Adrenergic modulation of focal adhesion kinase protects human ovarian cancer cells from anoikis

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Chronic stress is associated with hormonal changes that are known to affect multiple systems, including the immune and endocrine systems, but the effects of stress on cancer growth and progression are not fully understood. Here, we demonstrate that human ovarian cancer cells exposed to either norepinephrine or epinephrine exhibit lower levels of anoikis, the process by which cells enter apoptosis when separated from ECM and neighboring cells. In an orthotopic mouse model of human ovarian cancer, restraint stress and the associated increases in norepinephrine and epinephrine protected the tumor cells from anoikis and promoted their growth by activating focal adhesion kinase (FAK). These effects involved phosphorylation of FAKY397, which was itself associated with actin-dependent Src interaction with membrane-associated FAK. Importantly, in human ovarian cancer patients, behavioral states related to greater adrenergic activity were associated with higher levels of pFAKY397, which was in turn linked to substantially accelerated mortality. These data suggest that FAK modulation by stress hormones, especially norepinephrine and epinephrine, can contribute to tumor progression in patients with ovarian cancer and may point to potential new therapeutic targets for cancer management.

Introduction

There is growing evidence supporting the role of stress biology in cardiovascular (1), cancer (2, 3), and other disease states. Several epidemiological and experimental studies have suggested that behavioral stress factors may accelerate growth of existing tumors (4); however, the underlying mechanisms are not fully understood. Much research has suggested that neuroendocrine stress mediators might enhance cancer genesis by inhibiting antitumor immune responses (5), and we recently demonstrated that sympathetic nervous system (SNS) activity can also directly enhance the pathogenesis of ovarian carcinoma by upregulating angiogenic pathways in the tumor microenvironment (6). The latter effects were mediated through activation of tumor cell β2-adrenergic receptors (ADRB2) and the associated cAMP/PKA signaling pathway. Additional signaling and pathogenetic pathways may also be involved but are not fully understood at present.

Based on the effects of some neuropeptides, such as bombesin, on focal adhesion kinase (FAK) (7), we considered whether FAK might be involved in the tumor-promoting effects of chronic stress. FAK is a non-receptor protein tyrosine kinase that localizes to focal adhesions (8) and mediates physical attachment of cells to their ECM (9). It is not known whether stress hormones such as norepinephrine and epinephrine can activate FAK or what functional significance those dynamics might have for tumor cell survival. Normal cells enter apoptosis when separated from ECM and neighboring cells (a process known as anoikis) (10). In the present study, we sought to determine whether chronic stress and associated neuroendocrine dynamics could affect anoikis in ovarian cancer cells, and if so, what signaling pathways might mediate these effects. Analysis of cellular models and an orthotopic mouse model of human ovarian cancer showed that catecholamines can protect ovarian cancer cells from anoikis and these effects are mediated by FAK phosphorylation through ADRB2-dependent activation of Src. Parallel results were observed in human ovarian cancer in vivo, linking increased levels of stress/depression to increased FAK activation and showing accelerated disease progression in patients with high levels of FAK activity.

Results

Catecholamines protect tumor cells from anoikis. Tumor cells develop resistance to anoikis, which allows for their survival during the process of metastasis. FAK is known to promote tumor cell survival and may play a significant role in avoidance of anoikis (11–13). We have previously demonstrated that norepinephrine promotes ovarian cancer growth via stimulation of angiogenic pathways. To determine whether norepinephrine and epinephrine might also inhibit anoikis via FAK activation, we analyzed ovarian can-
cer cells maintained in poly-HEMA–coated tissue culture plates, which allows for anchorage-independent growth. These cells grew predominantly as a single-cell suspension, with 48-hour anoikis rates of 23.0% for SKOV3 cells and 51.3% for EG cells and 72-hour anoikis rates of 62.0% and 72.6%, respectively (Figure 1A). However, exposure to either epinephrine or norepinephrine resulted in significant inhibition of anoikis (Figure 1, A and B).

FAK activation is known to play a role in protecting cells from anoikis, and we have previously demonstrated that ovarian cancer cells express higher levels of FAK compared with nontransformed epithelial ovarian cells (14). To determine whether norepinephrine-mediated inhibition of anoikis might be mediated by FAK activation, we examined phosphorylation of all FAK tyrosine sites after treating SKOV3 cells with norepinephrine. Increased phosphorylation at pFAKY397 was noted in response to norepinephrine treatment, but no significant changes were noted at the other sites (Supplemental Figure 1; available online with this article; doi:10.1172/JCI40802DS1). Similar effects were observed in EG cells (Figure 2A) and after epinephrine treatment (Figure 2A), and subsequent experiments therefore focused on pFAK Y397. Norepinephrine-induced FAK tyrosine phosphorylation was detectable within 10 minutes and peaked at 30 minutes (Supplemental Figure 2, A and B). Similar effects occurred after the loss of cell attachment, with both SKOV3ip1 and EG cells showing norepinephrine-induced FAK Y397 phosphorylation even when cells were maintained in suspension (Figure 2B). Immunofluorescence analyses verified that norepinephrine induced a dose-dependent increase in pFAK Y397, localized specifically to focal adhesions in SKOV3ip1 cells (Figure 2C).

To confirm that FAK mediated the effects of norepinephrine on anoikis, we used siRNA to suppress FAK protein levels by 80% (Supplemental Figure 3). Norepinephrine continued to inhibit anoikis in ovarian cancer cells treated with a control siRNA, but FAK siRNA completely abrogated the effect of norepinephrine on ovarian cancer cell survival under anchorage-independent conditions (Figure 2D).

FAK activation by catecholamines is dependent on ADRB and Src. Our previous studies have shown that norepinephrine’s effects on ovarian cancer cell production of angiogenic factors are mediated specifically by tumor cell β-adrenergic receptors (6, 15). To identify the receptor subtype mediating norepinephrine effects on FAK activation and anoikis, we examined the effects of both α- and β-agonists. The nonspecific beta blocker propranolol (1 μM) blocked norepinephrine-induced FAK activation in both SKOV3ip1 (Figure 3A) and EG (Figure 3B) cells. Propranolol also blocked norepinephrine effects on anchorage-independent apoptosis (Figure 3C), which were similar to the effects observed with FAK siRNA. The ADRB1-specific inhibitor atenolol had minimal effects on norepinephrine-induced pFAK Y397, whereas the ADRB2-specific blocker butoxamine (1 μM) abrogated the effects of norepinephrine (Figure 3, A and B). The beta blockers had no effect on FAK or pFAK Y397 levels in the absence of norepinephrine (Supplemental Figure 4). Similar effects were observed using siRNA to inhibit expression and downstream signaling of specific β-adrenergic receptors (Supplemental Figure 5, A and B). ADRB2-targeted siRNA blocked the effects of norepinephrine on FAK activation, but ADRB1-targeted siRNA had no effect (Figure 3D). α-Adrenergic blockers had no effect on norepinephrine-mediated FAK activation (Supplemental Figure 6). Consistent with the effects on FAK activation, propranolol and ADRB2-siRNA reversed the protective effects of norepinephrine on anoikis (Figure 3C), but inhibition of other adrenergic receptor subtypes had no effect (Supplemental Figures 7 and 8). Consistent with the role of ADRB2, norepinephrine-mediated protection against anoikis was not observed in the ADRB2-null A2780-PAR and RMG-II cells (Supplemental Figure 9A). To further support the role of ADRB2 in mediating the effects of norepinephrine on anoikis, we transfected either ADRB2 (RMG-II-ADRB2) or empty vector (RMG-II-neo) into the RMG-II cells. The RMG-II-ADRB2 cells had significantly lower rates of anoikis compared with the controls upon stimulation with isoproterenol (Supplemental Figure 9B).

The major target of norepinephrine-induced FAK phosphorylation, Y397, is a high-affinity binding site for the SH2 domain of Src (16). To determine whether Src could directly induce FAK Y397 phosphorylation, we performed in vitro kinase assays. In these experiments, Src induced phosphorylation of either FAK or a kinase-dead FAK (i.e., mutation at K454M, resulting in alteration of the ATP-binding site; Supplemental Figure 10). Moreover, the Src inhibitor AP23846 prevented Src-induced FAK Y397 phosphorylation, further demonstrating the effect of Src. β-Adrenergic activation of G proteins can directly stimulate Src activation (17), so we pretreated ovarian cancer cells with the Src family kinase inhibitor PP2 (18) (or its inactive congener PP3) for 30 minutes prior to norepinephrine exposure. PP2 (10 μM) completely blocked the norepinephrine-mediated increases in pFAK Y397 (Figure 4A), whereas pretreatment with PP3 had no effect (Figure 4A). Similar experiments were also performed with Src siRNA and demonstrated abrogation of norepinephrine-mediated FAK Y397 phosphorylation after Src gene silencing (Figure 4A). We also examined cells null for Src, Yes, and Fyn (SYF), and norepinephrine failed to stimulate FAK phosphorylation in these cells at Y397 (Figure 4B) or any other site (Supplemental Figure 11). Moreover, when Src was transiently introduced, norepinephrine treatment resulted in FAK Y397 phosphorylation (Figure 4B).

To determine whether norepinephrine enhanced direct interactions between Src and FAK, we performed coimmunoprecipitation assays of SKOV3ip1 cell lysates obtained 10 minutes after norepinephrine exposure. Immunoprecipitation of Src followed by anti-FAK Western blot analysis revealed a direct association
between the 2 molecules that was significantly increased by nor-
epinephrine (Figure 4C). Parallel effects were observed when FAK
immunoprecipitates were assayed for Src (Figure 4C).

Norepinephrine is also known to increase intracellular calcium
levels (19, 20), which can lead to phosphorylation of tyrosine
kinases. To determine whether the stimulation of FAK phosphor-
ylation by norepinephrine was mediated by intracellular Ca^{2+},
we treated cells with the Ca^{2+}-ATPase inhibitor thapsigargin
for 30 minutes prior to norepinephrine exposure. Thapsigargin abol-
ished norepinephrine-induced calcium flux (data not shown), but it
did not block norepinephrine’s effects on FAK-Src complex for-
mation or FAK^{Y397} phosphorylation (Figure 4D). Similar effects
were observed when extracellular calcium was chelated with EGTA
(Figure 4D). To determine whether norepinephrine-induced FAK
activation required an intact actin skeleton, we exposed ovarian
cancer cells to the actin polymerization inhibitor cytochalasin D
for 2 hours and then stimulated them with norepinephrine (21).
Cytochalasin D substantially diminished the norepinephrine-
induced increase in pFAK^{Y397} (Figure 4E).

Effect of FAK silencing on norepinephrine-mediated tumor growth. To
assess the effects of catecholamine-induced FAK activation on in
vivo tumor growth, we analyzed the effects of restraint stress on
the growth and anoikis of ascites-producing 2774 ovarian cancer
cells implanted into the peritoneal cavity of nude mice. Animals
exposed to 2 hours of daily restraint stress had a significantly lower
rate of tumor cell apoptosis in ascites (P ≤ 0.01; Figure 5A), indi-
cating lower levels of anoikis. Similar effects were observed with
the β-agonist isoproterenol (P ≤ 0.01; Figure 5B), and those effects
were blocked by propranolol. Similar findings were noted with
the SKOV3ip1 model (data not shown). Both chronic stress and
isoproterenol also increased phosphorylation of FAK^{Y397} in tumor
cells, and these effects were blocked by propranolol (Supplemental

**Figure 2**
Effect of catecholamines on FAK activation. (A) SKOV3ip1 or EG cells were plated and treated with either norepinephrine (NE) or epinephrine (Epi) and then subjected to Western blot analysis for FAK or pFAK^{Y397}. (B) SKOV3ip1 cells kept in suspension were treated with norepinephrine, followed by Western blot for FAK and pFAK^{Y397}. (C) SKOV3ip1 cells were treated with norepinephrine, then subjected to immunofluorescence analysis for pFAK^{Y397} and actin. Quantification of pFAK^{Y397} staining is shown in the graph. Scale bars: 10 μm. (D) Effect of 10 μM norepinephrine with or without FAK silencing with siRNA on anoikis. Results represent the mean ± SEM. *P ≤ 0.01.
Figure 12 and data not shown). Next, to confirm the functional role of FAK activation as a mediator of stress-induced protection from anoikis, we delivered human FAK-specific siRNA (Supplemental Figure 13) in vivo by incorporation into 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) nanoliposomes (22, 23). Control siRNA-DOPC had no effect on SKOV3ip1 tumor growth (Figure 5C), but FAK-specific siRNA-DOPC completely blocked stress-induced increases in tumor growth. Similar effects of FAK silencing were observed in the HeyA8 model (Figure 5D). In these experiments, FAK siRNA-DOPC also blocked the protective effects of stress on tumor cell apoptosis (Figure 5E). We also performed similar experiments with the 2774 model to examine effects of propranolol (Figure 5F) or FAK siRNA-DOPC (Figure 5F) on tumor cell apoptosis in ascites as a measure of anoikis. Treatment with either propranolol or FAK siRNA-DOPC completely blocked the protective effects of daily restraint stress or isoproterenol on tumor cell apoptosis in ascites. On the basis of our in vitro findings regarding the role of Src in stress-mediated pFAK\(^{Y397}\) activation, we examined the effects of Src silencing using the restraint stress model. Mice bearing SKOV3ip1 tumors were exposed to daily restraint stress and treated with either control siRNA-DOPC or Src siRNA-DOPC. In comparison to the controls, tumors from Src siRNA-treated animals had significantly lower pFAK\(^{Y397}\) levels (Supplemental Figure 14).

**Discussion**

The key findings from our study are that norepinephrine and epinephrine protect ovarian cancer cells from anoikis via a FAK-mediated signaling pathway that is initiated by ADRB2 and involves subsequent Src-associated phosphorylation of FAK\(^{Y397}\). Norepinephrine-induced FAK activation was also found to play a significant role in the effects of chronic stress on ovarian cancer growth in vivo in an orthotopic mouse model. Additional studies of human clinical tumors showed that both depression and tumor norepinephrine content were associated with increased
FAK activation and that increased FAK activation was associated with substantially accelerated mortality times. Thus, these studies identify norepinephrine- and epinephrine-induced FAK activation as a novel mechanism by which stress might accelerate the pathogenesis of ovarian cancer.

FAK signaling is critical in many biological pathways, including embryonic development (26), cell migration and invasion (14, 27, 28), proliferation (27, 29), and apoptosis (11, 24). FAK is a particularly important regulator of signaling processes between the ECM and tumor cells (30, 31). FAK phosphorylation at Y397 follows integrin stimulation or ligand binding by growth factor receptors (32). The present study identified the ADRB2/Src signaling axis as a molecular pathway for inhibiting ovarian cancer anoikis via FAK Y397 phosphorylation. Avoidance of anoikis provides a selective advantage for metastatic cancer cells to allow for transit to new sites for attachment (33). The present studies suggest that inhibition of the ADRB2/Src/FAK pathway by beta blockade or siRNA silencing with siRNA was examined on norepinephrine-mediated FAK activation. (B) SYF-null cells transfected with either empty vector (EV) or Src (WT Src) were stimulated with 10 μM norepinephrine, followed by immunoblotting for FAK and pFAK Y397. (C) SKOV3ip1 cells treated with 10 μM norepinephrine were subjected to immunoprecipitation for Src (left) or FAK (right), followed by immunoblotting for FAK and pFAK Y397. (D) Cells stimulated with 10 μM norepinephrine were treated with thapsigargin followed by Western blot for pFAK Y397 and FAK (left). Cells stimulated with 10 μM norepinephrine were treated with EGTA followed by immunoprecipitation for FAK, then immunoblotting for FAK and Src (right). (E) SKOV3ip1 cells stimulated with 10 μM norepinephrine were treated with cytochalasin D (Cyt D), followed by Western blot for pFAK Y397 and FAK. In A, D, and E, the immunoblot is shown at the top, and quantification of band intensity relative to total FAK intensity is shown below.

FAK activation and increased expression of FAK in ovarian epithelial cells is critical for tumor growth and metastasis. It is possible that stress hormone–mediated activation of FAK-dependent resistance to anoikis might play a role in normal tissue remodeling or development.

The present study expands the scope of molecular pathways involved in effects of stress on cancer growth and progression (4). In addition to potential effects of stress on immune response in cancer (4, 5), we and others have demonstrated that stress mediators such as norepinephrine and epinephrine from the SNS and glucocorticoids from the hypothalamic-pituitary-adrenal axis can directly regulate the function of human cancer cells in ways that promote their survival and metastasis (4, 6, 40). In the case of the SNS, these effects are mediated by ADRBs expressed on ovarian, mammary, and other cancer cells (15, 41, 42). Norepinephrine activation of ADRB2 has been shown to enhance tumor growth in part via induction of VEGF-dependent angiogenesis (6). Dopamine, an important member of the catecholamine family, appears to play an opposing role against the angiogenic effects of VEGF but is present at reduced levels under chronic stress conditions (43, 44). Other studies have identified additional mechanisms by which stress might contribute to cancer pathogenesis, including impaired DNA repair (45, 46) and modulation of matrix metalloproteinases (47–49). The current

**Figure 4**
Mechanism of norepinephrine-mediated (NE-mediated) FAK activation. (A) SKOV3ip1 cells stimulated with 10 μM norepinephrine were treated with either the Src inhibitor PP2 or its inactive counterpart PP3 followed by immunoblotting for FAK and pFAK Y397. (B) SKOV3ip1 cells treated with 10 μM norepinephrine were subjected to immunoprecipitation for Src (left) or FAK (right), followed by immunoblotting for FAK and pFAK Y397. (C) SKOV3ip1 cells treated with 10 μM norepinephrine were treated with EGTA followed by immunoprecipitation for FAK, then immunoblotting for FAK and Src (right). (D) Cells stimulated with 10 μM norepinephrine were treated with thapsigargin followed by Western blot for pFAK Y397 and FAK (left). Cells stimulated with 10 μM norepinephrine were treated with EGTA followed by immunoprecipitation for FAK, then immunoblotting for FAK and Src (right). (E) SKOV3ip1 cells stimulated with 10 μM norepinephrine were treated with cytochalasin D (Cyt D), followed by Western blot for pFAK Y397 and FAK.
study identifies a new pathway by which stress biology can impact tumor growth and progression via ADRB2-dependent activation of FAK and the resulting protection of cells from anoikis. Resistance to anoikis is a hallmark of malignant transformation, affording tumor cells increased survival times in the absence of matrix attachment and facilitating migration, reattachment, and colonization of secondary sites (50, 51). Overexpression of oncogenes such as ras, raf, and src as well as the downregulation of tumor suppressor genes such as PTEN and p53 (TP53) contribute to protection from anoikis (52). This study identifies a novel neuroendocrine pathway by which behavioral stress factors can exert similar effects. These findings also imply that the neuroendocrine “macroenvironment” may play a significant role in shaping cellular activity in the tumor microenvironment in ways that ultimately facilitate cancer progression. Thus, protective interventions targeting the neuroendocrine system might simultaneously modulate multiple molecular pathways involved in tumor metastasis (e.g., anoikis, angiogenesis, and invasion).

Methods
Reagents. Norepinephrine, epinephrine, propranolol, butoxamine hydrochloride, dobutamine, leupeptin, aprotinin, poly-HEMA, thapsigargin, EGTA, ATP, and cytochalasin D were obtained from Sigma-Aldrich.

Figure 5
Effect of (A) chronic stress or (B) isoproterenol on tumor cell apoptosis in ascites as a reflection of in vivo anoikis using the 2774 model. Effects of control siRNA-DOPC or FAK siRNA-DOPC on stress-induced in vivo (C) SKOV3ip1 or (D) HeyA8 tumor growth. (E) Effect of stress on tumor cell apoptosis in the SKOV3ip1 model. (F) Effect of propranolol or FAK siRNA-DOPC on tumor cell apoptosis in ascites as a measure of anoikis using the 2774 model. Results represent the mean ± SEM; n = 10 mice per group. * P < 0.01.
(RS)-Atenolol and SR59230A were purchased from Tocris Cookson Inc. PP2, PP3, forskolin, and KT5720 were acquired from Calbiochem. Rabbit (polyclonal) anti-FAK (pY397) phosphospecific antibody, unconjugated, was obtained from Biosource International Inc.

Cell culture. The derivation and source of the established ovarian cancer cell lines SKOV3ip1, HeyA8, 2774, A2780-PAR, RMG-II, and EG have been reported previously (53). A2780 and RMG-II cells are known to be negative for ADRB2 expression based on mRNA and protein analysis and lack of intracellular cAMP response to norepinephrine or isoproterenol (6). These cells were maintained and propagated in vitro by serial passage in RPMI 1640 supplemented with 15% FBS and 0.1% gentamicin sulfate (Gemini Bioproducts). The SYF-null cells were maintained in DMEM with 10% FBS and 0.1% gentamycin sulfate. All of the cell lines are routinely screened for Mycoplasma species (GenProbe detection kit; Fisher). All experiments were performed with 70%–80% confluent cultures.

Immunoprecipitation and Western blot analysis. Cells were lysed with modified 1x RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, and 0.5% deoxycholate) containing 25 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM sodium orthovanadate, and 2 mM EDTA. Samples were removed from culture dishes by cell scraping and centrifuged at 15,000 g for 30 minutes. The protein concentration of the samples was determined using a bicinchoninic acid Protein Assay Reagent kit (Pierce), and whole cell lysates were analyzed by 7.5% SDS-PAGE and stained with Coomassie BBR-250 to ensure equal loading (data not shown). Samples were transferred to nitrocellulose. Blots were blocked with 5% nonfat milk at room temperature for 1 hour. Blots were incubated with the polyclonal antibody (1:1,000 dilution; Biosource) at room temperature, with agitation for 1 hour, followed by incubation with a horseradish peroxidase–conjugated anti-mouse secondary antibody (1:6,000; The Jackson Laboratory). Blots were developed using an enhanced chemiluminescence detection kit (ECL; Pierce).

Immunoprecipitation experiments, 300 µg of cell lysate was incubated with the Src antibody at 4°C for 1 hour. Protein-antibody complexes were incubated for 1 hour with protein A/G plus-agarose–conjugated beads (Upstate). Protein-antibody complexes were collected by centrifugation, washed 3 times with the modified RIPA buffer, and boiled in Laemmli sample buffer. Protein was resolved in SDS-PAGE gels, and immunoblotting was performed as described above.

Anoikis assays. Cells (5 × 10^4 per well) were cultured on either plastic or poly-HEMA–treated 6-well tissue culture plates for 24–72 hours at 37°C in 5% CO2 atmosphere. After incubation, adherent cells were detached with 0.5% trypsin/0.1% EDTA in PBS. Detached and suspended cells were harvested in complete RPMI 1640 medium and centrifuged at 500 g for 10 minutes. Pellets were washed with PBS and fixed with ice-cold 75% ethanol (v/v) overnight at 4°C. After fixation, cells were washed

Figure 6
Clinical significance of FAK activation in ovarian carcinoma. (A) Representative images of human ovarian tumors with low or high immunohistochemical staining for FAK and pFAK\textsuperscript{Y397}. Original magnification, ×200. (B) Kaplan-Meier curves of disease-specific mortality for patients with epithelial ovarian carcinoma based on FAK or pFAK\textsuperscript{Y397} expression. The log-rank test (2-sided) was used to compare differences between groups. Percentage of ovarian cancers with high FAK or pFAK\textsuperscript{Y397} expression based on CESD scores of at least 16 (C) or tumoral norepinephrine (NE) levels (greater versus less than median value of 0.84 pg/mg) (D).
with PBS and stained with 500 μl propidium iodide (PI) solution (50 μg/ml in PBS) containing 25 μg/ml RNase A. Cells were incubated at 37°C for 30 minutes and analyzed by flow cytometry on an Epics XL flow cytometer (Coulter).

In vivo anochis assay. Apoptotic rate of tumor cells from ascites fluid was determined by dual staining with PI and epithelial surface antigen tagged with FITC (ESA-FITC). Nude mice inoculated with the 2774 cells were left undisturbed until they developed detectable ascites. At this point, mice were divided into 2 groups, receiving either isotretinoin (10 mg/kg) or PBS. After 5 days of treatment, ascites fluid was drawn out from the peritoneal cavity and rapidly centrifuged at 500 g for 10 minutes. Pellets were washed first with a red blood cell lysis buffer and reconstituted in PBS. Suspended cells were then incubated with ESA-FITC (1:30 dilution) for 30 minutes at room temperature. After incubation, cells were washed and fixed overnight at 4°C with ice-cold 75% ethanol. After fixation, cells were washed and stained with a PI solution (50 μg/ml). Cells were then incubated for 30 minutes at 37°C and analyzed by flow cytometry on an Epics XL flow cytometer (Coulter).

Kinase assays. HEK 293 cells transiently expressing Flag-tagged FAK, Flag-tagged kinase-dead FAK, or Flag-tagged Src were individually lysed, and protein-antibody complexes were precipitated. The kinase reaction was carried out by adding 25 μl kinase buffer containing ATP and incubating for 30 minutes. The reaction was stopped by adding 25 μl of Laemmli sample buffer and boiling samples. Afterward, samples were subjected to Western blot analysis.

Immunohistochemistry. Formalin-fixed, paraffin-embedded human tumor samples were stained for total FAK and phospho-FAK (Y397). Slides were heated overnight at 65°C, deparaffinized, and hydrated with PBS. Antigen retrieval for total FAK was done with target solution (Dako) in 1 time with Tris pH 7, and 1 time with kinase buffer with no ATP. The antigen retrieval for total FAK was done with target solution (Dako) in 1 time with Tris pH 7, and 1 time with kinase buffer with no ATP. The kinase reaction was carried out by adding 25 μl kinase buffer containing ATP and incubating for 30 minutes. The reaction was stopped by adding 25 μl of Laemmli sample buffer and boiling samples. Afterward, samples were subjected to Western blot analysis.

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In vivo model of chronic stress. We purchased siRNAs targeted against human FAK (target sequence 5′-AACCACCCGCGCAGTATATTAT-3′) or Src (target sequence 5′-GGCTGAGGATGTTTATTTT-3′) from QIAGEN and incorporated them into a neutral liposome (DOPC), as previously described (22, 23).

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