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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 FAK^{Y397}, 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 pFAK^{Y397}, 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 pathogenesis 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 β_2 -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-

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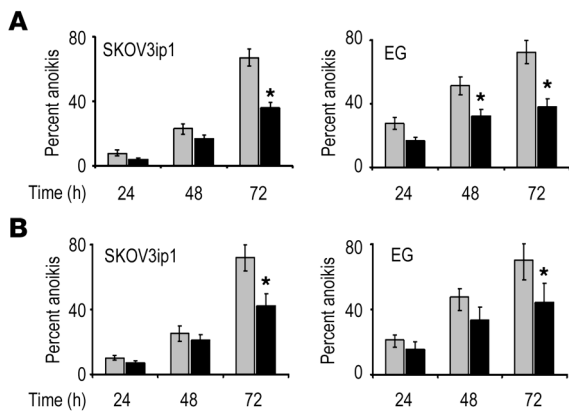


Figure 1 Effect of (A) 10 μ M norepinephrine or (B) 10 μ M epinephrine on anoikis in ovarian cancer cells. Gray bars, vehicle treatment; black bars, norepinephrine (A) or epinephrine (B) treatment. Results represent the mean \pm SEM. * $P < 0.05$.

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 pFAK^{Y397} 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/JCI140802DS1). 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 medi-

ated 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 -antagonists. The nonspecific beta blocker propranolol (1 μ M) blocked norepinephrine-induced FAK activation in both SKOV3ip1 (Figure 3A) and EG (Figure 3B) cells. Propranolol also blocked norepinephrine's 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 μ M) 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 μ M) 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

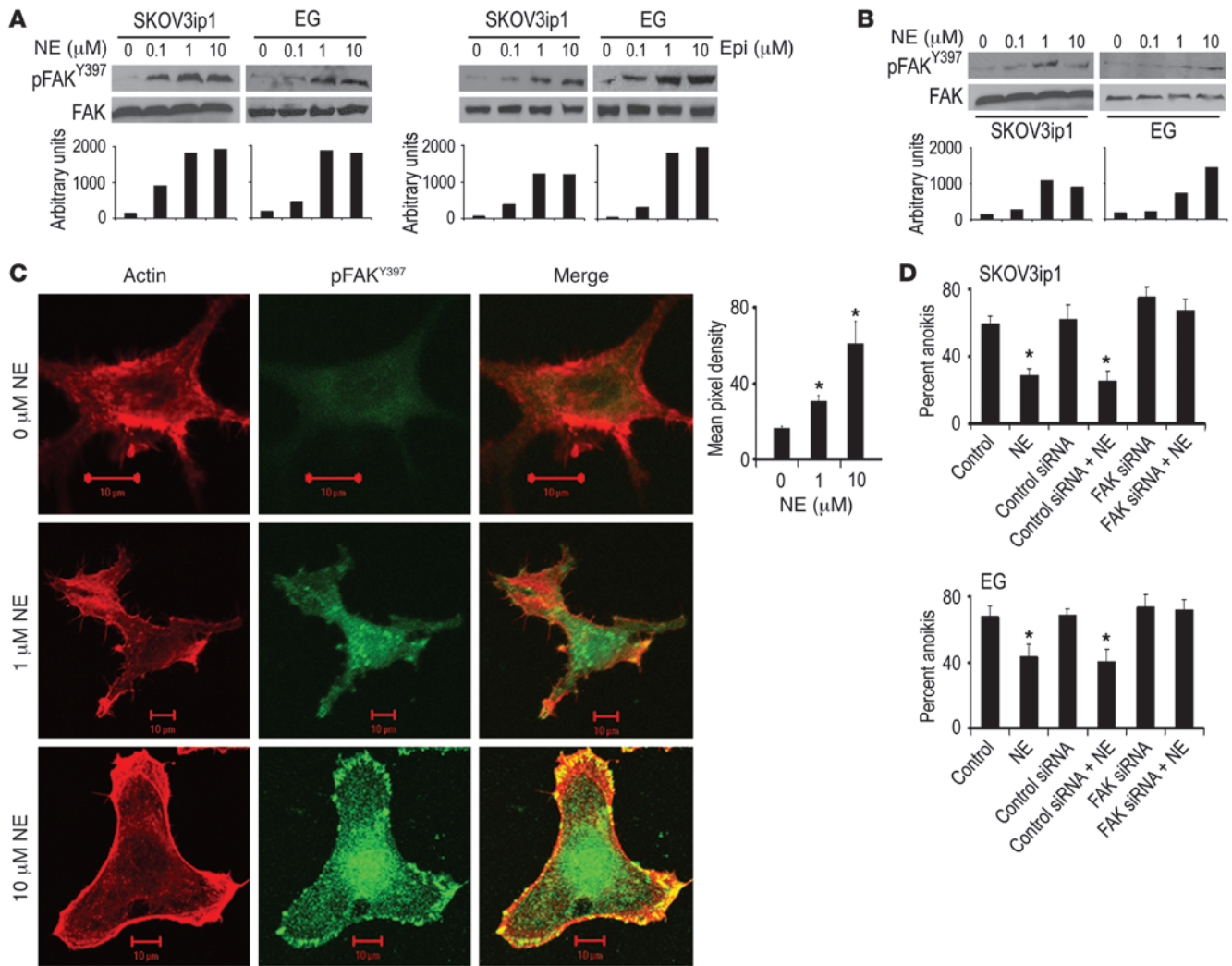


Figure 2

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.

between the 2 molecules that was significantly increased by norepinephrine (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 phosphorylation by norepinephrine was mediated by intracellular Ca²⁺, we treated cells with the Ca²⁺-ATPase inhibitor thapsigargin for 30 minutes prior to norepinephrine exposure. Thapsigargin abolished norepinephrine-induced calcium flux (data not shown), but it did not block norepinephrine's effects on FAK-Src complex formation 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), indicating 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

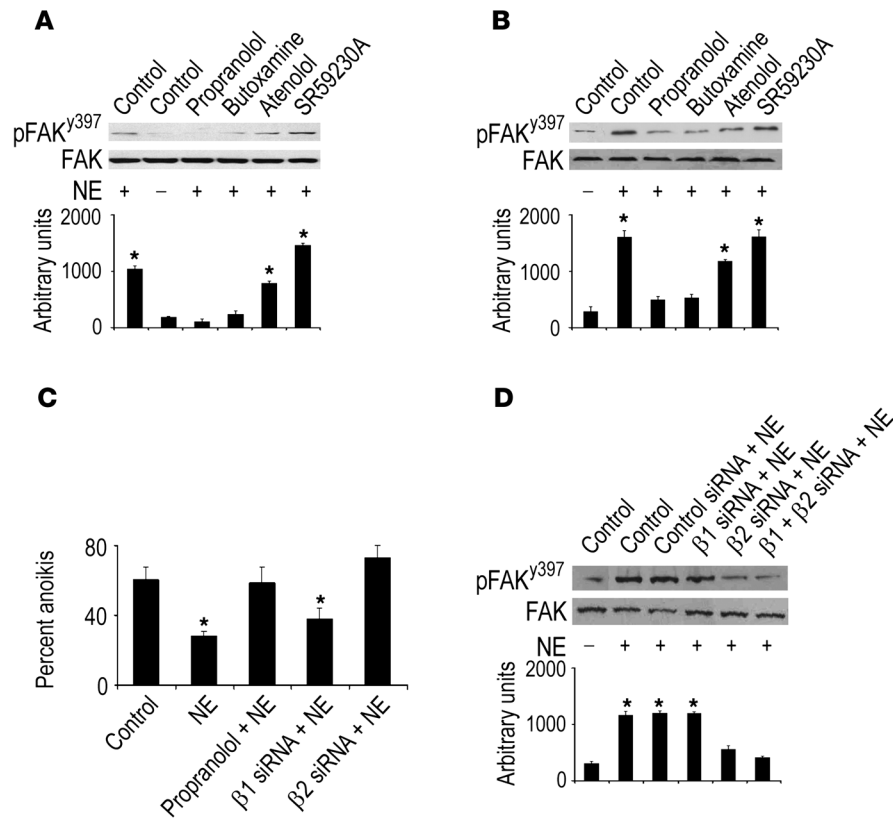


Figure 3

Effect of propranolol (nonspecific β -antagonist), atenolol (β_1 antagonist), butoxamine (β_2 antagonist), or SR59230A (β_3 -antagonist) on FAK and pFAK^{Y397} in (A) SKOV3ip1 and (B) EG cells. (C) Effect of 10 μ M norepinephrine with or without propranolol or ADRB1- or ADRB2-targeted (β_1 or β_2) siRNA in SKOV3ip1 cells on anoikis. (D) Effect of β_1 and β_2 targeted siRNA on pFAK^{Y397} and FAK in SKOV3ip1 cells. In A, B, and D, the immunoblot is shown at the top, and quantification of band intensity relative to total FAK intensity is shown below. For all panels, results represent the mean \pm SEM of triplicate experiments. * $P < 0.01$.

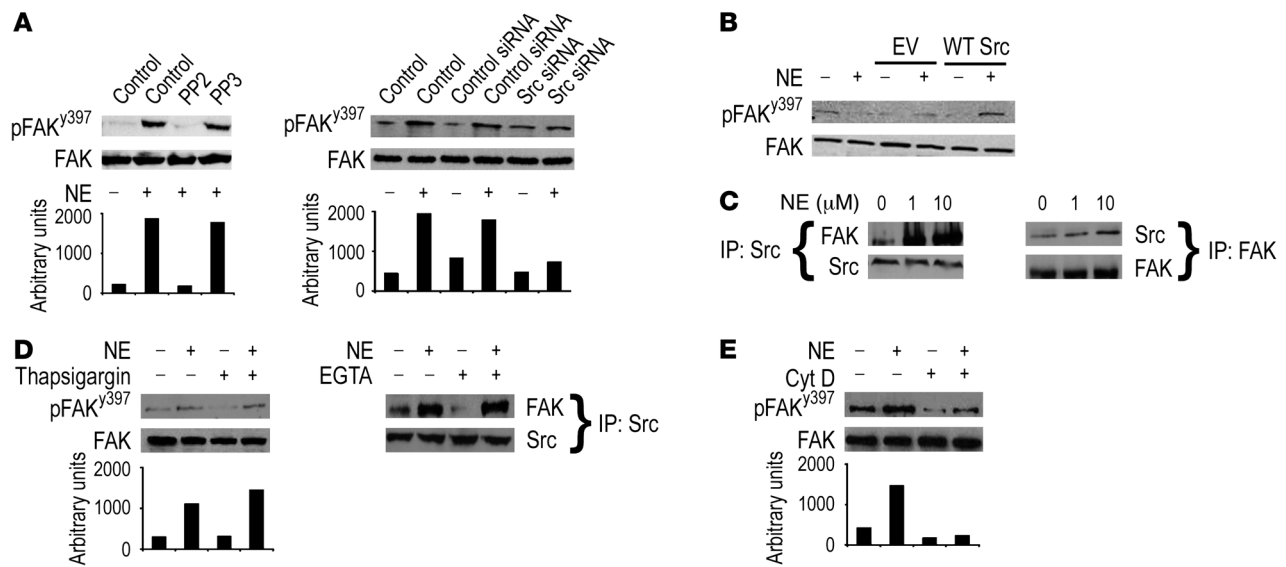
Figure 12 and data not shown). Next, to confirm the functional role of FAK activation as a mediator of stress-induced protection from anoikis, we delivered human FAK-specific siRNA (Supplemental Figure 13) in vivo by incorporation into 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) nanoliposomes (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).

FAK expression in human ovarian carcinoma. To assess the significance of stress-induced FAK activation for human clinical cancer, we examined FAK activity in 80 cases of invasive epithelial ovarian cancer. Consistent with our previous data (11, 14, 24), increased FAK expression was noted in 67% of the tumors. Increased levels of pFAK^{Y397} were observed in 50% of tumors (Figure 6A), and both

increased FAK expression and increased FAK^{Y397} phosphorylation were associated with poor overall patient survival (Figure 6B). The pFAK^{Y397} scores were normalized to total FAK scores, and high expression of normalized pFAK^{Y397} remained significantly associated with poor overall survival (Supplemental Figure 15). To assess the role of adrenergic signaling in these relationships, we grouped patients based on high versus low levels of depression, which has been linked to differential adrenergic signaling (25) in ovarian cancer patients. High levels of depression (Center for Epidemiological Studies Depression [CESD] scale ≥ 16) were associated with a marginally significant increase in FAK expression ($P = 0.08$) and a highly significant increase in the level of phosphorylated FAK^{Y397} ($P = 0.003$; Figure 6C). We also measured norepinephrine levels in 51 tumors and found increased FAK^{Y397} phosphorylation ($P < 0.04$; Figure 6D) and marginally increased levels of total FAK ($P = 0.06$; Figure 6D) in tumors with above-median norepinephrine content (>0.84 pg/mg). The box plots of FAK and pFAK^{Y397} scores based on CESD and norepinephrine levels are shown in Supplemental Figure 16.

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

**Figure 4**

Mechanism of norepinephrine-mediated (NE-mediated) FAK activation. **(A)** SKOV3ip1 cells stimulated with 10 μ M norepinephrine were treated with either the Src inhibitor PP2 or its inactive counterpart PP3 followed by immunoblotting for FAK and pFAK^{Y397}. In addition, the effect of Src 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 μ M norepinephrine, followed by immunoblotting for FAK and pFAK^{Y397}. **(C)** SKOV3ip1 cells treated with 10 μ M norepinephrine were subjected to immunoprecipitation for Src (left) or FAK (right), followed by immunoblotting for FAK and Src. **(D)** Cells stimulated with 10 μ M norepinephrine were treated with thapsigargin followed by Western blot for pFAK^{Y397} and FAK (left). Cells stimulated with 10 μ M norepinephrine were treated with EGTA followed by immunoprecipitation for FAK, then immunoblotting for FAK and Src (right). **(E)** SKOV3ip1 cells stimulated with 10 μ M 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 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 inhibition might provide a novel strategy for suppressing tumor growth and metastasis in clinical settings.

All 3 major catecholamines (norepinephrine, epinephrine, and dopamine) are known to be present in the ovary, with norepinephrine being the most abundant (34, 35). Ovarian norepinephrine levels are substantially higher than those in circulating blood (36, 37), and SNS activity can further enhance those levels to induce precystic follicles (38, 39). The doses of norepinephrine used for our study were selected to reflect the physiologic conditions of this hormone at the level of the tissue microenvironment. Studies suggest that within the parenchyma of the ovary, concentrations may reach as high as 10 μ mol/l (39). Although the physiological role of norepi-

nephrine and epinephrine in the ovarian tissue environment is not yet fully known, the present data imply that these signaling molecules might contribute to disease pathogenesis in the context of an emerging tumor. Further analysis of the roles of stress hormones in normal ovarian physiology would provide valuable insights into the basis for the presently observed effects in tumor cells. Particularly important in these future studies will be defining the physiological role of norepinephrine and epinephrine in modulating cellular dependence on neighboring cells and ECM. 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

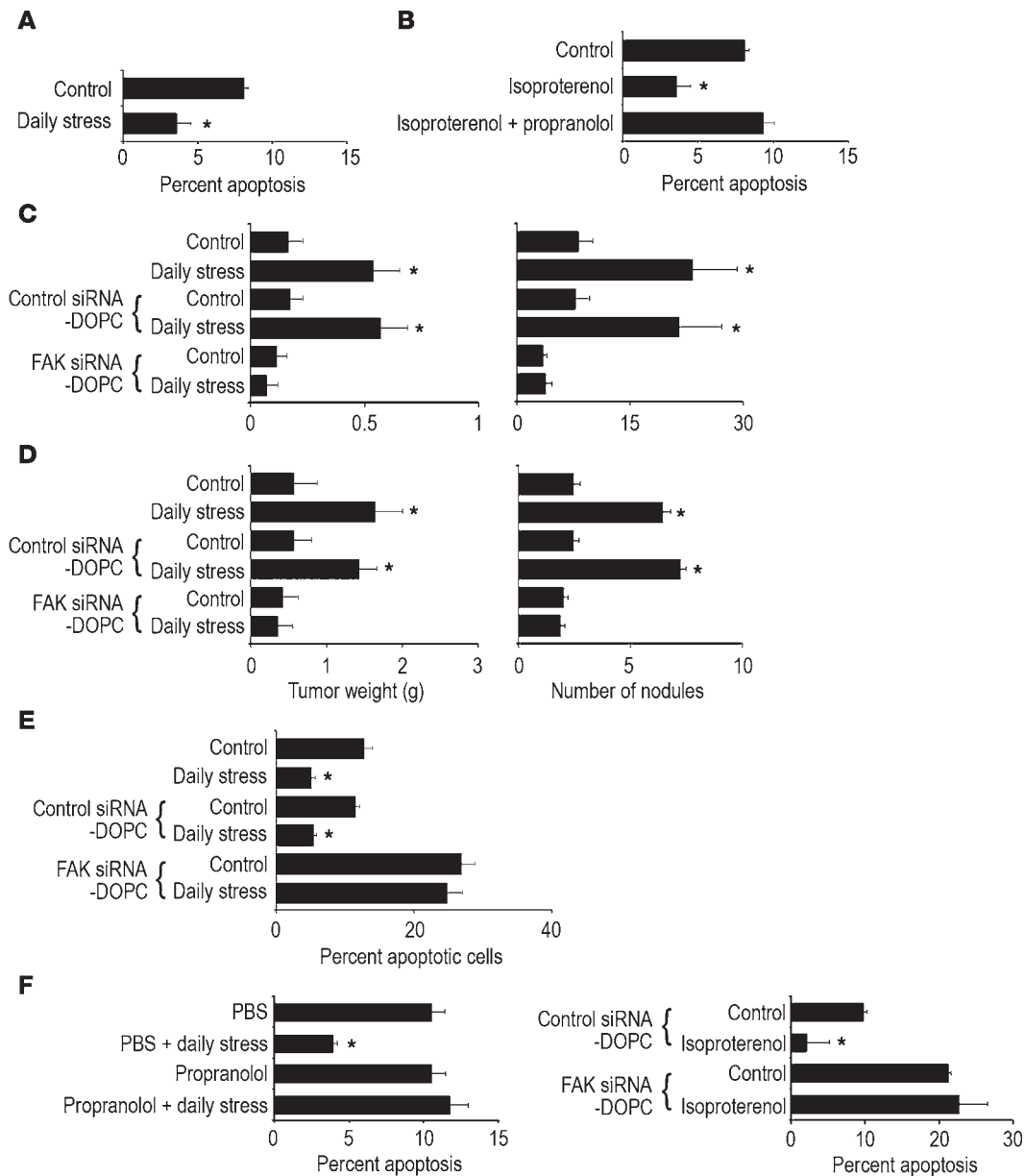


Figure 5 Effect of (A) chronic stress or (B) isoproterenol on tumor cell apoptosis in ascites as a reflection of in vivo anoikis using the 2774 model. Effects of control siRNA-DOPC or FAK siRNA-DOPC on stress-induced in vivo (C) SKOV3ip1 or (D) HeyA8 tumor growth. (E) Effect of stress on tumor cell apoptosis 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.

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.

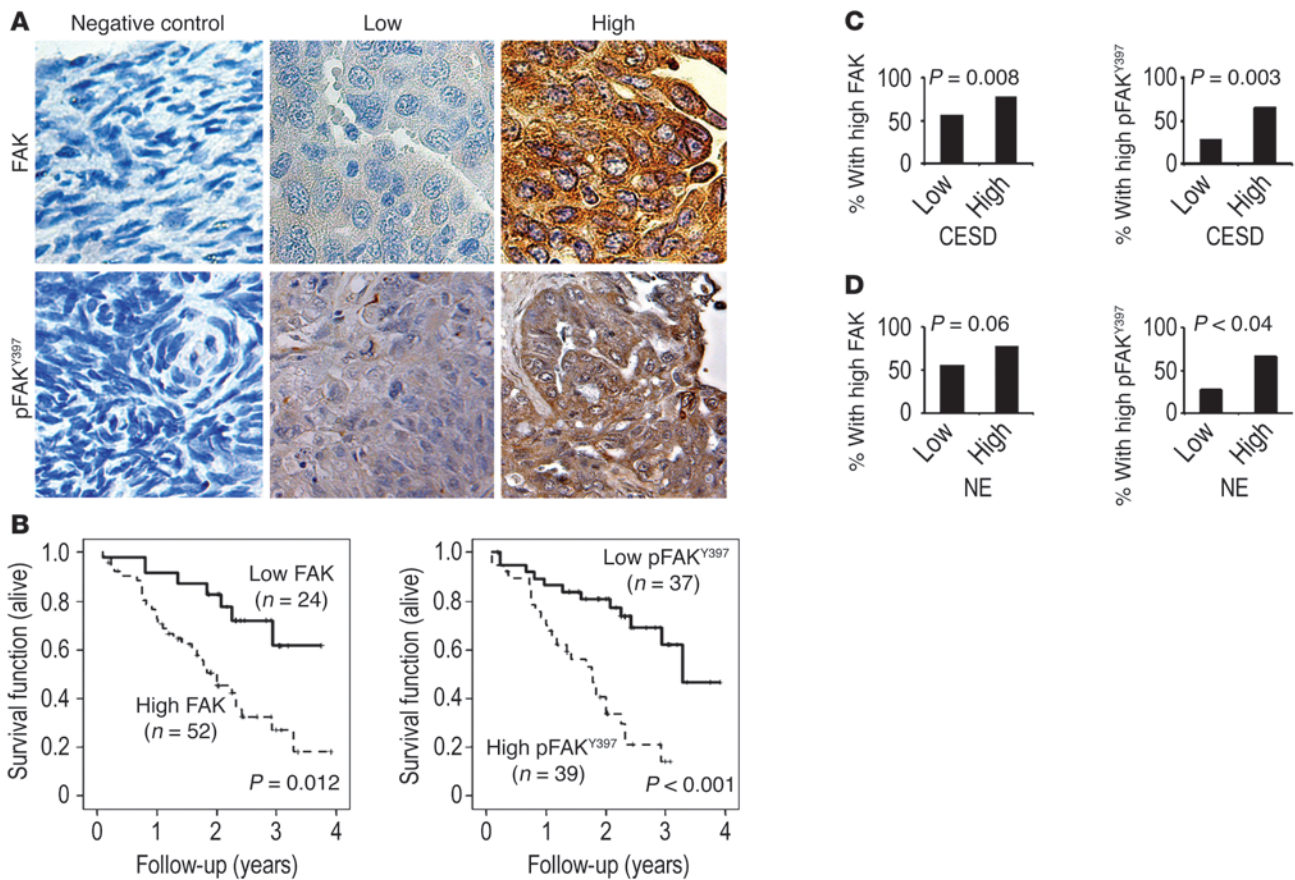


Figure 6

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^{Y397}. Original magnification, ×200. **(B)** Kaplan-Meier curves of disease-specific mortality for patients with epithelial ovarian carcinoma based on FAK or pFAK^{Y397} expression. The log-rank test (2-sided) was used to compare differences between groups. Percentage of ovarian cancers with high FAK or pFAK^{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)**.

(RS)-Atenolol and SR59230A were purchased from Tocris Cookson Inc. PP2, PP3, forskolin, and KT5720 were acquired from Calbiochem. Rabbit (polyclonal) anti-FAK (p^{Y397}) 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% gentamicin 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 1× 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).

For 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 at 4°C 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 × 10⁵ per well) were cultured on either plastic or poly-HEMA-treated 6-well tissue culture plates for 24–72 hours at 37°C in a 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



with PBS and stained with 500 μ l propidium iodide (PI) solution (50 μ g/ml in PBS) containing 25 μ g/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 anoikis 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 isoproterenol (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 μ g/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 the protein concentration for each sample was determined. Samples were incubated overnight with a Flag antibody. Protein-antibody complexes were precipitated for 1 hour using agarose-conjugated mouse secondary antibody. Immune complexes were washed 3 times with RIPA buffer, 1 time with Tris pH 7, and 1 time with kinase buffer with no ATP. The kinase reaction was carried out by adding 25 μ l kinase buffer containing ATP and incubating for 30 minutes. The reaction was stopped by adding 25 μ l 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 a vegetable steamer for 40 minutes. Antigen retrieval was not performed for pFAK staining to maintain adequately low background. After PBS wash, slides were incubated in 3% H₂O₂ for 12 minutes to block endogenous peroxidase, washed with PBS, and blocked with 5% normal horse serum (NHS) for 30 minutes at room temperature. Slides were then incubated with primary antibody at 1:25 overnight at 4°C. After PBS wash and additional blocking with 5% NHS, slides were incubated with anti-mouse IgG secondary antibody (MACH4 kit, BioCare Medical) for 20 minutes at room temperature, washed in PBS, and incubated with polymer-linked horseradish peroxidase (MACH4 kit, BioCare Medical) for 20 minutes at room temperature. Slides were again washed and incubated with 3,3'-diaminobenzidine (DAB; Phoenix Biotechnologies) for 10 minutes at room temperature to visualize antibody staining. Slides were counterstained with Gill No. 3 hematoxylin (Sigma-Aldrich) for 15 seconds and washed with distilled water. All samples were reviewed by a gynecologic pathologist who was blinded to the clinical outcome of the patients. FAK or pFAK³⁹⁷ expression was determined by assessing semiquantitatively the percentage of stained tumor cells and the staining intensity, as described previously (14). Briefly, the percentage of positive cells and staining intensity were rated on a scale of 1 to 4; points for expression and percentage of positive cells were added, and an overall score (OS) was assigned. Tumors with an OS in the upper tertile were considered as having protein overexpression. The stained slides were also scored on the basis of the histochemical score (with a score >100 defined as high expression and \leq 100 as low expression), according to the method described by McCarty et al., which considers both the intensity of staining and the percentage of cells stained (54–56).

Immunohistochemical staining of orthotopic tumors from therapy experiments was performed in a similar manner, with the exception of protein blocking. Nonspecific epitopes were blocked in fragment block (1:10; Jackson ImmunoResearch Laboratories) in 5% NHS for 5 hours at room temperature, followed by incubation with primary antibody at 4°C overnight.

Patient samples. Human epithelial ovarian cancer specimens were obtained from women enrolled in a prospective study, as described previously (57). The tumor samples were collected after consent was obtained from patients. This research was approved by the Institutional Review Boards of the University of Iowa and University of Texas MD Anderson Cancer Center. Among the 80 tumor samples, 75 (94%) were of serous histology and 5 (6%) were endometrioid or mucinous.

Behavioral measures. Patients completed psychosocial questionnaires between their initial preoperative appointment and surgery. The CESD scale is a 20-item measure designed to assess depressive symptomatology and has frequently been used in studies of cancer patients (25). Scores of 16 or higher are associated with clinical depression.

Assessment of tumor norepinephrine levels. Norepinephrine levels in tumor extracts were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ED) as previously described (25). Chromatograph peak areas for norepinephrine were compared with the average peak areas determined from the injection of 100 μ g pure standard and corrected for extract dilutions and tissue wet weights.

siRNA preparation. We purchased siRNAs targeted against human FAK (target sequence 5'-AACCACCTGGGCCAGTATTAT-3') or Src (target sequence 5'-GGCTGAGGAGTGGTATTTT-3') from QIAGEN and incorporated them into a neutral liposome (DOPC), as previously described (22, 23).

In vivo model of chronic stress. We obtained female athymic nude mice from the National Cancer Institute. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas MD Anderson Cancer Center. For the chronic stress model, we used a physical restraint system previously used by our group (6). In our model, mice ($n = 10$ per group) were injected intraperitoneally with ovarian cancer cells 7 days after starting stress. Treatment with siRNA designed against human FAK began 3 days later and continued for 3 weeks. Mice were necropsied 21 days after tumor cell injection, and tumors were harvested for immunohistochemistry and Western blot analysis.

Statistics. We compared continuous variables with either 2-tailed Student's *t* test or ANOVA and compared categorical variables with the χ^2 test. We used nonparametric tests (Mann-Whitney *U* test), if appropriate, to compare differences. Kaplan-Meier survival curves and the log-rank test were used to examine the effects on patient disease-specific survival. A *P* value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 12 for Windows statistical software (SPSS Inc.).

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