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Role of c-Src and focal adhesion kinase in progression and metastasis of estrogen receptor-positive breast cancer

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Abstract

The non-receptor tyrosine kinases c-Src and focal adhesion kinase (Fak) mediate signal transduction pathways that regulate cell proliferation, survival, invasion, and metastasis. Here, we investigated whether c-Src and Fak are activated during progression of hormone-dependent breast cancer. Maximally active c-Src was overexpressed in a subset of tamoxifen-resistant variants and in metastases of recurrent hormone-treated breast cancer. Active Fak was also frequently observed in these tumors. We also show that estrogen receptor (ER) can bind to Fak and that estrogen can modulate Fak autophosphorylation supporting a cross-talk between these two pathways. Inhibition of c-Src activity blocked proliferation of all tamoxifen-resistant variants, suggesting that inhibitors of c-Src–Fak activity may delay or prevent progression and metastasis of ER-positive tumors. These studies also raise the possibility that fully active forms of c-Src and Fak in breast tumors may be biomarkers to predict tamoxifen resistance and/or risk of recurrence in ER-positive breast cancer.

Keywords: Estrogen receptor; c-Src tyrosine kinase; Focal adhesion kinase; Tamoxifen resistance; Breast cancer; Metastasis; Estrogen; Tumor biomarker

Hormone-dependent breast cancer is currently treated with therapies that block estrogen receptor-α (ER) function. Commonly used hormonal therapies include the anti-estrogen tamoxifen or, in postmenopausal women, aromatase inhibitors that reduce estrogen production [1]. Unfortunately, many breast cancers recur after surgery and adjuvant therapy. Breast cancer tends to recur at distant sites and becomes practically incurable at this metastatic stage. In this advance stage, many tumors respond to hormonal therapies but, after a period of response, tumors will develop resistance to the particular hormonal therapy used. Various models have been proposed to explain the resistance of breast cancer cells to tamoxifen or other hormonal therapies [2–4]. In particular, different signal transduction pathways such as overexpression of Her-2/neu could lead to decreased responsiveness to hormonal therapies in the advance setting [5]. Understanding the mechanisms that lead to progression and metastatic growth of hormone-dependent breast cancer may provide better ways to increase overall survival rates by preventing or delaying distant recurrence.

ER regulates many different processes by orchestrating a plethora of non-genomic and genomic pathways [6]. ER-dependent proliferation has been shown to require the non-receptor tyrosine kinase c-Src [7–9]. Recent studies suggest that c-Src, or its viral counterpart v-Src, can modulate tamoxifen agonist/antagonist activity [10,11]. This effect of c-Src/v-Src is due to modulation of ER function by upregulation of ER phosphorylation, and, independently, by enhancement of the activity of several transcription coactivators [10,11]. Many receptor tyrosine kinases, known to affect tamoxifen-sensitivity in breast cancer cells,

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can also activate c-Src [12]. Thus, c-Src's role in mediating ER proliferation and affecting tamoxifen response makes this tyrosine kinase a potential mediator of tamoxifen-resistant growth. Moreover, many studies have already linked overexpression and enhanced activity of c-Src to progression and metastasis of different types of cancer [13,14].

In addition to c-Src, the non-receptor tyrosine kinase focal adhesion kinase (Fak) can also mediate signal transduction pathways that modulate proliferation, survival, cell adhesion, migration, and invasion [15–18]. Fak overexpression has recently been associated with aggressive, ER-negative breast cancer [19]. However, overexpression of Fak per se did not predict outcome in node-negative breast cancer [20]. Overexpression of Fak does not necessarily indicate activation of Fak. Fak activity, similar to that of c-Src, can be regulated by multiple factors such as integrins and growth factors [16]. Changes in the extracellular microenvironment exert profound effects on growth of tumor cells at both primary and metastatic sites. In addition to paracrine factors from the stroma or neighboring cells, the extracellular matrix (ECM) affects proliferation and survival of cancer cells. Blocking ECM-mediated signals via integrins can partially reverse the transformed phenotype of MCF-7 cells, an ER-positive breast cancer cell line [21]. Moreover, different integrin ligands can have a profound effect on ER expression and function. For example, laminin-1 or collagen-IV can partially prevent the decrease in ER expression observed upon plating primary mouse mammary epithelial cells on plastic [22]. In breast cancer cells, estrogen-induced proliferation can be decreased by the presence of laminin but not of collagen type I or fibronectin [23]. In addition, cellular localization of ER and ligand-independent ER-mediated transcription can also be stimulated by integrin function [22,24], in a manner that is dependent on c-Src [24]. Therefore, it is possible that activation of c-Src and/or Fak may stimulate tamoxifen-resistant and/or estrogen-independent growth of breast cancer cells. In these studies, we investigated whether the non-receptor tyrosine kinases c-Src and Fak were fully activated during progression and distant relapse of ER-positive breast cancer.

Materials and methods

Cell culture. Tamoxifen-resistant variants derived from ER-positive MCF-7 breast cancer cells were grown in 5% charcoal/dextran-stripped fetal bovine serum (CSS) (Hyclone, Logan, UT) in the presence of 1 μM tamoxifen (Sigma, St. Louis, MO) [25]. All experiments were conducted using uncoated tissue culture plates. Hormonal treatments were performed for three days in the presence of 5% CSS except for tamoxifen-dependent arrest (two-day treatment) [26]. Cells were harvested after a rinse with phosphate-buffered saline (PBS) by either denaturing conditions to assess total levels of expression (1% SDS in 10 mM Tris, pH 7.4, with 1 mM sodium orthovanadate, Figs. 1A and 2A only) or using mPer (Pierce, Rockford, IL) with a cocktail of protease and phosphatase inhibitors [26]. Similar amounts of total protein were loaded in each lane (5–20 μg). Thymidine incorporation in the presence or absence of PP2 (Tocris, Ellisville, MO) was performed as described [26].

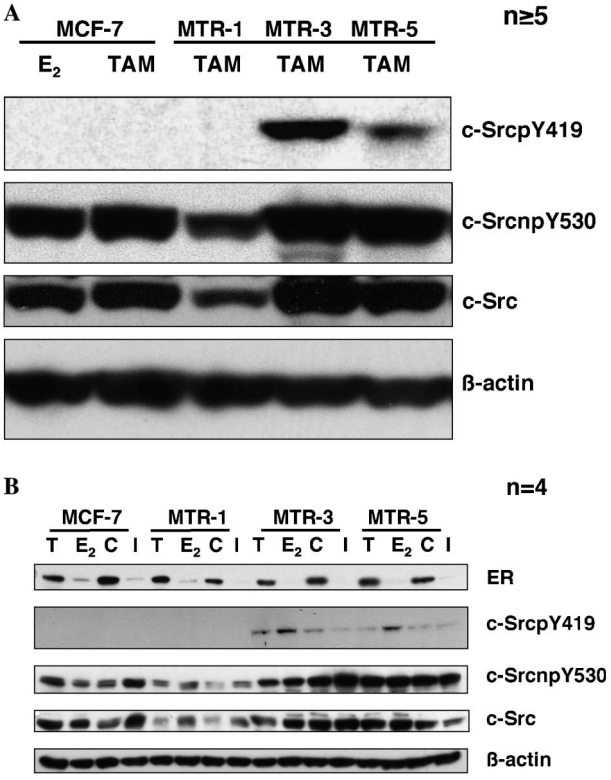

Fig. 1. Expression and hormonal regulation of active c-Src in tamoxifen-resistant variants. (A) Expression of c-Src and its phosphorylated forms in MCF-7 cells and tamoxifen-resistant variants (MCF-7 tamoxifen resistant, MTR-1, MTR-3, and MTR-5) using denaturing conditions. (B) Hormonal regulation of c-Src evaluated using non-denaturing conditions. T, 1 μM tamoxifen; E_2, 5 nM 17β-estradiol; C, control (5% charcoal-stripped fetal bovine serum); I, 100 nM ICI182780.

Antibodies and immunoprecipitation. Phosphospecific antibodies used for immunoblotting were against c-Src (Cell Signaling, Technology, Beverly, MA) and Fak (Biosource International, Camarillo, CA). FakpY576 was also obtained from Santa Cruz Biotechnology (Santa Cruz, CA) while FakpY925 was from Cell Signaling. Antibodies to c-Src, ER, and polyclonal to Fak were from Santa Cruz, a monoclonal to Fak was from Upstate Biotechnology (Lake Placid, NY), and β-actin was from Sigma. Antibodies for immunohistochemistry were against FakpY576, from Biosource; ER (1D5), from Dako (Carpinteria, CA); while c-SrcnpY527 and c-SrcpY416 were from Cell Signaling. Antibodies for immunoprecipitation of ER were from Cell Signaling while for Fak (C-20) and for c-Src and Src family kinases (SRC2) were from Santa Cruz. IgG controls were from Calbiochem (La Jolla, CA). Immunoprecipitations were conducted overnight with equal amounts of protein ($\geqslant$500 μg). Immunoprecipitates were washed 2–3 times with PBS containing 0.1% NP-40 and 2 mM EDTA.

Tissue samples and immunohistochemistry. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded archival tissue. Tissue samples were from breast cancer metastases of patients who had received hormonal therapy after removal of their primary breast cancer (adjuvant hormonal therapy) and, subsequently, developed recurrence at distant sites. Biopsies of their metastatic lesions were performed as part of their standard clinical care. These studies were approved by our Institutional Review Board. Tissue sections (3 μm thick) were subjected to heat-induced antigen retrieval by steaming in 0.01 M sodium citrate buffer, pH 6.0 (for FakpY576 only), or in 1 mM EDTA, pH 8.0 (all other antibodies), for 20 min. Immunostaining was performed on a Dako Autostainer Plus using DakoCytomation EnVision + detection system. Titration of antibodies was done using tissues, such as breast or colon, that were previously

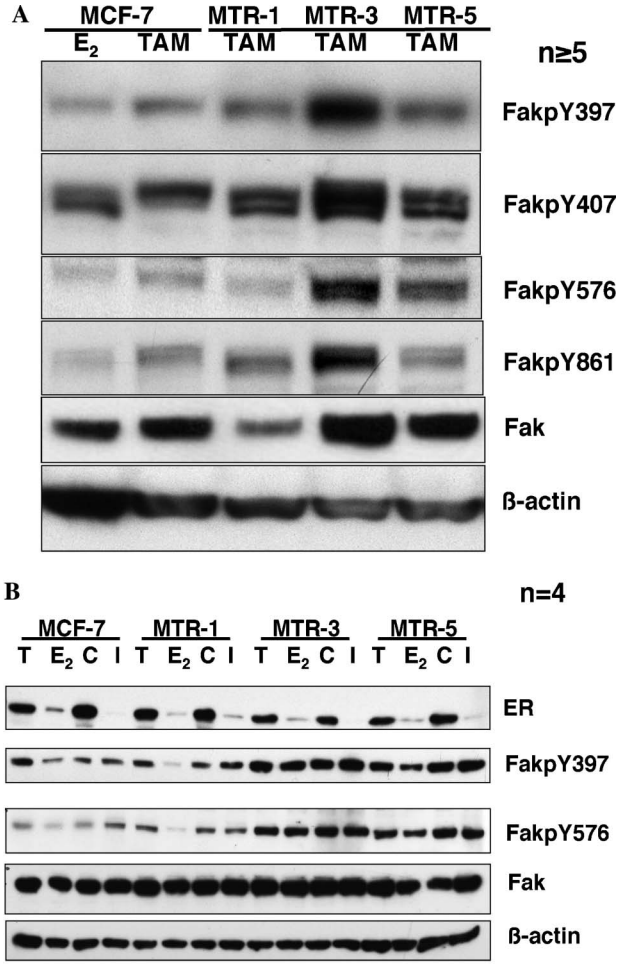

Fig. 2. Fak signaling in tamoxifen-resistant variants. (A) Expression of Fak and its phosphorylated forms using denaturing conditions. FakpY577 and FakpY925 were not detected. (B) Hormonal regulation of Fak tyrosine phosphorylation as described in Fig. 1 evaluated using non-denaturing conditions.

reported to be positive for the specific antibody by immunohistochemistry [20,27].

Statistical analyses. Data were evaluated using unpaired, two-tailed Student's *t* test between PP2 treated and control cells.

Results

Activation of c-Src during acquisition of tamoxifen-resistant growth in cell culture

To study potential mechanisms of tamoxifen-resistant cells, we developed a cell culture model of acquisition of tamoxifen-resistant growth [25]. This cell culture model consisted of three independent tamoxifen-resistant variants obtained from the same parental tamoxifen-sensitive MCF-7 cell line after long-term culture in the presence of tamoxifen [25]. The tamoxifen-resistant variants are called MTR (MCF-7, Tamoxifen-Resistant). Using this cell culture model we determined whether c-Src expression or activity was altered during acquisition of tamoxifen resistance. To assess changes in c-Src function, we determined its state of tyrosine phosphorylation. Phosphorylation at tyrosine-530 locks human c-Src in an inactive conformation. When tyrosine-530 is not phosphorylated, the enzyme is partially active. Maximal c-Src catalytic activity requires phosphorylation at tyrosine-419 in addition to absence of phosphorylation at tyrosine-530. Using phosphospecific antibodies against the active form of c-Src (c-SrcpY419) and denaturing conditions to extract all c-Src, we found that two of three tamoxifen-resistant variants (MTR-3 and MTR-5), but not parental MCF-7 cells, expressed fully active c-Src (Fig. 1A). Levels of the partially active form of c-Src were assessed by using an antibody that detects c-Src only when tyrosine-530 is unphosphorylated. All tamoxifen-resistant variants and parental MCF-7 cells expressed partially active c-Src (c-SrcnpY530) (Fig. 1A). The levels of c-SrcnpY530 correlated well with the levels of total c-Src expression. However, upregulation of fully active c-Src (c-SrcpY419) could not be explained by changes in the levels of c-Src or c-SrcnpY530. These results indicate that overexpression of fully active c-Src occurs frequently, although not always, during acquisition of tamoxifen resistance in cell culture. Thus, enhanced c-Src activity is one potential mechanism leading to tamoxifen-resistant growth.

Treatment of estrogen-deprived MCF-7 with estrogen transiently activates c-Src [7,24,28]. We investigated whether enhanced c-SrcpY419 expression in tamoxifen-resistant variants was dependent on ER function. To block ER function in tamoxifen-resistant cells, we used the ER downregulator ICI 182,780 (ICI). To better compare our results with most studies in the literature, this time we used non-denaturing conditions to harvest cells. Expression of ER was evaluated as control for successful hormonal effects. ER levels, as expected, were downregulated by estrogen and the ER downregulator but not by tamoxifen or estrogen deprivation (Fig. 1B). Using non-denaturing conditions, we again observed overexpression of fully active c-Src in two out of three tamoxifen-resistant cells, suggesting that active c-Src is present in both soluble and non-soluble (cytoskeleton-associated) fractions. Although levels of active c-Src were highest in the presence of estrogen in both tamoxifen-resistant cells, the fact that fully active c-Src (c-SrcpY419) was still detectable in the presence of ICI (Fig. 1B) suggested that activation of c-Src was, at least partially, independent of ER function. Although others have detected increased activity of c-Src in estrogen-treated MCF-7 cells by using kinase assays [24,28,29], our inability to detect c-SrcpY419 in MCF-7 cells after estrogen treatment may be due to the lower sensitivity of our assay (~10 μg of total cell extracts vs. ~1 mg of total cell extract for immunoprecipitation followed by kinase assay). These data suggest that, in parental MCF-7 cells, only a small pool of c-Src is activated by addition of estrogen. Nevertheless, our results argue that two out of three tamoxifen-resistant variants overexpress maximally active c-Src and that estrogen treatment can further increase the total levels of active c-Src. Thus, upregulation

of active c-Src in tamoxifen-resistant variants may be the result of ER-dependent and -independent mechanisms.

Enhanced Fak signaling during acquisition of tamoxifen-resistant growth in cell culture

Many different signaling pathways can activate c-Src [14]. As integrin function can regulate c-Src via Fak, we investigated whether changes in Fak signaling were present in tamoxifen-resistant cells overexpressing active c-Src. Fak can bind and regulate c-Src activity when tyrosine-397 of Fak is phosphorylated (FakpY397). FakpY397, the autophosphorylation site of Fak, is upregulated by integrin signaling. However, maximal catalytic activity of Fak, in a way similar to c-SrcpY419, may correlate better with phosphorylation of tyrosine-576 (FakpY576), a site found in the activation loop [30]. We first determined the levels of Fak tyrosine phosphorylation in parental cells and tamoxifen-resistant variants using phosphospecific antibodies and denaturing conditions. Enhanced levels of active Fak were detected in both tamoxifen-resistant variants with active c-Src (Fig. 2A). Thus, these results support the idea that c-Src-Fak activation is a common, although not unique, mechanism leading to tamoxifen resistance.

As estrogen can decrease tyrosine phosphorylation of Fak [31], we investigated whether enhanced levels of FakpY397 and FakpY576 were dependent on ER function. Even though estrogen could decrease the levels of Fak tyrosine autophosphorylation and of FakpY576, both tamoxifen-resistant variants with active c-Src (MTR-3 and MTR-5) consistently showed enhanced tyrosine phosphorylation of Fak when compared to parental cells treated in a similar way (Fig. 2B). Therefore, Fak signaling, as judged by phosphorylation of tyrosine-397 and tyrosine-576, is enhanced in tamoxifen-resistant variants independently of ER function. Moreover, the ability of estrogen to downregulate active Fak indicates that ER function can modulate integrin signaling and, by simultaneously activating c-Src, estrogen, perhaps, may increase the turnover of focal adhesions. The high levels of active Fak in ICI-treated cells contrast with the lower levels of active c-Src observed in Fig. 1B for MTR-3 and MTR-5 cells. Possibly, as has been shown recently in colon cancer cells by using c-Src kinase deficient mutants [32], c-Src kinase-dependent and -independent mechanisms regulate FakpY576 phosphorylation. Nevertheless, increased levels of active Fak and c-Src during acquisition of tamoxifen resistance could explain the enhanced motility and invasion of tamoxifen-resistant cells [33]. Thus, enhanced tyrosine phosphorylation of Fak is most likely driven by ER-independent mechanisms.

Association of Fak with ER in breast cancer cells

As integrin signaling can modulate ER function [22–24], and ER activation by estrogen can modulate Fak, we explored the possibility that ER interacts with Fak. Several signaling molecules that interact with Fak can also interact with ER and mediate non-genomic functions of ER [6,34]. We, therefore, tested whether ER binds Fak. ER-immunoprecipitates from tamoxifen-arrested cells and short-term estrogen-treated cells contained Fak (Fig. 3A). The ligand-independent interaction of ER with Fak is reminiscent of a recently described association of ER with the focal adhesion protein vinexin [35]. We also tested whether ER and Fak associate in asynchronously growing tamoxifen-resistant variants. In all tamoxifen-resistant variants, Fak was found in ER immunoprecipitates (Fig. 3B). Similar levels of ER/Fak complexes were observed in parental cells treated for three days with estrogen or tamoxifen, despite lower levels of total ER in estrogen-treated cells. These results may indicate that the pool of ER complexed to Fak is not subjected to downregulation after

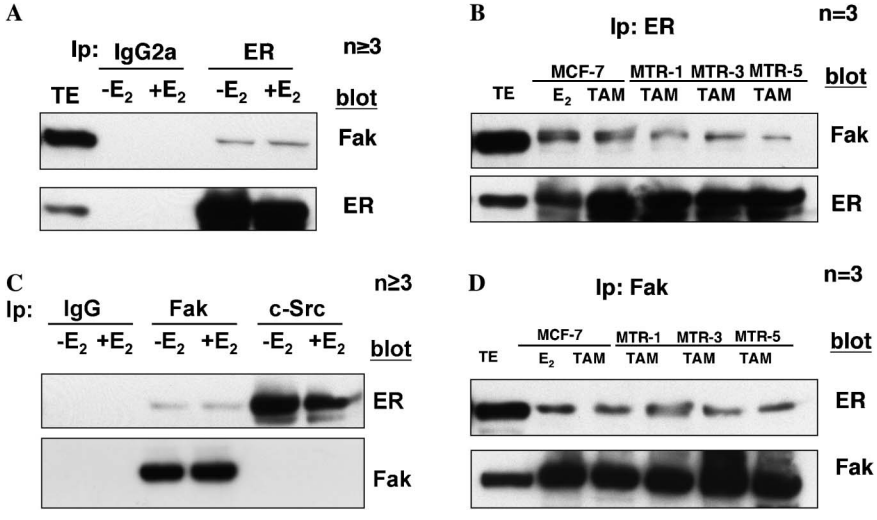

Fig. 3. Interaction of ER with Fak in breast cancer cells. (A) Immunoprecipitation of endogenous ER from tamoxifen-arrested cells after addition of 0.1% ethanol ($-E_2$) or 5 nM 17β-estradiol ($+E_2$) in 0.1% ethanol for 10 min. TE, total extract. (B) ER immunoprecipitation from MCF-7 and MTR cells growing asynchronously. (C) Immunoprecipitation of endogenous Fak and Src family tyrosine kinases from tamoxifen-arrested cells in the presence or absence of 17β-estradiol as in (A). (D) Fak immunoprecipitation from MCF-7 cells and tamoxifen-resistant variants as in (B).

estrogen-treatment as seen for nuclear ER [36]. Lack of downregulation of ER after estrogen binding has been observed using forms of ER targeted to the membrane that cannot go to nuclei or induce gene transcription [37]. Association of ER with Fak was also detected by immunoprecipitation of Fak (Fig. 3C). However, levels of ER in Fak complexes were lower than those in c-Src immunoprecipitates. As Fak was not detected in c-Src immunoprecipitates, our data suggest that ER/Fak complexes may be a small subset of ER/c–Src complexes or can be formed independently of c-Src. Again, similar complexes between Fak and ER could be detected in all tamoxifen-resistant variants (Fig. 3D). The association between Fak and ER may explain ECM effects in ER-dependent transcription and proliferation, estrogen's effect in Fak phosphorylation and, most likely, the controversial effects of ER in cell motility [24,38,39]. Thus, binding of ER to Fak argues for cross-talk between ECM and ER and may explain some of the non-genomic effects of estrogen.

Proliferation of tamoxifen-resistant breast cancer cells is blocked by inhibiting the activity of Src family tyrosine kinases

ER-dependent proliferation in tamoxifen-sensitive cells requires c-Src [7]. To evaluate whether c-Src activity is required for proliferation of tamoxifen-resistant cells, we blocked c-Src function using a compound, PP2 (Tocris), that selectively inhibits c-Src and Src family tyrosine kinases [40]. In cellular assays, this compound can inhibit cell proliferation of fresh human peripheral blood lymphocytes with an IC_{50} that depends on the stimuli used for inducing proliferation (range between 0.6 and 18 μM) [40]. Considering that tamoxifen-resistant cells are grown in the presence of serum and overexpress active c-Src, we used 10 μM PP2. A similar dose of a related compound (PP1) was previously shown to successfully block steroid-induced non-genomic signaling in breast cancer cells [41]. All tamoxifen-resistant variants were growth inhibited by PP2 as judged by inhibition of thymidine incorporation, a marker for progression through the S-phase of the cell cycle (Fig. 4A). Comparison of all tamoxifen-resistant variants indicated that c-Src or Src family kinase activity was required for proliferation of all tamoxifen-resistant cells (Fig. 4B). The fact that MTR-1 was also inhibited, in spite of not overexpressing maximally active c-Src, suggests that all tamoxifen-resistant cells, as parental cells do [7], depend on Src family tyrosine kinases for growth. These results provide a rationale for blocking c-Src and related tyrosine kinases to prevent tamoxifen-resistant growth of ER-positive breast tumors.

Active c-Src and Fak are frequently found at the metastatic site upon relapse of hormone-treated early breast cancer

In an experimental model of breast cancer metastasis, Fak is required for the early stages of metastasis [42].

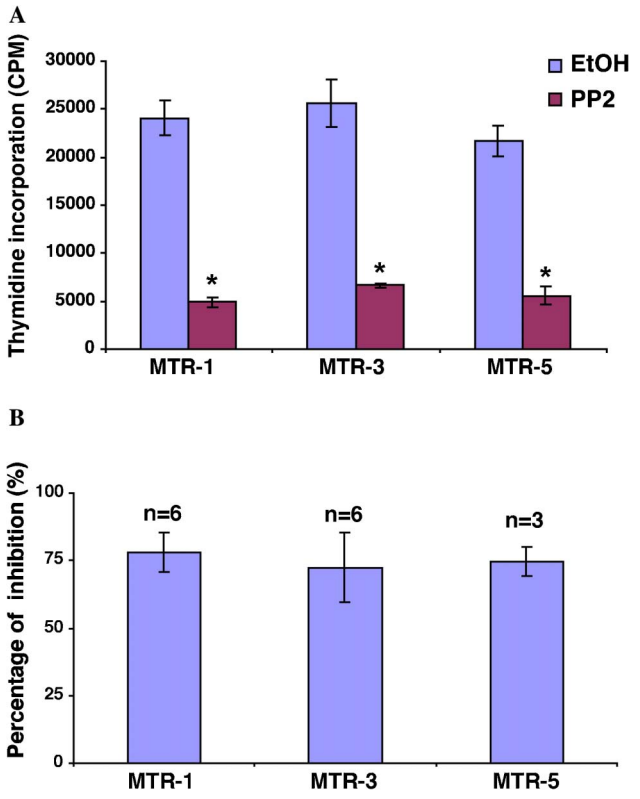

Fig. 4. Role of Src family tyrosine kinases in proliferation of tamoxifen-resistant variants. (A) Changes in thymidine incorporation after treatment with 10 μM PP2 (Tocris), a specific tyrosine kinase inhibitor for Src family tyrosine kinases, for 24 h. Error bars represent standard deviation from triplicate wells. $^{*}P < 0.001$. (B) Average values for inhibition of cell cycle progression by 10 μM PP2 after 24 h of treatment. Error bars represent the standard deviation from the indicated independent experiments.

If breast tumors that recur at distant sites also need maximal c-Src and Fak catalytic activity for growth at the metastatic site, we should be able to identify fully active forms of c-Src and Fak in a subset of breast cancer samples obtained after distance recurrence of ER-positive breast cancer. To determine whether fully active forms of c-Src and Fak were present after breast cancer progression in clinical settings, we identified breast cancer metastases from patients who received either tamoxifen (19/21) or other hormonal therapies (2/21) as their adjuvant therapy and had experienced relapse. All the tissue biopsies from distant recurrences were stained for ER, c-SrcnpY530, c-SrcpY419, and FakpY576. Tumors were assigned a score for each staining: 0 (no staining), 1+ (low), 2+ (moderate), or 3+ (strong). All scored samples were positive for ER whereas all except one were positive for c-SrcnpY530. Over 50% of cases (11/21) scored 2+ or 3+ for c-SrcpY419, whereas over two-thirds (15/19) of breast cancer metastases had similar scoring for FakpY576. High levels of c-SrcpY419 and FakpY576 were also found in a case of recurrent breast cancer after therapy with second-generation aromatase inhibitors, suggesting that activation of c-Src–Fak is

not specific to acquisition of resistance to tamoxifen. Interestingly, all metastases with strong staining (3+) for active Fak were from patients under age 50, a finding that may reflect the more aggressive nature of ER-positive breast cancer in younger women.

Different signaling pathways regulated via integrins, growth factor receptors, G-protein-coupled receptors, and cell–cell adhesion molecules activate both Fak and c-Src. Besides cytoplasmic staining, we observed a case with active c-Src and Fak at areas of cell-matrix contact (Figs. 5A and B) supporting the role of the ECM/stroma in c-Src–Fak signaling. Nevertheless, staining at cell–cell borders was more common, suggesting that growth factor receptors and/or cell–cell adhesion molecules may be activating c-Src–Fak (Fig. 5C). In addition, several breast cancer metastases had distinctive nuclear staining for active Fak (Fig. 5D). The presence of active Fak in the nuclei supports the observations that certain forms of Fak translocate to the nuclei [43]. Varied cellular localization of active molecules supports the concept that more than one mechanism could lead to activation of c-Src and Fak in metastatic breast cancer. Our results argue that activation of c-Src and Fak is a frequent event in distant recurrences of hormone-treated ER-positive breast cancer. In addition, high levels of c-SrcpY419 and/or Fakpy576 or their specific localization in primary breast cancer may be potential biomarkers to predict tamoxifen-resistance and/or early recurrence of ER-positive breast cancer.

Discussion

These studies provide evidence for enhanced c-Src and Fak signaling during progression and distant recurrence of ER-positive breast cancer. These results support observations that progression of hormone-dependent breast cancer in experimental models is associated with an increased migratory and metastatic capacity of tumor cells [33,44]. Interestingly, other molecules regulated by c-Src and Fak that mediate motility, such as p130Cas; or activate c-Src, such as HER-2/neu, have already been associated with tamoxifen resistance and high risk of recurrence of primary breast cancer [45,46]. However, increased expression of p130Cas was not observed in breast tumors that acquired resistance to tamoxifen [47]. Our findings offer insights into one potential mechanism leading to metastatic progression of hormone-dependent breast tumors. We propose a model (Fig. 6) in which multiple signals emanating from either growth factors; ECM; chemokines, such as stromal-derived factor-1 (SDF-1); or cell–cell adhesion molecules can increase c-Src and Fak activity. These ER-independent increases in c-Src–Fak signaling can lead to ligand-independent tamoxifen-resistant proliferation and, simultaneously, promote motility, invasion, and metastatic growth of ER-positive breast cancer cells resulting in distant recurrence.

Physical association of ER with Fak argues for a cross-talk between ER and possibly other steroid receptors [48] with several signaling pathways via Fak and c-Src.

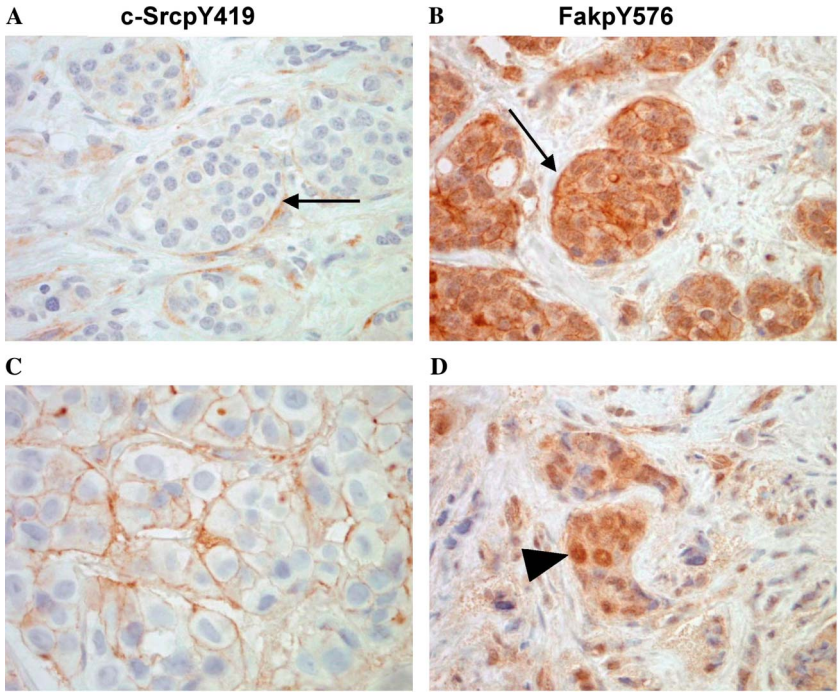

Fig. 5. Expression of active c-Src and Fak in metastases of recurrent ER-positive breast cancer. (A) Expression of active c-Src in bone metastasis showing case with staining at cell-matrix junction (arrow). (B) Same bone metastasis as in (A) showing active Fak at cell–matrix junction (arrow) and in the cytoplasm. (C) Skin metastasis with active c-Src present at cell-cell borders. (D) Another bone metastasis showing fully active Fak present in nuclei of some cells (arrowhead).

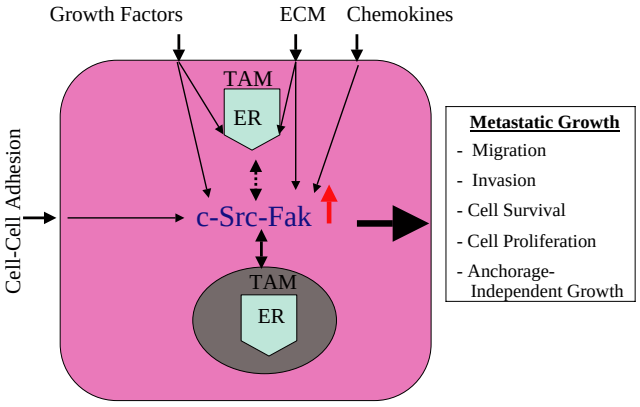

Fig. 6. Cross-talk among ER, c-Src, and Fak in ER-positive breast cancer. ER can regulate c-Src and Fak activity while c-Src–Fak signaling can affect ER function. Multiple ER-independent pathways can activate c-Src–Fak signaling leading to ligand-independent ER function and tamoxifen-resistant growth. At the same time, increased c-Src–Fak signaling promotes motility, invasion, and metastatic growth of ER-positive breast cancer cells. Therefore, combining hormonal therapies with drugs that inhibit c-Src–Fak signaling may prevent or delay progression and metastasis of ER-positive breast cancer.

Recently, p130Cas was found to bind ER transiently in an estrogen-dependent manner. Thus, the association of ER with the c-Src–Fak–p130Cas signaling pathway in breast cancer cells provides a framework to allow coordination between hormones and ECM in proliferation, survival, and migration of breast cancer cells at the primary or metastatic site [21]. As Fak regulates many different signaling pathways, ER binding to Fak may also explain the ability of estrogen to modulate different signaling transduction pathways in a cell context-dependent manner.

Inhibiting c-Src–Fak signaling may prevent or delay metastasis [15] and acquisition of tamoxifen-resistance. It may also reduce the growth-promoting effects of tamoxifen in the uterus [11]. Activation of c-Src can promote bone and lung metastasis in an animal model of breast cancer [49]. Therefore, combining hormonal therapy with inhibition of c-Src–Fak activity may not only decrease recurrences in the adjuvant setting but may extend overall survival in the advance stages of breast cancer by inhibiting the growth of breast cancer cells at the metastatic site. In support of this idea, a recent study has shown that blocking c-Src activity in tamoxifen-resistant cells affects IGF-1R cross-talk with EGF-R and EGF-R signaling [50].

Although our studies were done using only biopsies from breast cancer after relapse, it will be interesting in the future to determine the role of active c-Src and Fak in the response to hormonal therapy in early or advanced breast cancer. It is possible that in early stage ER-positive breast tumors, the presence of high levels of fully active forms of c-Src (c-SrcpY419) and Fak (FakpY576), or their specific cellular localization, may represent biomarkers of a more aggressive, hormone-resistant ER-positive breast cancer with a high risk of recurrence. A study using antibodies against c-SrcnpY530 has already shown the frequent presence of this partially activated form of c-Src in hormone-dependent breast cancer [51]. Moreover, a recent study that could not show an impact of Fak overexpression in node-negative breast cancer on clinical outcome, detected fully active c-Src in 25 out of 140 primary breast tumors [20]. Therefore, fully activated c-Src is present in a subset of early stage breast tumors. Surprisingly, c-SrcpY419 did not correlate with Her-2/neu status as not all tumors overexpressing Her-2/neu showed fully active c-Src. Thus, it will be interesting to evaluate whether coexpression of Her-2/neu and c-SrcpY419 better predicts resistance to tamoxifen than expression of Her-2/neu alone. Nevertheless, inhibition of c-Src with PP2 can reduce Her-2/neu mediated invasion in vitro and metastasis in an animal model [52]. In addition, the growth inhibitory effect of gefinitib, a selective EGF-R tyrosine kinase inhibitor, in ER-positive breast cancer cells may also depend on its ability to reduce c-Src activity [50,53]. Thus, it is possible that the effect of different signal transduction inhibitors on c-Src–Fak activity may better predict their clinical efficacy in the treatment of ER-positive breast tumors.

In summary, we have identified enhanced c-Src and Fak signaling during acquisition of tamoxifen-resistant growth in cell culture and upon distant relapse of hormone-treated breast cancer. Activation of c-Src–Fak in tamoxifen-resistant variants suggests that these signaling pathways can overcome the inhibition of cell proliferation and survival mediated by tamoxifen in cell culture. The high frequency of active c-Src and Fak expression in distant recurrences suggest that these pathways are mediating progression of hormone-dependent breast cancer leading to invasion and metastatic growth. Therefore, drugs that block c-Src–Fak signaling may cooperate with hormonal therapy to prevent or decrease growth at the metastatic site.

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