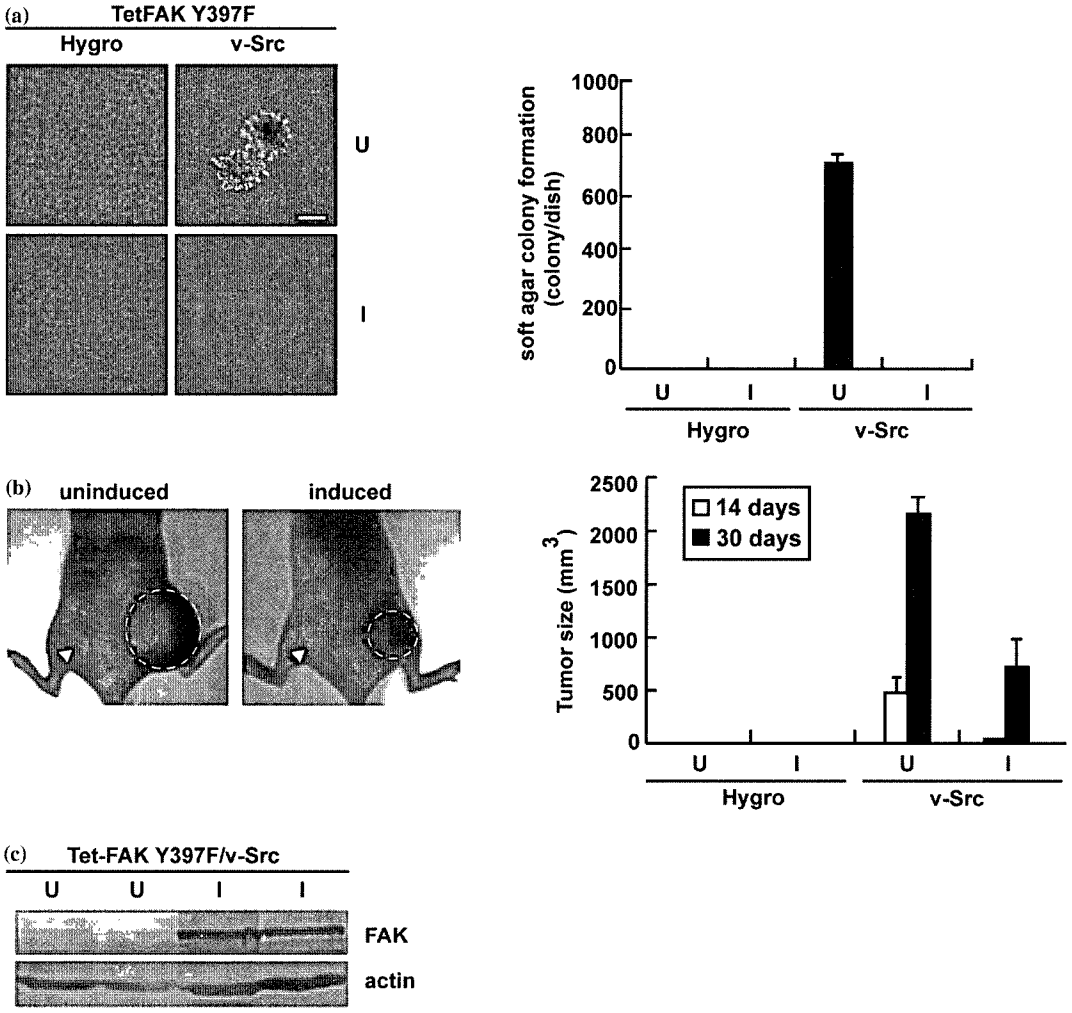


Figure 4. FAK Y397F mutant suppresses v-Src-stimulated anchorage-independent cell growth *in vitro* and tumor formation in nude mice. (a) The expression of the FAK Y397F mutant was induced (*I*) or un-induced (*U*) in Tet-FAK-Y397F/Hygro cells and Tet-FAK-Y397F/v-Src cells. Forty-eight hours after induction, the cells were subjected to a soft agar-colony formation assay in the presence or absence of tetracycline. Twenty-one days later, cell colonies were enumerated. The experiments were performed in duplicate. The value (mean ± SE) was from six data points from three experiments. (b) Forty-eight hours after induction, the cells were subcutaneously injected to nude mice. Each mouse received two injections with Tet-FAK-Y397F/Hygro cells on the left and Tet-FAK-Y397F/v-Src cells on the right. To control the expression of the FAK Y397F mutant *in vivo* , nude mice were fed with water containing with or without tetracycline (150 μg/ml). At 14 or 30 days after injection, the size (length × width × height) of tumor was measured by a caliper. The value (mean ± SE) of each experimental group was from 15 mice from three separate experiments. Arrowhead indicates the location where Tet-FAK-Y397F/Hygro cells were injected. Dashed line circles the tumors formed by Tet-FAK-Y397F/v-Src cells. (c) Thirty days after injection, the tumors formed by Tet-FAK-Y397F/v-Src cells were excised from the nude mice, homogenized, and analyzed by immunoblotting with monoclonal anti-FAK or anti-actin. The representative result shown includes four tumor samples; two of which were excised from mice fed with tetracycline-containing water, and the other two were from mice fed with tetracycline-free water. Note that no detectable expression of the FAK Y397F mutant in the tumors from the mice fed with tetracycline-containing water.

promote v-Src-stimulated invasion (Figure 5b) and MMP-2 expression (Figure 5c). To examine the role of AKT in this process, a dominant-negative version of AKT was expressed in Tet-FAK-Y397F/v-Src cells, leading to decreased invasion promoted by the Y397F mutant (Figure 5d). These results together suggest that the activation of the PI3K/AKT pathway by the FAK Y397F mutant is critical for v-Src-induced invasiveness.

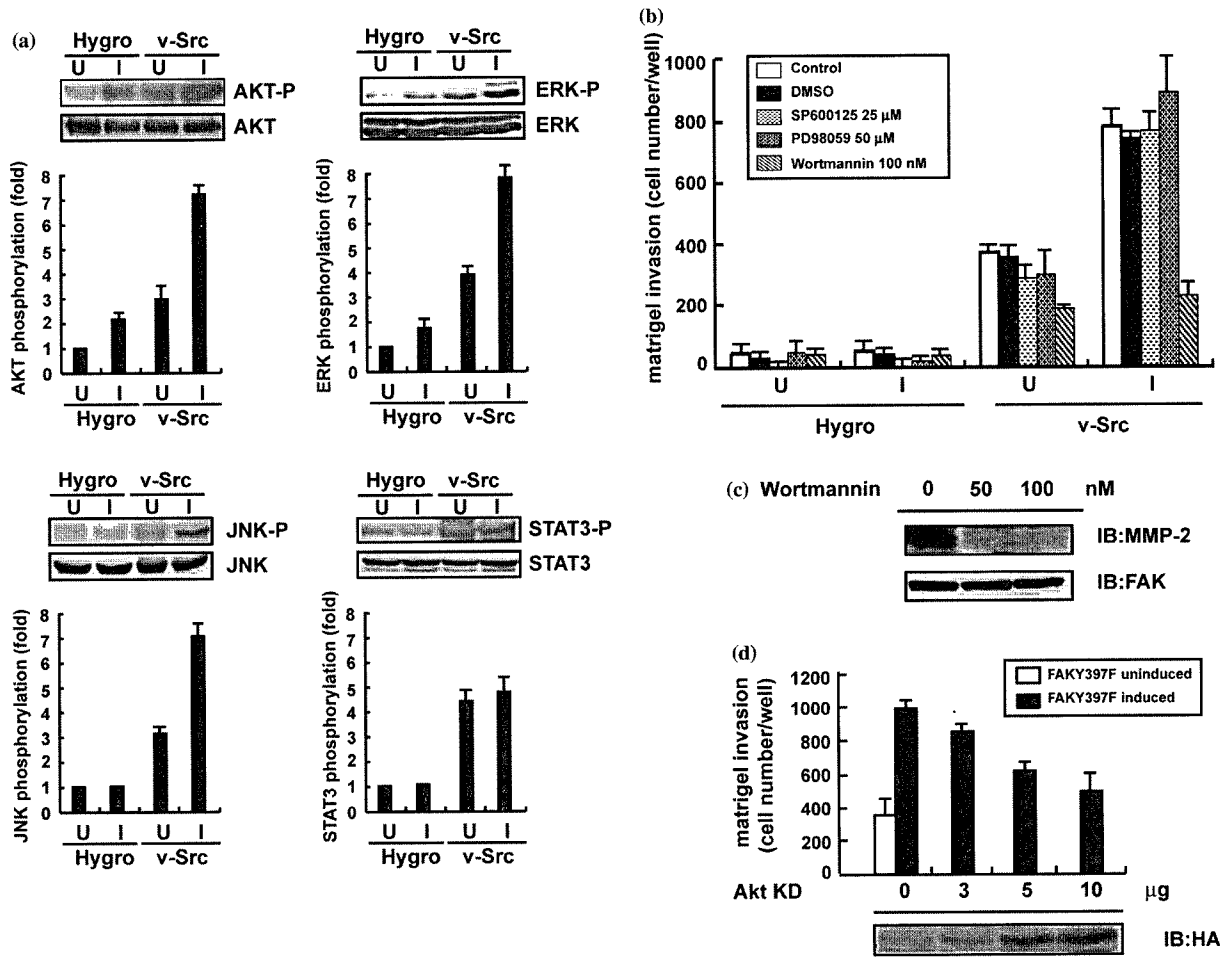


Figure 5. The PI3K/AKT pathway is important for the FAK Y397F mutant to promote v-Src-stimulated Matrigel invasion. (a) The expression of the FAK Y397F mutant was induced (*I*) or un-induced (*U*) in Tet-FAK-Y397F/Hygro cells and Tet-FAK-Y397F/v-Src cells. Forty-eight hours later, the cells were lyzed and equal amounts of the cell lysates was analyzed by immunoblotting with antibodies specific to the molecules as indicated. The levels of phosphorylated AKT, ERK, JNK, and STAT3 were measured and expressed as – fold relative to those in Tet-FAK-Y397F/Hygro cells without induction of the Y397F mutant. The value (mean ± SE) was from three experiments. (b) Forty-eight hours after induction, the cells as described in the panel A were subjected to a Matrigel invasion assay in the presence or absence of SP600125 (the JNK inhibitor), PD98059 (the MEK inhibitor), wortmannin (the PI3K inhibitor), or the control solvent Me_2SO (DMSO). The experiments were performed in duplicate. The value (mean ± SE) was from six data points from three experiments. (c) Tet-FAK-Y397F/v-Src cells were allowed to express the Y397F mutant in the medium with 10% serum. Twenty-four hours later, the medium was replaced by serum-free medium with or without wortmannin. Twelve hours later, the conditioned medium was collected and analyzed for the expression of MMP-2 by immunoblotting. The cell lysates were analyzed by immunoblotting with anti-FAK. (b) Increasing amounts of the HA-tagged kinase-deficient (KD) mutant of AKT were transiently expressed in Tet-FAK-Y397F/v-Src cells in tetracycline-free medium. Forty-eight hours later, the cells were subjected to a Matrigel invasion assay or lysed to verify the expression of HA-tagged KD mutant of AKT by immunoblotting with anti-HA. The quantitative value (mean ± SE) of the Matrigel invasion assay was from three experiments.

Induction of the FAK Y397F expression increases the susceptibility of v-Src-expressed cells to anoikis

To examine whether the inhibitory effect of the FAK Y397F mutant on v-Src-stimulated tumor growth was because of its inhibitory effect on cell proliferation, the growth rate of the cells with or without FAK Y397F induction was measured. The results showed that the growth rate of Tet-FAK-Y397F/v-Src cells was faster

than Tet-FAK-Y397F/Hygro cells regardless of induction of the Y397F mutant (Figure 6a), rendering it unlikely that the inhibitory effect of FAK Y397F mutant on v-Src-stimulated tumor growth was due to its effect on cell proliferation. In fact, the failure of Tet-FAK-Y397F/v-Src cells to grow in soft agar upon FAK Y397F induction (Figure 4a) suggested that the FAK Y397F mutant might render v-Src-transformed cells susceptible to anoikis, a type of cell death induced by the loss of cell-matrix adhesion. To examine this possibility, Tet-FAK-Y397F/v-Src cells with or without induction of the FAK Y397F mutant were subjected to an anoikis assay by suspending the cells in methylcellulose, as described previously [52]. Without the FAK Y397F induction, the survival rate of Tet-FAK-Y397F/v-Src cells was approximately 50% higher than that of Tet-FAK-Y397F/Hygro cells (Figure 6b), supporting a role for v-Src in suppressing anoikis, as described [53]. Importantly, upon induction of the Y397F mutant, the survival rate of Tet-FAK-Y397F/v-Src cells was 40% decreased (Figure 6b), indicating that FAK Y397 mutant is able to antagonize the anti-anoikis potential of v-Src.

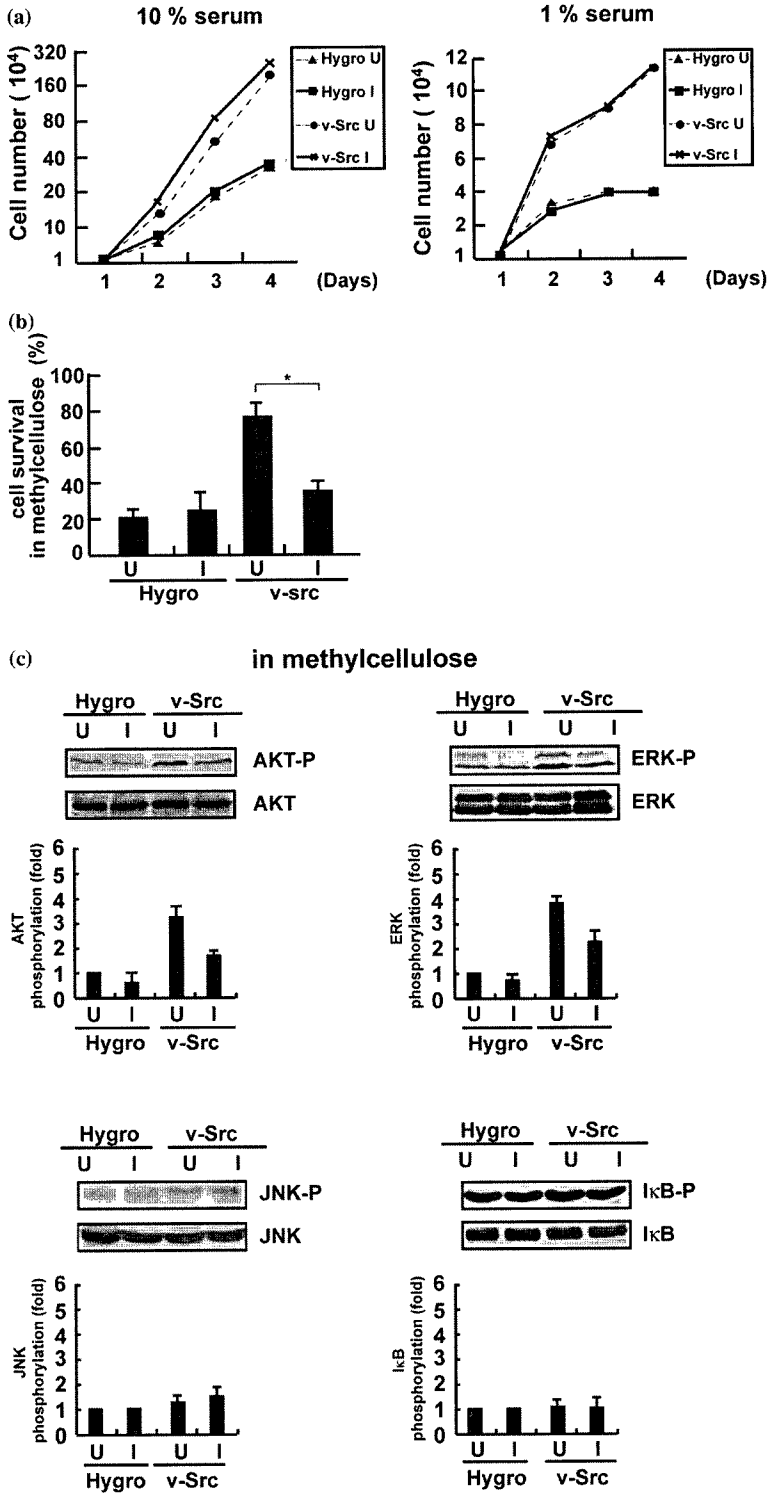
To examine the mechanism under this phenomenon, the effect of FAK Y397F mutant on the activation of AKT, ERK, JNK, and NFκB was examined. We found that although the FAK Y397 mutant selectively promoted the activation of AKT and ERK in attached cells (Figure 5a), it inhibited v-Src-stimulated activation of both molecules in the cells suspended in methylcellulose (Figure 6c). The phosphorylation of JNK was hardly detected in suspended cells (Figure 6c). In addition, we found that the phosphorylation of IκB was not changed during anoikis, suggesting that the NFκB pathway is not involved in the cell regulation for anoikis. These results indicate that FAK Y397F mutant could play either positive or negative role in v-Src-stimulated activation of certain signaling molecules, such as AKT and ERK, depending on whether cells are attached or not. The significance of the AKT and ERK activation in v-Src-stimulated resistance to anoikis was demonstrated by using the specific inhibitors of PI3K and MEK, which suppressed v-Src-stimulated cell survival in methylcellulose (data not shown).

The induction of the Y397F mutant leads to a decreased level of p130 ^{Cas} in v-Src-transformed cells during anoikis

To understand how the FAK Y397F mutant exerts its dominant negative effect on v-Src-stimulated activation of AKT and ERK in the cells kept in suspension, we compared the tyrosine phosphorylation level of the FAK Y397F mutant between attached cells and suspended cells. We found that the phosphorylation of the Y397F mutant was much lower in the suspended cells than in attached cells (Figure 7a), suggesting that cell adhesion is required for efficient phosphorylation of FAK by v-Src. However, because v-Src could still stimulate activation of AKT and ERK in the cells even without FAK expression, the result in Figure 7a did not explain why the Y397F mutant possesses a dominant negative effect on v-Src-stimulated activation of AKT and ERK. Next, we turned to examine whether the Y397F mutant modulates the intracellular levels of certain focal adhesion proteins during anoikis. Among the molecules examined in this study, we found that the induction of the Y397F mutant selectively led to a decreased level of p130^{Cas}, but not other focal adhesion proteins, including talin, vinculin, and paxillin, in suspended Tet-FAK-Y397F/v-Src cells (Figure 7b), suggesting that FAK may be able to modulate the level of p130^{Cas} when the cells encounter anoikis stress.

Discussion

In this study, we demonstrate that v-Src is capable of inducing oncogenic transformation of MEF FAK$^{-/-}$ cells, characterized by morphological transformation and acquisition of invasiveness, anchorage-independent growth, and tumorigenicity. Our results support the notion that FAK is not essential for v-Src to induce cell transformation, as proposed by Roy et al. [54]. However, the current study provides the first evidence that the FAK Y397F mutant has both positive and negative effects on the transforming potential of v-Src; it promotes v-Src-stimulated invasion, but suppresses v-Src-stimulated anchorage-independent cell growth *in vitro* and tumorigenicity *in vivo*. To exclude the possibility that the observed changes in the transformed phenotypes of



Tet-FAK-Y397F/v-Src cells might be caused by the Tet transactivator rather than the Y397F mutant, we had established a control v-Src-transformed $FAK^{-/-}$ cell line harboring the plasmid encoding the Tet transactivator. We confirmed that the transformed characteristics of v-Src-transformed

Figure 6. FAK Y397F mutant increases the susceptibility of v-Src-transformed cells to anoikis. (a) 10^4 of Tet-FAK-Y397F/Hygro cells or Tet-FAK-Y397F/v-Src cells were seeded on 100-mm culture dishes in the medium supplemented with 10% or 1% serum in the presence or absence of tetracycline. The number of cells was counted every day for three days. The value is the average of three experiments. (b) The expression of the FAK Y397F mutant was induced (*I*) or uninduced (*U*) in Tet-FAK-Y397F/Hygro cells and Tet-FAK-Y397F/v-Src cells. Forty-eight hours after induction, the cells were suspended in 0.5% methylcellulose in the presence or absence of tetracycline. Ten hours later, the cells were harvested by centrifugation and their survival rate was determined by trypan blue exclusion. (c) The cells as described in the panel B were harvested and lyzed. An equal amount of cell lysates was analyzed by immunoblotting with antibodies specific to molecules as indicated. The levels of phosphorylated AKT, ERK, JNK, and IκB were measured and expressed as – fold relative to those in Tet-FAK-Y397F/Hygro cells without induction of the FAK Y397F mutant. The value (mean ± SE) was from three experiments.

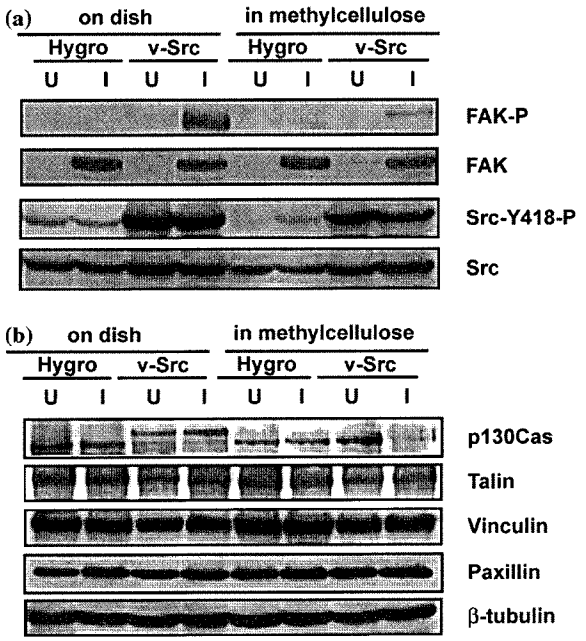


Figure 7. FAK Y397F mutant decreases the level of $p130^{Cas}$ in v-Src-transformed cells during anoikis. (a) The expression of the FAK Y397F mutant was induced (*I*) or un-induced (*U*) in Tet-FAK-Y397F/Hygro cells and Tet-FAK-Y397F/v-Src cells. Forty-eight hours after induction, the cells were suspended in 0.5% methylcellulose. Ten hours later, the cells were collected and lyzed. An equal amount of cell lysates from the cells suspended in methylcellulose and those attached on culture dishes was subjected to analysis for the expression and phosphorylation of FAK and Src, as described in Figure 1a. (b) An equal amount of cell lysates as described in the panel A was analyzed by immunoblotting for the expression of $p130^{Cas}$, talin, vinculin, paxillin, and β-tubulin.

$FAK^{-/-}$ cells were not affected by the Tet transactivator no matter whether tetracycline was present (data not shown).

Although we thought about comparing the effects of wt FAK and its Y397F mutant on v-Src-stimulated cell transformation on the basis of the same study model, we had difficulties in doing this. The major challenge was that the induced level of wt FAK was much lower than the level of the Y397F mutant in v-Src-transformed $FAK^{-/-}$ cells (data not shown). Although we had examined several cell clones derived from separate experiments, we failed to obtain cells that express wt FAK at the level similar to that of the Y397F mutant in v-Src-transformed $FAK^{-/-}$ cells upon tetracycline withdrawal. Because v-Src-induced cell transformation has previously been shown to be accompanied by calpain-mediated cleavage of FAK [55], we speculate that the wt FAK may be more vulnerable to degradation than the Y397F mutant in v-Src-transformed $FAK^{-/-}$ cells.

Previous studies have shown that FAK plays an important role in v-Src-stimulated cell invasion [56, 57]. In particular, Hsia et al. [57] showed that stable re-expression of wt FAK was required for v-Src to induce an invasive phenotype of $FAK^{-/-}$ cells. Notably, they found that adenovirus-mediated expression of the FAK Y397F mutant in v-Src-expressed $FAK^{-/-}$ cells failed to promote invasion. In contradiction with their results, we found that induction of the FAK Y397F mutant in v-Src-transformed $FAK^{-/-}$ cells markedly elevated their invasiveness (Figure 3). The answer for this discrepancy is currently unknown. It could be due to clonal variation of the $FAK^{-/-}$ cells used in both studies or differences between both studies in the approach used for the expression of the Y397F mutant, the expression level of the Y397F mutant in the cells, the efficiency of the Y397F mutant being phosphorylated by v-Src, and the effect of the Y397F mutant on cell morphology. We showed that the Y397F mutant was highly phosphorylated in v-Src-transformed $FAK^{-/-}$ cells (Figure 1) and localized in focal adhesions (Figure 2), indicating that the Tyr-397 of FAK is not required for its targeting to focal adhesions and phosphorylation by v-Src, as described previously [58]. Importantly, the Y397F mutant was able to reverse the long spindle morphology of the v-Src-transformed $FAK^{-/-}$cells into a more spread cell shape, concomitant with a more organized

actin cytoskeleton and an increased formation of focal adhesions (Figure 2). Although the underlying mechanisms for those phenomena are not clear yet, it is possible that the reversion of v-Src-transformed cell morphology by the Y397F mutant may underscore the observed changes described in this report.

It has been shown that the ability of FAK to promote v-Src-stimulated cell invasion is at least partially through its stimulatory effect on the expression of MMPs [56, 57]. Similarly, we found that elevated invasiveness of v-Src-transformed $FAK^{-/-}$cells by the Y397F mutant was correlated with increased expression of MMP2 and, possibly, MMP9 (Figure 3). In our attempt to identify the signal transduction pathways important for the Y397F mutant to promote v-Src-stimulated cell invasion and MMP2 expression, we found that the PI3K pathway was essential for both events (Figure 5). The role of PI3K in cell invasion has been extensively studied [59, 60]. It has been shown to promote cell invasion through activation of Rac, a small GTPase Rho family protein known to play a key role in the formation of lamellipodia and invasion [59, 60]. In addition, the PI3K/AKT signaling pathway was reported to transcriptionally activate the expression of MMP through the activation of NFκB [61]. Thus, it was not too surprising for us to find the involvement of PI3K in v-Src-stimulated cell invasion. The question is how the Y397F contributes to the PI3K activation. Given the fact that the Tyr397 of FAK is required for its binding of the SH2 domain of the regulatory subunit of PI3K [31], the synergistic effect of the Y397F mutant on v-Src-stimulated activation of the PI3K/AKT signaling pathway is likely through an indirect mechanism rather than its direct binding to PI3K.

We hypothesized that the Y397F mutant may facilitate the interaction of certain cellular proteins with v-Src and/or their phosphorylation by v-Src, thereby leading to activation of PI3K. One of the candidates fit with our hypothesis is the docking protein $p130^{Cas}$. It has previously been shown that $p130^{Cas}$ is highly phosphorylated in v-Src-transformed cells and plays an essential role in v-Src-induced cell transformation [46, 62]. In addition, expression of the SH3 domain of $p130^{Cas}$ blocks v-Src-stimulated cell transformation, correlated with decreased phosphorylation of endogenous $p130^{Cas}$ in those cells [63]. We found in this study that v-Src formed stable complexes with $p130^{Cas}$ in v-Src-transformed $FAK^{-/-}$cells and that the tyrosine phosphorylation of $p130^{Cas}$ was further increased upon induction of the Y397F mutant (data not shown). The effect of the Y397F mutant on promoting $p130^{Cas}$ phosphorylation may be through its binding to the SH3 domain of $p130^{Cas}$, bring v-Src and $p130^{Cas}$ in juxtaposition and/or stabilizing their interaction, thereby leading to more efficient phosphorylation of $p130^{Cas}$ by v-Src. The phosphorylation of $p130^{Cas}$ has been shown to link to multiple signal transduction pathways, including PI3K [64], ERK [65], and JNK [66].

Mounting evidence has indicated that the cell adhesion is required for growth factors to transmit appropriate signals into the cell [67]. Similarly, our results here suggest that cell adhesion is also required for v-Src to activate some, but not all, its downstream signaling molecules. In this study we found that JNK was only activated in attached v-Src-transformed $FAK^{-/-}$cells (Figure 5a), but not in the suspended ones (Figure 6c). In contrast, AKT and ERK were activated by v-Src no matter whether the cells were attached or not (Figure 6c). Moreover, the Y397F mutant markedly elevated the potential of v-Src to activate AKT, ERK, and JNK in attached v-Src-transformed $FAK^{-/-}$cells (Figure 5a). Unexpectedly, in suspended v-Src-transformed $FAK^{-/-}$cells, the Y397F mutant inhibited rather than increased the v-Src-stimulated activation of AKT and ERK (Figure 6c). This inhibition in AKT and ERK by the Y397F mutant may partially explain its inhibitory effect on anoikis resistance of v-Src-transformed $FAK^{-/-}$cells (Figure 6b). The mechanism by which the Y397F mutant exerts such a dominant negative effect on v-Src-stimulated activation of AKT and ERK and anoikis resistance is not clear. In this study, we found that the amount of $p130^{Cas}$ was selectively decreased in suspended v-Src-transformed $FAK^{-/-}$ cells upon induction of the Y397F mutant (Figure 7b). The result from semi-quantitative reverse transcription-polymerase chain reaction revealed that the mRNA of $p130^{Cas}$ was not decreased during anoikis (data not shown), suggesting the decreased amount of $p130^{Cas}$ was not due to a suppression at the transcriptional level. In fact, a recent report [68] described that $p130^{Cas}$ specifically underwent cleavage during anoikis and that overexpression of the $p130^{Cas}$ cleavage product induced apoptosis. Our results shown in Figure 7b suggest

that FAK may facilitate the $p130^{Cas}$ cleavage during anoikis.

Another interesting finding in this study is that the Y397F mutant inhibits v-Src-stimulated anchorage-independent growth of $FAK^{-/-}$cells (Figure 4). In fact, the role of FAK in anchorage-independent cell growth has been previously studied. Expression of a constitutively active version of FAK (CD2-FAK) by anchoring it on the plasma membrane conferred epithelial cells an anchorage-independent growth potential [16]. Our laboratory previously demonstrated that hepatocyte growth factor stimulated FAK independently of cell adhesion [22] and that the synergistic effect of FAK overexpression and hepatocyte growth factor stimulation allowed epithelial cells to grow in soft agar [51]. These studies support the ideal if the activation of FAK abnormally bypasses the requirement of cell adhesion, it promotes anchorage-independent cell growth. On the other hand, it has been shown that expression of wt FAK in v-Src-transformed $FAK^{-/-}$cells suppressed their potential to grow in soft agar [54, 69]. Similarly, expression of a FAK mutant with five substitutions at tyrosines Y407, Y576, Y577, Y861, and Y925 blocked v-Src-stimulated anchorage-independent cell growth [70]. Together with our current results derived from the Y397F mutant, it seems that FAK antagonizes the anchorage-independent cell growth independently of its autophosphorylation site or known Src-phosphorylation sites. As discussed above, FAK may inhibit anchorage-independent growth by facilitating the cleavage of $p130^{Cas}$, which is already known to be required for v-Src-induced cell transformation [46].

In summary, our results here convey a possibility that FAK may have a dual function in oncogenic transformation. It could promote cell invasion through multiple mechanisms, for instance, by promoting cell spreading, $p130^{Cas}$ phosphorylation, and MMP expression. In contrast, it could inhibit tumor cell growth by increasing cell susceptibility to anoikis. The dual function of FAK in oncogenic transformation may provide an explanation for certain cancerous cells showing a tendency to invade surrounding tissues rather than form a large tumor mass *in situ*. The cells inside a large tumor mass have less chance to contact with extracellular matrix proteins and more likely to undergo anoikis. Experiments are in progress to examine the mechanisms by which FAK exerts its pro-anoikis function.

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