Activated Signal Transducer and Activator of Transcription (STAT) 3: Localization in Focal Adhesions and Function in Ovarian Cancer Cell Motility

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ABSTRACT

Constitutive activation of the Janus-activated kinase/signal transducer and activator of transcription (STAT) pathway promotes the proliferation and survival of cancer cells in culture and is associated with various cancers, including those of the ovary. We found that constitutively activated STAT3 levels correlated with aggressive clinical behavior of ovarian carcinoma specimens. Furthermore, inhibition of STAT3 reduced the motility of ovarian cancer cells in vitro. Surprisingly, we found that activated STAT3 localized not only to nuclei but also to focal adhesions in these cells. Activated STAT3 communoprecipitated with phosphorylated paxillin and focal adhesion kinase and required paxillin and Src for its localization to focal adhesions. These results suggest that Janus-activated kinase/STAT signaling may contribute to ovarian cancer cell invasiveness.

INTRODUCTION

The Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway is best characterized as a highly conserved mechanism for communicating signals from the cell membrane to the nucleus. JAK/STAT signaling is required for diverse processes during embryonic development and in the adult. In addition, constitutive activation of this pathway has been associated with cancer (1, 2).

The signaling pathway is activated when an extracellular ligand, typically a cytokine of the interleukin (IL) family, binds to its receptor (3). JAKs are tyrosine kinases that constitutively bind to the receptor. Ligand binding activates JAK, resulting in its autophosphorylation and phosphorylation of specific tyrosine residues on the receptor. STAT proteins are recruited to the receptor by binding to phosphotyrosine residues via an Src homology 2 domain in the STAT protein. STAT then is phosphorylated by JAK, dimerizes, and shuttles into the nucleus, where it functions as a transcription factor.

Components of the JAK/STAT pathway are present in a variety of multicellular organisms, ranging from the slime mold Dictyostelium to invertebrates, such as Drosophila, and in mammals, including humans. Whereas Dictyostelium and Drosophila have one JAK and one STAT, humans have four JAKs, six STATs, and many receptors and ligands. Mammalian STATs have unique expression patterns and appear to have distinct physiologic functions, as evidenced by the different phenotypes of STAT knockout mice. For example, mice lacking STAT5 are viable and have defects in mammary gland development and impaired growth (4). In contrast, STAT3, which is widely expressed, is required for embryonic viability (5). However, STAT3 and STAT5 have been shown to promote cell proliferation and prevent apoptosis in different cell types (3).

A significant body of evidence suggests that JAK/STAT signaling is involved in promoting tumorigenesis. Constitutive activation of either STAT or JAK occurs in many cancers (2). A translocation of the NH2 terminus of the TEL fusion factor with the COOH terminus of JAK results in a constitutively active JAK that is associated with T-cell leukemia (6). STAT3 is constitutively activated in many different tumor cell lines and primary tumors, including prostate, breast, and head and neck cancer (2, 7, 8). Interestingly, a constitutively active form of STAT3 has been demonstrated to transform fibroblasts in vitro and to induce tumor formation in nude mice (1). The mechanisms by which activated STAT3 can promote tumorigenesis are as yet unclear but appear to involve, at least in part, deregulated cell proliferation and/or prevention of apoptosis (7).

Recent evidence indicates a role for STATs in cell motility and survival. The requirement for STAT3 in mouse gastrulation is intriguing because this is the first epithelial to mesenchymal transition in embryonic development (5). In zebrafish embryos undergoing gastrulation, STAT3 is essential for migration of sheets of cells, independent of a requirement in cell fate specification (9). In addition, mouse keratinocytes, in which STAT3 has been conditionally removed, exhibit migration defects in a monolayer wounding assay (10). STAT also is necessary for the migration of a subset of epithelial follicle cells, called the border cells, in the Drosophila ovary (11). Furthermore, ectopic activation of the JAK/STAT pathway in the Drosophila ovary causes extra cells to become invasive without affecting cell proliferation.

The human ovary, like that of Drosophila, is surrounded by a simple epithelium comprising a single layer of cells. Recent experimental evidence supports the long-held notion that epithelial ovarian cancers originate from cells of the ovarian surface (12). Epithelial ovarian cancer is the most lethal gynecologic malignancy and the fifth major cause of cancer death among women in the United States. A major mechanism by which ovarian carcinomas are thought to metastasize is by the seeding of clusters of cells throughout the peritoneal cavity. This process may share some similarities with the invasive, migratory behavior of epithelial cells in the Drosophila ovary. Several of the genes that control Drosophila epithelial follicle cell migration are homologous to genes that have been implicated in promoting ovarian carcinoma progression (13).

Recent studies have found that STAT3 is constitutively activated in ovarian cancer cell lines and clinical specimens (14–16). These findings, together with evidence that STAT3 is required for cell motility in several contexts, raise the possibility that activated STAT3 promotes dissemination of ovarian carcinoma cells. In this study, we found that activated STAT3 is more frequently activated in high-grade ovarian carcinomas that are typically diagnosed at late stages of disease than in low-grade carcinomas and organ-confined borderline tumors. Furthermore, we demonstrate that depletion of STAT3 inhibits migration of ovarian carcinoma cells. Interestingly, we show that activated STAT3 is a novel component of focal adhesions within these cells and mouse fibroblasts. Together, these findings raise the possibility that activated STAT3 contributes to ovarian cancer cell motility and invasion by responding to changes in cell adhesion and/or affecting the cytoskeleton.
MATERIALS AND METHODS

Tissue Microarray Construction and Analysis. For tissue microarray construction, two core biopsies (0.6 mm in diameter) were taken from histologically identified representative regions of formalin-fixed, paraffin-embedded tissues of a given case. The number of cases per group was the following: high-grade carcinomas, n = 21 (5 clear cell carcinomas, 5 high-grade endometrioid carcinomas, and 11 high-grade serous carcinomas); low-grade carcinomas, n = 15 (5 low-grade endometrioid carcinomas and 10 low-grade serous carcinomas); borderline tumors, n = 17 (9 serous borderline tumors and 8 mucinous borderline tumors); cystadenomas, n = 12 (10 serous cystadenomas and 2 mucinous cystadenomas); and normal ovary, n = 8. The phospho-STAT3 antibody was used at 1:20, and the total STAT3 antibody was used at 1:200. Slides were viewed under a Nikon TS-100F microscope (Tokyo, Japan) with a DXM1200 high-resolution digital imaging system (Nikon). Phospho-STAT3 staining was calculated as a percentage of positive cells in a given case where two cores per case and two fields per core were examined (each field comprising a minimum of 200 preserved cells). Scoring of immunohistochemical staining was conducted independently of histopathologic diagnosis to eliminate bias in scoring. P values were calculated using a Mann-Whitney nonparametric U test.

Pharmacologic Treatment and siRNA Transfection. For pharmacologic treatment, cells were plated and grown overnight to ~60% confluence in 0.5% FCS to reduce basal levels of activated STAT3. The following day, either DMSO or AG490 (Santa, St. Louis, MO) was added to the cells overnight or for 4 h. For siRNA treatment, cells were plated and grown overnight to ~30% confluence. Cells were transfected with siRNA oligos made against the following sequences: STAT3(1), AACUCCAGACCGCGACAAAA; STAT3(2), AAAGUCGG-UUGCGGUAACAAA; lamina A/C, CUGGACUCCAGAAGAACA; STAT1, AAGCGUAAUCUCAGGAUAAU; STAT5B(1), AAGCGUAGCACUGU-AAGACU; and STAT5B(2), AAUGAUAAUGCGGGAAGACU.

Purified RNA oligos were purchased from Dharmacon (Lafayette, CO), along with the Scramble II Duplex siRNA. Cell transfection was performed using Oligofectamine (Invitrogen, Carlsbad, CA) in serum-free media using the RNA oligos at ~260 nm final concentration. Transfection was carried out for 4 h, after which time serum was added back to the cells. Transfections were repeated the following day using the same protocol.

Transfection of cells with all of the siRNAs, including the scrambled siRNA, resulted in cells that were ~60% confluent (untreated cells were ~75–80% confluent). Treatment of cells with AG490 or DMSO resulted in ~60–70% confluency for all of the samples. After treatment with AG490 inhibitor or siRNAs, cells were tested for viability by trypan blue exclusion. In all of the cases, cell culture samples were used only when viability was >95%. Cells were diluted 1:5 and counted in at least 10 large squares using a hemacytometer. In all of the cases, cell number was uniform regardless of treatment.

Motility Assay. Cells were used following pretreatment with either DMSO or AG490 overnight, for 4 h, or 3 days after initial transfections with siRNAs. A total of 2.5 × 10^4 cells (at ~70% confluency) were put in a solution containing media plus 0.1% BSA. This was placed on the top of 8-well transwell chambers (Corning Costar, Cambridge, MA). The following chemoattractants were used: media containing 2.5% FCS placed in the bottom of the chamber, fibronectin (10 μg/ml) bound to the underside of the membrane and replaced with media alone, or media alone as a negative control. Migration was allowed to proceed for 4 h, after which time the membranes were fixed and stained using Diff-quick (Baxter, Deerfield, IL). The tops of membranes were cleaned of bound cells, and membranes were mounted for imaging using Permount (Fisher Scientific, Hampton, NH). The number of cells in each of 20× fields was counted. The percentage of migration was scored relative to untreated cells. Experiments were performed in triplicate, and at least three independent experiments were performed for each point on each graph. P values were calculated using a Student’s t test by comparing samples with the scrambled siRNA control.

Immunolocalization. The cells were plated either in 10% FCS or on cover slips coated with 15–25 μg/ml fibronectin (BD Biosciences, San Jose, CA). They were then fixed in 3.7% paraformaldehyde for 10 min followed by 5 min of 0.1% Tween treatment. Stainings were performed using the following antibodies: rabbit total STAT3, 1:100; phospho-TYK2, 1:200; mouse phospho-paxillin, 1:100 (Cell Signaling, Beverly, MA); rabbit phospho-STAT3, 1:200 (Cell Signaling and Biosource International, Camarillo, CA); mouse vinculin, 1:200 (Sigma); mouse lamin A/C, 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit phospho-JAK2, 1:100; mouse focal adhesion kinase (FAK), 1:200 (UBI, Lake Placid, NY); and mouse paxillin 1:200 (BD Transduction, Lexington, KY). Cells were visualized using an Ultraview confocal microscope (Perkin Elmer, Wellesley, MA. Peptides used for competition studies were purchased from Biosource International.

Immuneoprecipitations. OVCA3 cells or null MEF cells were grown in either 100-mm or 250-mm plates to confluence and lysed in 1 ml of immuneoprecipitation buffer for 30 min on ice, with occasional swirling. One ml of immuneoprecipitation buffer lacking detergent then was added to each plate, and cells were scraped and put through a 25-gauge needle. The following immuneoprecipitation buffer was used: 0.5% 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 150 mM NaCl, 50 mM Tris–HCl, 2 mM EDTA, 2 mM EGTA, 2 mM NaVO₄, 2 μM DTT, and protease inhibitor mixture. Cells were then spun for 20–30 min at 20,000 × g, and the supernatant were used. Antibodies were added at a dilution of 1:100 to a total of 1 ml, and immuneoprecipitations were performed at 4°C overnight with constant mixing. Fifty to 100 μl of protein A or G beads were added, and the solution was allowed to mix for 1–3 h at 4°C. Immuneoprecipitations were washed using the immuneoprecipitation buffer 4× and then brought up in 2× Laemli buffer for protein electrophoresis and Western blot analysis. Supernatants were loaded at one-fifth the volume of the pellet.

For STAT activation assays, cells were placed in media containing low serum overnight, trypsinized, and replated onto 100-mm plates coated with 8–24 μg/ml fibronectin (BD Biosciences). After overnight incubation, total STAT3 protein was immunoprecipitated from the cells as described previously.

Western Blot Analysis. The following antibodies were used: phospho-TYK2, phospho-STAT1, STAT3, STAT5, and STAT6; total-STAT3; phospho-ezrin/radixin/moesin; phospho-paxillin; phospho-actin1; (Cell Signaling, all rabbit, at 1:1000); mouse α-tubulin, 1:1000 (Sigma); phospho-FAK997, 1:1000; JAK3, 1:400; STAT1, 1:1000 to 1:10000 (Biosource International; all rabbit); mouse FAK, 1:1000; rabbit TYK2, 1:500; rabbit JAK2, 1:100; phospho-JAK2, 1:350 (UBI); rabbit JAK1, 1:100; mouse lamin A/C, 1:100 (Santa Cruz Biotechnology); mouse STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6, 1:250; rabbit phospho-STAT4, 1:1000 (Zymed, San Francisco, CA); and mouse paxillin, 1:10000 (BD Transduction).

Cell Culture. SKOV3 cells were maintained in McCoy’s 5A medium containing 2% glutamine, penicillin/streptomycin, and 10% FCS. OVCA3 cells were maintained in RPMI 1640 medium containing 2% glutamine, penicillin/streptomycin, 10% HEPES, 1 mM sodium pyruvate, and 20% FCS. ALTS cells were maintained in DMEM containing penicillin/streptomycin and 15% FCS. All of the cell lines were obtained from American Type Culture Collection (Manassas, VA), except paxillin rescued and paxillin null cells, which were a gift of Sheila Thomas.

RESULTS

STAT3 Phosphorylation Is Associated with Aggressive Epithelial Ovarian Carcinomas. Epithelial ovarian cancer is not a single disease but rather encompasses a heterogeneous group of tumors that exhibit distinct clinicopathologic features. Recent studies have found that STAT3 is constitutively activated in several ovarian carcinoma cell lines and clinical specimens (15, 16). However, it is unclear whether STAT3 activation is associated with the aggressiveness of the clinical behavior of ovarian cancers. Using an antibody specific for the phosphorylated form of STAT3, we compared expression of STAT3 in a tissue microarray comprising core biopsies of ovarian carcinomas, borderline ovarian tumors, benign ovarian cystadenomas, and normal ovary (Fig. 1, A and B). Consistent with Western blot analysis findings of Huang et al., activated STAT3 was not detected in surface epithelial cells of normal ovaries. In cystadenomas, activated STAT3 was detected in <25% of epithelial cells for all of the cases examined. Borderline ovarian tumors have been described as being of low malignant potential and are distinguished from carcinomas by their lack of stromal invasion. No significant
A difference in the degree of STAT3 activation was observed between cystadenomas and borderline tumors (Fig. 1B). In contrast, the percentage of epithelial cells positive for activated STAT3 was significantly higher in carcinomas than in borderline tumors (Fig. 1B). Furthermore, activated STAT3 was present in a significantly greater proportion of cells in aggressive, high-grade carcinomas than in low-grade carcinomas that behave more indolently ($P < 0.005$). No significant differences were observed in STAT3 activation between different histologic subtypes. We confirmed that STAT3 was constitutively activated in five high-grade carcinoma specimens by Western blot analysis (data not shown). Interestingly, the distribution of cells positive for activated STAT3 was heterogeneous as has been observed in various other types of cancers (17). In contrast to the results using phospho-STAT3 antibody, total STAT3 protein, as detected using a phospho-independent antibody, was expressed uniformly in epithelial cells of normal ovaries, cystadenomas, borderline tumors, and carcinomas (Fig. 1A). These findings suggest that activation of STAT3 is associated with the aggressiveness of the clinical behavior of epithelial ovarian cancers.

**Depletion of STAT3 Inhibits Ovarian Cancer Cell Migration.** Because activation of STAT3 correlated with ovarian carcinomas demonstrating stromal invasion and previous evidence implicated STAT3 in cell motility, we investigated whether STAT3 was required for ovarian cancer cell migration. We first examined expression of STAT3 and other JAK/STAT components in two established human ovarian carcinoma cell lines, SKOV3 and OVCAR3. Western blot analysis showed that STAT3 was expressed and constitutively activated in both of these cell lines (Fig. 2A and Supplementary Fig. 1). In addition, we found that STAT1 and STAT4 also were expressed in both lines, whereas neither STAT2 nor STAT6 was detectable in either line (Supplementary Fig. 1). STAT5A was primarily expressed in OVCAR3 cells, whereas STAT5B was primarily in SKOV3 cells. STAT5 was constitutively tyrosine phosphorylated in OVCAR3 cells. All of the four JAK family members were expressed in the ovarian cancer cell lines. JAK2 was constitutively activated in both cell lines, whereas TYK2 was activated primarily in OVCAR3 cells. We were unable to assess the phosphorylation states of JAK1 and JAK3 because of lack of appropriate antibodies.

We assessed chemotaxis of SKOV3 cells toward 2.5% FCS using transwell migration assays (see “Materials and Methods”). We used a pharmacologic inhibitor of JAK2, AG490, to inhibit STAT3 activation (2). Treatment of SKOV3 cells with either 50 or 100 μM AG490 overnight caused a reduction in phospho-STAT3 levels (Fig. 2A). As shown in Fig. 2B, AG490 treatment of SKOV3 cells inhibited their
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migration toward FCS in a 4-h transwell migration assay in a dose-dependent manner. For example, cells pretreated with high levels of the inhibitor for either 4 h or overnight showed dramatically reduced migration compared with untreated cells. These effects are unlikely to be caused by reduced cell survival or proliferation because of the short time course of the migration assay (see “Materials and Methods”).

Because pharmacologic agents can have nonspecific effects, we inhibited STAT3 directly by pretreating SKOV3 cells with siRNAs, using two different STAT3 sequences (see “Materials and Methods”). Equal numbers of cells were harvested from control cultures and from STAT3 siRNA-treated cultures, and the ability of the cells to migrate was evaluated in transwell migration assays. Cells pretreated with STAT3 siRNA showed a specific and dramatic reduction in total STAT3 protein levels (Fig. 2C and Supplementary Fig. 2). Four h after beginning the assay, a dramatic reduction in the migration of the STAT3 siRNA-treated cells was observed compared with untransfected cells (Fig. 2D; $P < 0.0001$ and $P < 0.003$). As a negative control, cells were treated with siRNA for lamin A/C or a scrambled sequence. These treatments caused specific reductions in the targeted proteins (Supplementary Fig. 2) but had only a slight effect on migration (Fig. 2D). Inhibition of STAT5B had a similar mild effect, whereas reduction of STAT1 caused a more significant inhibition of migration, although not to the same extent as STAT3 siRNA treatment ($P = 0.04$; Fig. 2D and Supplementary Fig. 2). Together, these data suggest that, among the STATs, STAT3 is the most significant for SKOV3 ovarian cancer cell migration.

**Activated STAT3 Localizes to Focal Adhesions.** We examined the subcellular localization of STAT3 in SKOV3 and OVCAR3 cells. In cells cultured with 10% FCS, STAT3 localized within the nucleus and at lower levels in the cytoplasm as expected, yet also was detectable at low levels at the cell membrane (Fig. 3A). The STAT3 membrane staining also was evident when cells were plated onto fibronectin-coated coverslips (Fig. 3C–E). In contrast, another nuclear protein, lamin A/C, did not localize to the cell membrane when plated on fibronectin (Fig. 3B). These findings indicate that fibronectin activation may induce STAT3 to localize at the cell membrane.

We examined the localization of activated STAT3 in the ovarian cancer cells and NIH mouse fibroblast cells (3T3) using an antibody that specifically recognizes the tyrosine phosphorylated protein (Fig. 4 and Fig. 5A). Surprisingly, we observed that phospho-STAT3 was not only present in the cell nucleus but also concentrated in focal adhesions (Fig. 4). The focal adhesion staining did not appear to represent nonspecific immunoreactivity with phosphotyrosine residues on other proteins because it was effectively competed using 1 ng/ml of the phospho-STAT3 peptide antigen but not the same concentration of phospho-peptides from FAK, PP2A, or paxillin (Fig. 4, A–C; data not shown). This also is consistent with the finding that the phospho-STAT3 antibody recognized predominately one band in Western blot analyses (Fig. 5A).

We detected colocalization of phospho-STAT3 and several focal adhesion markers, including vinculin, paxillin, and FAK in SKOV3, OVCAR3, and NIH 3T3 cells (Fig. 4, D–F, and Fig. 6; data not shown). Phospho-STAT3 localized to classical focal adhesions and to focal complexes, which may serve as precursors of focal adhesions (18). A second antibody against phospho-STAT3 showed the same staining pattern (data not shown). The focal adhesion localization was observed in unstimulated cells and in cells stimulated with FCS, IL-6, IL-10, or fibronectin (data not shown), consistent with the constitutive activation of the JAK/STAT pathway in these cells. We also detected focal adhesion localization of phospho-JAK2 and phospho-TYK2 in both ovarian cancer cell lines (Fig. 4, G–I). Nuclear staining also was evident using phospho-TYK2 and total TYK2 antibodies. Although these antibodies recognized one major band by

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**Fig. 2.** Inhibition of signal transducer and activator of transcription 3 (STAT3) reduces SKOV3 cell migration. A, Western blot analyses depicting phospho-STAT3 levels (top) and total STAT3 levels (bottom) in SKOV3 cells treated overnight with AG490. B, transwell migration assays using AG490-treated SKOV3 cells showing chemotaxis toward 2.5% FCS. C, Western blot analyses depicting total STAT3 levels (top) and tubulin levels (bottom) in SKOV3 cells transfected 3 days previously, with the listed siRNAs. D, transwell migration assays using siRNA-transfected SKOV3 cells showing chemotaxis toward 2.5% FCS. For A, the ratio of phospho-STAT3 to total STAT3 levels, and for C, the ratio of total STAT3 to tubulin levels were determined by densitometry, and the levels were normalized to the untreated cells. For transwell migration assays in B, the average of three representative experiments is shown, and for D, the average of five representative experiments is shown. SE bars are depicted, and values for which the Student’s t test, $P < 0.05$, are indicated by asterisks.

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**Fig. 3.** Total signal transducer and activator of transcription 3 (STAT3) localizes to the cell membrane. Confocal micrographs of SKOV3 or OVCAR3 cells plated on A, FCS overnight, or B–E, fibronectin for 2 h. Cells were stained with antibodies against A, total STAT3 (green) and vinculin (red); B, lamin A/C (red) and vinculin (green); C, total STAT3 (red); D, total STAT3 (red) and phospho-tyrosine (green); and E, total STAT3 (red). Membrane staining is indicated by filled arrowhead; scale bar, 50 μm.

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**Fig. 4.** Localization of activated STATs to focal adhesions. A, Western blot analyses depicting phospho-STAT1 levels (top) and total STAT1 levels (bottom) in SKOV3 cells treated overnight with AG490. B, transwell migration assays using AG490-treated SKOV3 cells showing chemotaxis toward 2.5% FCS. C, Western blot analyses depicting total STAT3 levels (top) and total STAT5B levels (bottom) in SKOV3 cells transfected 3 days previously, with the listed siRNAs. D, transwell migration assays using siRNA-transfected SKOV3 cells showing chemotaxis toward 2.5% FCS. For A, the ratio of phospho-STAT3 to total STAT3 levels, and for C, the ratio of total STAT3 to total STAT5B levels were determined by densitometry, and the levels were normalized to the untreated cells. For transwell migration assays in B, the average of three representative experiments is shown, and for D, the average of five representative experiments is shown. SE bars are depicted, and values for which the Student’s t test, $P < 0.05$, are indicated by asterisks.
Western blot analysis, additional studies are necessary to determine whether this nuclear staining is significant. Together, our results suggest that components of the JAK/STAT pathway localize to focal adhesions in immortalized fibroblasts and ovarian cancer cells.

**Activated STAT3 Interacts Physically with Phosphorylated Paxillin and FAK.** We also investigated the subcellular localization of phospho-STAT3 by testing for coimmunoprecipitation with other focal adhesion proteins from ovarian cancer cell extracts. We initially used Western blot analysis to confirm that antibodies against total and phosphorylated STAT3 specifically recognized bands at $M_r$ 92,000, the predicted size of STAT3 (Fig. 5A). Using the phospho-STAT3 antibody, we immunoprecipitated all of the detectable activated STAT3 from OVCAR3 cells, which represented only a fraction of the total STAT3 (Fig. 5, B and C). Whereas the phospho-STAT3 peptide antigen specifically competed immunoprecipitation of phospho-STAT3, the phospho-FAK peptide antigen did not (Fig. 5B). As additional controls, we found that no phospho-STAT3, phospho-FAK, or phospho-paxillin coimmunoprecipitated with c-myc or with beads alone (Fig. 5B; data not shown).

We performed coimmunoprecipitations using the phospho-STAT3 antibody and probed these with antibodies against abundant focal adhesion components (Fig. 5C). Phospho-FAK$^{977}$ coimmunoprecipitated with phospho-STAT3. In addition, we detected two tyrosine-phosphorylated isoforms of paxillin, P31 and P118, in this complex (Fig. 5C; data not shown). Interestingly, all of the detectable phospho-paxillin coimmunoprecipitated with phospho-STAT3. In contrast, we did not detect phospho-ezrin/radixin/moesin, a family of membrane-associated proteins, nor tubulin, an abundant protein in these cells, in this complex.

We confirmed the interactions by immunoprecipitating phospho-FAK$^{977}$ and phospho-paxillin$^{31}$. Phospho-STAT3 coimmunoprecipitated with both focal adhesion components (Fig. 5D and Supplementary Fig. 3). A second antibody to phospho-STAT3 also recognized STAT3 and gave similar coimmunoprecipitation results (data not shown; Supplementary Fig. 3). In contrast, little STAT3, FAK, or paxillin coimmunoprecipitated when antibodies that recognize the unphosphorylated forms of the proteins were used (data not shown). This suggests that the STAT3/FAK and STAT3/paxillin interactions were phosphorylation dependent.

Because we found that phospho-STAT3 was in a protein complex with phosphorylated forms of paxillin and FAK, we tested whether phospho-STAT3 depends on these proteins for its localization to focal adhesions. We examined embryonic fibroblasts derived from knockout mice and control littersmates. FAK-deficient fibroblasts are smaller and more rounded than control fibroblasts but have larger focal adhesions (19). We found that in the absence of FAK, phospho-STAT3 still colocalized with paxillin in focal adhesions (Fig. 6, A and B), regardless of whether cells were stimulated with FCS or fibronectin. In addition, the levels of activated STAT3 were unaffected by the absence of FAK (Fig. 7A), indicating that activation and localization of phospho-STAT3 are independent of FAK.

We also examined phospho-STAT3 localization and activation in paxillin null fibroblasts (Fig. 6, C and D, and Fig. 7). In paxillin null cells that were rescued by expression of a transgene encoding wild-type paxillin, activated STAT3 localized to nuclei and focal adhesions. In contrast, in paxillin-deficient cells, we detected a marked reduction in phospho-STAT3 even though the cells still form focal adhesions (20). In addition, phospho-STAT3 localized normally to nuclei and the cytoplasm of paxillin null cells (Fig. 6D). We observed similar results whether cells were stimulated by FCS or by fibronectin. The overall levels of activated STAT3 were normal in paxillin-deficient cells stimulated with FCS as assessed by Western blot analysis (Fig. 7A). However, when the same cells were stimulated with fibronectin, activation of STAT3 was decreased (Fig. 7B). Together, this suggests that paxillin is necessary for STAT3 localization and for its activation in response to fibronectin.

Src family tyrosine kinases also are components of focal adhesions (21). The three members of this family, Src, Yes, and Fyn (SYF), are not essential for the formation of focal adhesions but are required for phosphorylation of other proteins in these complexes, such as FAK.
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In this study, we have provided evidence that activation of STAT3 correlates with increasing aggressive clinical behavior of ovarian cancer cells, that STAT3 activity is required for ovarian cancer cell migration, and that activated STAT3 accumulates in focal adhesions and in the nuclei of cultured cells. Together, these findings suggest that activation of JAK/STAT signaling may contribute to ovarian cancer and may do so by affecting cell motility, in addition to its well-characterized roles in cell survival and proliferation.

A New Localization for JAK/STAT Signaling in Focal Adhesions. The finding that activated STAT3 accumulated in focal adhesions was unexpected but was supported by numerous lines of evidence, including peptide competition and coimmunoprecipitation with known components of focal adhesions, including FAK and paxillin. We observed strong interactions between the tyrosine-phosphorylated forms of STAT3 and FAK and paxillin. However, we did not detect strong binding between unphosphorylated STAT3 and FAK or paxillin, suggesting that tyrosine phosphorylation is required for the interactions. We cannot completely rule out the possibility that the anti-phospho-STAT3 antibodies cross-reacted with other phosphotyrosine-containing epitopes, which may be present at high concentrations in focal adhesions. However, this is unlikely because we did not detect cross-reactivity of the anti-phospho-STAT3 antibody on Western blot analyses, even when other tyrosine-phosphorylated proteins were highly concentrated (e.g., when immunoprecipitations of phospho-FAK or phospho-paxillin were probed). In addition, tyrosine-phosphorylated peptides from paxillin, FAK, or PP2A did not compete the anti-phospho-STAT3 staining as the phospho-STAT3 peptide did. Furthermore, the findings that phospho-STAT3 coimmunoprecipitated with anti-phospho-FAK and anti-phospho-paxillin cannot be explained by cross-reactivity of the anti-phospho-STAT3 antibody. Together, these results suggest that phospho-STAT3 does localize to focal adhesions.

Previous studies have reported transient and relatively weak interactions between JAKs, STATs, and FAK. Fibronectin binding of 293 and A431 cells causes FAK to activate and interact directly with STAT1 (23). The kinase activity of JAK2 also has been shown to promote activation of FAK and paxillin in hematopoietic cells (24). In addition, FAK associates with JAK2 following growth hormone stimulation of mammalian cells (25). These reports provide support for the idea that JAKs and STATs may more generally associate with focal adhesion components. However, because we did not detect direct interactions between STAT3 and FAK, it remains to be seen whether there are direct and indirect interactions between STATs and FAK or whether individual STATs interact directly with different focal adhesion proteins.

The reduction of phospho-STAT3 labeling of focal adhesions in fibroblasts lacking paxillin is interesting because few other focal adhesion components have been reported to mislocalize in paxillin-deficient cells. For example, the localization of vinculin, which binds to paxillin, is not dependent on paxillin (20). Similarly, FAK is mislocalized in only a fraction of paxillin null cells. In contrast, we found that in the absence of paxillin, there was a consistent reduction in phospho-STAT3 localization to focal adhesions. Paxillin null cells have a migration defect (20); therefore, one possibility is that this defect is caused by loss of phospho-STAT3 from focal adhesions. Paxillin is thought to promote cell motility by acting as a scaffold for actin-binding proteins and kinases at the focal adhesions. (26) Although paxillin localization is normal in STAT3-depleted cells (data not shown), STAT3 may be important for transducing signals to proteins downstream of paxillin. STAT3 has been shown to be required for cell motility in a few other contexts. For example, a keratinocyte-specific knockout of STAT3 causes defects in keratinocyte motility and wound healing (10). Therefore, it is possible that migratory cells generally require activated STAT3 at focal adhesions, a possibility that merits additional investigation.

In addition, we found that the Src family of tyrosine kinases, and in particular Src itself, is essential for the localization of phospho-STAT3 to focal adhesions and for its interaction with paxillin.
a particularly interesting finding because three major focal adhesion proteins (FAK, paxillin, and vinculin) localize normally to focal adhesions in cells lacking SYF (22). Because Src also is essential for cell migration, this raises the possibility that STAT3 may be a major downstream target of Src that stimulates cells to move (21). In support of this, we found that Src family proteins were required for normal phosphorylation of STAT3 in response to fibronectin stimulation. In fact, Src family kinase activity is required for STAT3 activation in fibroblasts, melanoma, and breast cancer cells (27–29). In addition, recent evidence has suggested that Src activity is required for activation of STAT3 in SKOV3 cells (30). In contrast, whereas FAK is thought to be important for recruiting other proteins to focal adhesions, the studies with fibroblasts lacking FAK demonstrate clearly that FAK is not essential for the interaction of phospho-STAT3 with paxillin or for phosphorylation of STAT3 (26, 31). This finding is consistent with the observation that loss of FAK does not affect paxillin tyrosine phosphorylation (19). In addition, the results from this study and others suggest that in certain conditions, Src or downstream targets of Src activate STAT3 at the focal adhesions. Because paxillin is a known target of Src and also was required for STAT3 activation, the effect of Src on STAT3 may be mediated in part through paxillin activation (21). However, the loss of phospho-STAT3 from focal adhesions is more pronounced in Src-deficient cells than in paxillin null cells, suggesting that paxillin is not the only relevant target of Src.

In contrast to the fibronectin-stimulated cells, activation of STAT3 was independent of Src and paxillin activity in serum-stimulated cells. In this situation, STAT3 may be phosphorylated by one or more JAKs, such as JAK2 and TYK2, which were present and activated in focal adhesions. The observation that phospho-STAT3 focal adhesion staining was reduced in serum-stimulated paxillin and SYF null cells indicates that there is a tethering function for paxillin and Src kinases in the phospho-STAT3 localization that is independent of a role in STAT3 activation.

We found that depletion of STAT3 by siRNA inhibited SKOV3 cell migration in vitro. This effect could be caused by either loss of the...
nuclear function of STAT3 or loss of a focal adhesion function for STAT3, or a combination of the two. Focal adhesions are thought to promote cell migration by transducing extracellular signals into changes in cell adhesion, the cytoskeleton, and gene expression. FAK and Src have been implicated in regulating focal adhesion turnover and dynamics because null cells have enlarged focal adhesions (19, 32). In addition, paxillin also may promote focal adhesion dynamics because paxillin null cells have shorter focal adhesion structures than control cells (20). However, focal adhesions appear to be intact in cells depleted of STAT (data not shown), suggesting that STAT3 is not required to regulate focal adhesion turnover or assembly.

Thus, there are at least two distinct functions that STAT3 may perform at focal adhesions. STAT3 may have a completely separate function in focal adhesions, unrelated to its well-characterized transcriptional activation function. This would be analogous to Armadillo/β-catenin, which functions as an adapter in E-cadherin-mediated cell adhesion and, independently, as a transcriptional coactivator in Wingless/WNT signaling (33). Similarly, STAT3 may serve as an adapter protein in integrin-mediated cell adhesion. Alternatively, STAT3 could function as a sensor of adhesion, becoming activated in focal adhesions and then translocating to the nucleus to alter gene expression in response to cell adhesion. In contrast to the previous model in which STAT3 might affect cell migration relatively directly, this hypothesis would imply an indirect, transcriptional effect. This function would be analogous to that proposed for zyxin, which also localizes to focal adhesions under steady-state conditions and shuttles to the nucleus (34, 35). Although STAT3 is known to function as a direct transcriptional activator, zyxin is more likely to promote gene transcription by its interactions with other proteins in the nucleus (36). The Y-box transcription factor ZONAB localizes to epithelial tight junctions and in the nucleus, where it regulates gene expression (37). In addition, the membrane-associated guanylate kinase-like protein CASK and the coactivator JAB1 have been proposed to shuttle between the cell membrane and the nucleus (38, 39). Together, this suggests that perhaps STAT3 is a member of a broader class of proteins that translate changes in cell adhesion into changes in gene expression.

In Drosophila epithelial follicle cells where STAT has an essential function in promoting motility, there is clearly a requirement for transcriptional activation because the expression of multiple proteins that are required for border cell migration depends on STAT (11). However, this does not rule out the possibility of a transcription-independent function for STAT in border cell migration as well. There exist hypomorphic stat mutants in which migration is defective even though no alteration in downstream gene expression has been observed (11, 40). Furthermore, a temperature-sensitive allele of STAT appears to cause defective migration almost immediately upon shifting flies to the nonpermissive temperature.4 Such a rapid effect is more consistent with a direct effect on cell adhesion than a transcriptional response.

A Possible Role for STATs in Ovarian Cancer Invasion. It seems likely that cell motility and in particular dynamic regulation of cell adhesion could contribute to the spread of ovarian cancer cells to nearby tissues. We have found activated STAT3 in focal adhesions, structures known to be important in cell motility. In ovarian cancer cells, STAT3 could be activated by constitutively secreted cytokines, such as IL-6, IL-10, and oncostatin M (16, 41–43). In addition, FAK and Src are overexpressed in ovarian cancer cells (44, 45) and are associated with the progression of various human cancers and with promoting cell invasion (46, 47). Together, these results suggest that the elevation in Src and FAK levels may cause in part the constitutive activation of STAT3 and/or its localization to focal adhesions. Alternatively, the constitutive activation of STAT3 in ovarian cancer cells may lead to increased accumulation and/or activity of Src and FAK.

4 Unpublished observations.
Additional studies will be required to distinguish the precise relationships between these molecules in ovarian cancer cells.

Our results indicate that activated STAT3 contributes to ovarian cancer cell motility in vitro and suggest the possibility that this contributes to invasion and possibly metastasis in vivo. In clinical specimens, we found that activation of STAT3 is strongly associated with the aggressive clinical behavior of ovarian carcinomas. In addition, we have shown that signaling through the JAK/STAT pathway is required for normal SKOV3 cell motility. STATs have been implicated in metastasis of other types of cancers. In renal cell cancer, there is a strong correlation of activated STAT3 with aggressive cancers that have metastasized (48). Interestingly, blocking STAT3 in pancreatic cancer cells by expression of a dominant-negative form inhibited tumor growth and liver metastasis in mice, whereas expression of a constitutively dimerized form of STAT3 promoted pancreatic cancer metastasis (49). STAT5 also has been implicated recently in cell motility and in prostate cancer invasion and metastasis (50). Together with the findings reported here, this suggests that STATs may contribute generally to cell motility and tumor progression. Because dynamic regulation of cell adhesion is likely to play an important role in tumor invasion and motility, these studies also provide a possible mechanism as to how STAT5 may contribute to these processes.

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