Phosphoproteomic Analysis Identifies Focal Adhesion Kinase 2 (FAK2) as a Potential Therapeutic Target for Tamoxifen Resistance in Breast Cancer*

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Tamoxifen, an estrogen receptor-α (ER) antagonist, is an important agent for the treatment of breast cancer. However, this therapy is complicated by the fact that a substantial number of patients exhibit either de novo or acquired resistance. To characterize the signaling mechanisms underlying this resistance, we treated the MCF7 breast cancer cell line with tamoxifen for over six months and showed that this cell line acquired resistance to tamoxifen in vitro and in vivo. We performed SILAC-based quantitative phosphoproteomic profiling on the tamoxifen resistant and vehicle-treated sensitive cell lines to quantify the phosphorylation alterations associated with tamoxifen resistance. From >5600 unique phosphopeptides identified, 1929 peptides exhibited hyperphosphorylation and 409 peptides showed hypophosphorylation in the tamoxifen resistant cells. Gene set enrichment analysis revealed that focal adhesion pathway was one of the most enriched signaling pathways activated in tamoxifen resistant cells. Significantly, we showed that the focal adhesion kinase FAK2 was not only hyperphosphorylated but also transcriptionally upregulated in tamoxifen resistant cells. FAK2 suppression by specific siRNA knockdown or a small molecule inhibitor repressed cellular proliferation in vitro and tumor formation in vivo. More importantly, our survival analysis revealed that high expression of FAK2 is significantly associated with shorter metastasis-free survival in estrogen receptor-positive breast cancer patients treated with tamoxifen. Our studies suggest that FAK2 is a potential therapeutic target for the management of hormone-refractory breast cancers. Molecular & Cellular Proteomics 14: 10.1074/mcp.M115.050484, 2887–2900, 2015.

Approximately 70% of all breast tumors express estrogen receptor (ER) and are classified as estrogen receptor-alpha positive (ER+) breast cancers (1). Activation of ER by its ligand estrogen (E2), plays an essential role not only in regulating normal mammary gland development but also in the progression of hormone dependent breast cancer (2). Tamoxifen, a selective ER modulator (SERM), competes with E2 binding on ER and induces conformational changes leading to inactivation of ER (3, 4). Tamoxifen has been used for the treatment and prevention of breast cancer for more than three decades (5, 6). However, up to 40% of patients receiving tamoxifen adjuvant therapy develop recurrent disease within 5 years (7, 8). This resistance to tamoxifen and other endocrine therapy remains a major challenge in breast cancer management.

During the past two decades, a large battery of studies has been carried out to explore the mechanisms underlying resistance to endocrine therapy. One important mechanism for the development of resistance is a shift of tumor cells from growth dependent on estrogenic steroids to growth driven by growth factor signaling pathways which is independent of estrogenic steroids (9, 10). For example, activation of receptor tyrosine kinases, such as EGFR, HER2 and IGF-1R (11–15) and their downstream signaling pathways including PI3K/AKT and MAPK pathways have been linked to endocrine therapy resistance (16–18). However, early clinical trials combining...
tyrosine kinase inhibitors (TKIs), farnesyltransferase (RAS) inhibitor, or mammalian target of rapamycin (mTOR) inhibitors with endocrine therapy have been disappointing (19, 20). Therefore, there is an urgent need to further delineate the molecular mechanisms of endocrine resistance to identify novel therapeutic targets.

Recent advances in mass spectrometry have enabled researchers to identify and quantify thousands of proteins and phosphorylated peptides from in vitro and in vivo models. However, in contrast to a large number of genomic and transcriptomic studies investigating the mutational and gene expression changes involved in endocrine resistance, only a handful of proteomic-based studies have been reported (21–26) and only two of these studied the phosphoproteomic alterations in endocrine resistant cells (21, 22). In the current study, we employed stable isotope labeling by amino acids in cell culture (SILAC)-based quantitative mass spectrometry approaches to identify the signaling pathways activated in tamoxifen resistant breast cancer cells derived from the ER+ MCF7 breast cancer cell line with long-term (>6 months) tamoxifen treatment. In order to comprehensively profile the phosphoproteome of the tamoxifen resistant cells, we employed two phosphopeptide enrichment approaches: anti-phosphotyrosine antibody to capture tyrosine phosphorylated peptides and TiO2 beads to enrich for serine/threonine phosphorylated peptides prior to LC-MS/MS analysis. Our study identified and quantified 5640 unique phosphopeptides corresponding to 2189 proteins, thereby generating the largest quantitative phosphoproteomic data set to date for tamoxifen resistant breast cancer cells. This enabled us to investigate the signaling mechanisms of tamoxifen resistance in a much more comprehensive manner compared with other published studies. We identified multiple signaling pathways activated in tamoxifen resistant cells with the focal adhesion pathway being one of the most enriched pathways. More importantly, we discovered the non-receptor tyrosine kinase, PTK2B (more widely known as FAK2 or PYK2) to be hyperphosphorylated and up-regulated in cells with tamoxifen resistance. Suppression of FAK2 with siRNA knockdown or a small molecule pharmacological inhibitor significantly inhibits the resistant cell proliferation and tumor formation in a xenograft mouse model. Thus our study demonstrates the potential of FAK2 as a novel therapeutic target for the treatment of endocrine resistant breast cancer.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Establishment of Tamoxifen Resistant Cells—** MCF7 was purchased from American Type Culture Collection (ATCC, Manassas, VA). To establish the tamoxifen resistant cell line (MCF7-TamR), MCF7 cells were grown in RPMI 1640 medium with 5% FBS and 1 μM tamoxifen (Sigma, St. Louis, MO) for more than 6 months. MCF7 control cells (MCF7-CTRL) were cultured in RPMI 1640 medium supplemented with 5% FBS and 0.1% ethanol as vehicle. In order to label cells with stable isotopic amino acids, MCF7-CTRL and MCF7-TamR cells were propagated in RPMI 1640 SILAC media (Thermo Fisher Scientific, Waltham, MA) with 5% FBS supplemented with light lysine (K) and arginine (R) for light and 13C6-15N2-K and 13C6-15N2-R for heavy state labeling (Cambridge Isotope Laboratories, Tewksbury, MA). The labeling efficiency was confirmed by mass spectrometry analysis.

**Immunoblotting and siRNA Knockdown—** Cells were harvested and lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, and 1 mM sodium orthovanadate in the presence of protease inhibitors). Whole-cell protein extracts were denatured and separated in NuPAGE gels (Invitrogen, Grant Island, NY), transferred to nitrocellulose membranes, and probed with primary antibody followed by horseradish peroxidase-conjugated secondary antibodies. Primary antibodies used were pFAK1-Tyr576/577 (3281), FAK1 (4332), pFAK2-Tyr402 (3291), FAK2 (3480S), pPaxillin-Tyr118 (2541S), Paxillin (2542S), Claudin-1 (12355), E-Cadherin (3195), N-Cadherin (13116), Slug (9585), Snail (3879), TCF8/VEB1 (3396), Vimentin (5741), ZO-1 (8193) and β-Catenin (8480) purchased from Cell Signaling Technology (Danvers, MA), β-ACTIN (AS516, Sigma, St. Louis, MO) and 4G10 anti-phosphotyrosine antibody (Millipore, Belerica, MA). 50 nM siRNA were used for transfections with RNAiMax (Invitrogen, Grant Island, NY). Cells were harvested 48 h post transfection for assessing knockdown efficiency or other follow-up experiments.

**In-solution Trypsin Digestion—** Cell lysates were prepared in urea lysis buffer containing 20 mM HEPES pH 8.0, 9 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate and 5 mM sodium fluoride. Protein estimation was carried out using BCA protein assays. Equal amount of protein from heavy labeled MCF7-CTRL and light labeled MCF7-TamR cell lysates was mixed, reduced with 5 mM dithiothreitol and alkylated with 10 mM iodoacetamide. Lysates were then diluted to less than 2 M urea final concentration using 20 mM HEPES (pH 8.0) and in-solution digestion was carried out using TPCK-treated trypsin. The tryptic peptides were desalted using C18 reverse phase column (Waters, Milford, MA) and eluted peptides were lyophilized and subjected to phosphopeptide enrichment.

**Immunofluorescence Purification of Phosphotyrosine Peptides—** The phosphotyrosine peptide enrichment was performed according to the manufacturer’s protocol (Cell Signaling Technology). Briefly, 250 μg anti-phosphotyrosine antibody (pY100, Cell Signaling Technology) was used to immunoprecipitate (IP) tyrosine phosphorylated peptides in IP buffer containing 50 mM MOPS pH 7.2, 10 mM sodium phosphate, 50 mM NaCl. The enriched phosphopeptides were eluted using 0.1% TFA, and the eluted phosphopeptides were desalted using C18 STAGE tips, vacuum dried and kept at ~80 °C before liquid chromatography-mass spectrometry (LC-MS) analysis.

**TiO2-based Phosphopeptide Enrichment—** Peptides were fractionated by strong cation exchange (SCX) chromatography as described earlier (27). Briefly, 10 μg of lyophilized peptides mixture was resuspended in 1 ml of SCX solvent A (5 mM KH2PO4, pH 2.7, 30% ACN) and separated on a PolySULPHOETHYL A column (5 μm, 200 Å, 200 × 9.4 mm; PolyLC Inc., Columbia, MD) with an increasing gradient of SCX solvent B (5 mM KH2PO4, pH 2.7, 30% ACN, 350 mM KCl) on an Agilent 1100 HPLC system. In total, 15 fractions were collected. Each fraction was subjected to TiO2-based phosphopeptide enrichment as described earlier (28). Briefly, each fraction was re-suspended in DHB solution (80% ACN, 1% TFA, 3% 2,5-dihydroxybenzoic acid (DHB), and incubated with TiO2 beads for 2 h. Phosphopeptide-bound TiO2 beads were sequentially washed with DHB solution followed by 80% ACN in 1% TFA). Peptides were eluted with 40 μl of 2% ammonia into 10 μl of 2% TFA.

**Liquid Chromatography Tandem Mass Spectrometry—** Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of
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RESULTS

Establishment and Characterization of Tamoxifen-resistant MCF7 Breast Cancer Cells—In order to identify phosphorylation regulated signaling changes in tamoxifen resistant breast cancers, we first generated tamoxifen resistant MCF7 breast cancer cells (MCF7-TamR) with long-term treatment of 1 μM tamoxifen for more than 6 months. In parallel, another set of MCF7 cells were treated with 0.1% ethanol as vehicle control cells (MCF7-CTRL). To assess the tamoxifen resistance developed in MCF7-TamR cells, we performed proliferation assays using the MCF7-TamR and MCF7-CTRL cells with treatment of different concentrations of tamoxifen. We found that MCF7-TamR cells grew ~25% slower than MCF7-CTRL cells in the absence of tamoxifen treatment (Fig. 1A). However, inhibition induced by tamoxifen was dramatically attenuated in MCF7-TamR cells compared with MCF7-CTRL cells (Fig. 1A). We then subcutaneously transplanted MCF7-TamR cells and MCF7-CTRL cells into immunocompromised mice with the supplement of E2 pellets or E2 with tamoxifen pellets. We

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demonstrated that MCF7-TamR cells exhibited resistance to tamoxifen in vivo and formed significantly larger tumors than MCF7-sensitive control (MCF7-CTRL) cell line. Thus we show that we have established a tamoxifen-resistant cell line that appears to reflect hormone resistance acquired by tumors in patients treated with tamoxifen.

Phosphoproteomic Analysis of Tamoxifen Resistant MCF7 Cells—Deregulation of kinase-mediated protein phosphorylation signaling pathways has been demonstrated to be involved in tumor progression and resistance to therapy (31). Given the central role of protein kinases in cell signaling networks, phosphoproteomic profiling is an ideal approach to identify activated kinase pathways and to discover therapeutic candidates for tamoxifen resistant breast cancers. To survey the phosphoproteome alterations in tamoxifen resistant cells, we first compared protein tyrosine phosphorylation levels of MCF7-CTRL with MCF7-TamR cells using anti-phosphotyrosine (anti-pTyr) antibody. A, Inhibitory effect of different concentrations of tamoxifen on the proliferation of MCF7 tamoxifen-resistant (MCF7-TamR) cell line and MCF7 sensitive control (MCF7-CTRL) cell line. Cell proliferation was assessed using MTT assay after 7 days of treatment. Quantification was done relative to vehicle-treated MCF7-CTRL. Student’s t test was performed for statistical analysis. B, Tumor size of xenograft on mice grown with either MCF7-TamR or MCF7-CTRL cell lines with estrogen (E2) supplementation and treated with or without tamoxifen (TAM). Two-way Anova test was performed for statistical analysis. C, Immunoblot of tyrosine phosphorylated proteins using the anti-phosphotyrosine antibody (pTyr) on the cell lysates or after immunoprecipitation (IP) with pTyr antibody. β-actin, heavy (H) chain and light (L) chain serve as loading control.

![Image](image_url)
and light-labeled MCF7-TamR cells were lysed, mixed and digested with trypsin. In order to interrogate the observed elevation in tyrosine phosphorylation in MCF7-TamR cells, we performed antibody-based phosphotyrosine peptide enrichment. In addition, we also employed the TiO$_2$-beads enrichment method to enrich for phosphoserine/threonine peptides in order to obtain the global phosphorylation profiles for both the resistant and sensitive cell lines. We performed biological replicate experiments to increase the reliability of our phosphoproteomic analyses. The mass spectrometry data were processed and searched against databases using SEQUEST-HT algorithms through the Proteome Discoverer platform. Using a false discovery rate (FDR) cutoff of 1%, the Percolator algorithm generated 23,798 phosphopeptide spectrum-matches. The probability of phosphorylation for each Ser/Thr/Tyr site on each peptide was calculated by the phosphoRS algorithm. Overall, we identified 5640 unique phosphopeptides derived from 2189 proteins. 477 of these were tyrosine phosphorylated peptides derived from 329 proteins, and $\sim$90% (427) of them were identified through the antibody based phosphotyrosine peptide enrichment. In addition, we identified 4723 peptides with serine phosphorylation and 599 with threonine phosphorylation (Fig. 3A). Most of the phosphopeptides were singly or doubly phosphorylated (supple-
The SILAC ratios (MCF7-TamR cells versus MCF7-CTRL cells) of phosphopeptides obtained from the two replicate experiments showed a strong positive correlation ($r = 0.80$) for two independent biological replicates (Fig. 3B).

The relative abundance of phosphopeptides was quantitated based on the area under the MS peaks using the quantitation node in Proteome Discoverer. We found phosphorylation levels of 1529 peptides were increased (>twofold) in MCF7-TamR cells compared with MCF7-CTRL cells (Fig. 3C).
Among them, 187 up-regulated phosphopeptides were identified in phosphotyrosine antibody based enrichment and 1342 were identified in TiO2 based enrichment. 409 peptides exhibited decrease in phosphorylation (>0.5-fold) in MCF7-TamR cells compared with MCF7-CTRL cells. Of these, 76 were identified from phosphotyrosine antibody enrichment and 333 were identified from TiO2 based enrichment. These differentially phosphorylated peptides correspond to 1050 proteins. Of these, 850 proteins showed up-regulation in phosphorylation levels in MCF7-TamR cells, and 233 proteins showed down-regulation in phosphorylation levels in MCF7-TamR cells. 33 proteins contained both up- and down-regulated phosphorylation sites. These results are consistent with our phosphotyrosine immunoblot observation, where we saw substantial and global elevation of protein phosphorylation in MCF7-TamR cells (Fig. 1C). This suggests that there is a robust activation of kinases in the MCF7-TamR cells which could contribute to the development of resistance.

Pathway Analysis to Identify Activated Pathways Involved in Tamoxifen Resistance—To better understand the signaling pathways involved in tamoxifen resistance, we performed a KEGG pathway analysis using an integrated online functional annotation tool, DAVID, for the proteins with increased phosphorylation in MCF7-TamR cells. This analysis revealed that several tyrosine kinase-mediated signaling pathways, such as focal adhesion kinase, ERBB, neurotrophin and insulin signaling pathways, are highly enriched in MCF7-TamR cells (Fig. 3D). Some of these signaling pathways such as focal adhesion (33, 34), MAPK (35, 36), ERBB (15, 37, 38) and insulin signaling pathways (39) have been reported to be activated and contribute to the development of resistance to tamoxifen. Significantly, multiple pathways regulating cell migration/invasion including actin cytoskeleton regulation, focal adhesion and tight junction signaling pathways, were also found to be enriched in MCF7-TamR cells (Fig. 3D). This suggests that, in addition to developing resistance to tamoxifen, MCF7-TamR cells also acquired a migration/invasion advantage. In support of this, we observed that the MCF7-TamR cells form more invadopodia-like structures compared with the MCF7-CTRL cells (Fig. 3E). We then performed matrigel invasion assay and found that MCF7-TamR cells are significantly more invasive than MCF7-CTRL cells (Fig. 3F). These observations thus support our phosphoproteomic data reflecting changes consistent with increased motility in tamoxifen resistant cells. The increase of invasiveness and morphological changes imply that the MCF7-TamR cells might have undergone epithelial-mesenchymal transition (EMT) during the development of resistance to tamoxifen. However, when we examined the key regulators and markers of EMT, we did not observe significant expression changes of most EMT markers between the two cell lines, including TCF8, vimentin, β-catenin, E-cadherin, ZO-1 and N-cadherin. We did however observe up-regulation of SLUG and CLAUDIN1 and down-regulation of SNAIL (supplemental Fig. S1). These results suggest that development of tamoxifen resistance in MCF7 cells could be accompanied by EMT-like changes but the transition was not complete.

Activation of Focal Adhesion Kinases in Tamoxifen Resistant MCF7 Cells—One of the top most enriched signaling pathways as revealed by our analysis is the focal adhesion pathway. 31 proteins in this pathway showed regulation of phosphorylation levels in the MCF7-TamR cells. Twenty-seven of these proteins were hyperphosphorylated and four were hypophosphorylated (Table I). The hyperphosphorylated proteins include both of the focal adhesion kinases, PTK2 (FAK1) and PTK2B (FAK2), their upstream kinase, SRC and multiple downstream substrates and interaction partners including Shc, p130Cas, Paxillin and Talin (Fig. 4A). The representative MS spectra of up-regulated phosphopeptides belonging to key proteins in focal adhesion pathway are depicted in Fig. 4B–4E. Our phosphoproteomic data are consistent with studies reported by different groups showing that SRC and FAK1 kinases were activated in tamoxifen resistant breast cancer cells and inhibition of these kinases could suppress the proliferation and migratory ability of tamoxifen resistant cells (33, 34). Another member of the focal adhesion complex, p130Cas, encoded by the BCAR1 gene, has been shown to be up-regulated in breast cancer with tamoxifen resistance and is associated with increased relapse and aggressiveness of the disease (40, 41). As a scaffolding protein, p130Cas can be phosphorylated by multiple kinases including SRC, FAK1 and FAK2. Phosphorylation of p130Cas regulates its interaction with many of its downstream partners (42). For instance, p130Cas can be tyrosine phosphorylated by SRC and reciprocally, p130Cas can elevate SRC kinase activity (43, 44). Tyrosine phosphorylation of p130Cas by SRC can also be enhanced by the docking of both p130Cas and SRC to focal adhesion kinases (45). Further, the interaction between p130Cas, SRC and focal adhesion kinases can promote survival and migration of breast cancer cells with tamoxifen resistance (46, 47).

In order to confirm the activation of SRC-FAK signaling pathway in the MCF7-TamR cells, we performed Western blot analyses to examine the phosphorylation levels of the key kinases SRC, FAK1, and FAK2, and downstream protein Paxillin. We observed significant elevation of phosphorylation of SRC Y416, FAK1 Y576/Y577, FAK2 Y402, and Paxillin Y118 in tamoxifen resistant cells (Fig. 5A). Notably, short-term treatment with tamoxifen did not affect the phosphorylation levels of these signaling proteins in either MCF7-CTRL or MCF7-TamR cells, and these proteins retained hyperphosphorylation in MCF7-TamR cells even without tamoxifen treatment. This suggests that the increase in phosphorylation is stable and developed during the long-term exposure to tamoxifen. Interestingly, FAK2 also had a concomitant increase in protein expression levels in the MCF7-TamR cells compared with MCF7-CTRL cells (Fig. 5A). When we examined FAK2 expression in cells with different exposure time to tamoxifen, we observed that FAK2 expression level gradually increased dur-
ing the development of tamoxifen resistance (Fig. 5B). We sought to investigate whether the up-regulation of FAK2 expression was at the transcriptional or post-transcriptional level. Our quantitative real-time RT-PCR result showed that FAK2 mRNA level increased by more than 10-fold in MCF7-TamR cells compared with MCF7-CTRL cells (Fig. 5C).

As a member of the focal adhesion kinase family, FAK2 has been reported to be mainly localized at the focal adhesions and nuclei of cells (48). Autophosphorylation at tyrosine residue Y402 activates FAK2, which then functions as a docking site for the SH2 domain of Src (49). In order to further interrogate the activation status of FAK2 in MCF7-TamR cells, we performed immunofluorescence staining of the active form of FAK2 (pY402 FAK2). As demonstrated in Fig. 5D, we observed similar cytoplasmic staining patterns of pY402 FAK2 in both MCF7-CTRL and MCF7-TamR cells. However, there was a substantial increase in the staining of foci on the periphery of MCF7-TamR cells compared with MCF7-CTRL cells. Quan-

## Table I

| Gene symbol | Phosphopeptide sequence | Protein name | Site | TamR/CTRL |
|-------------|-------------------------|--------------|------|-----------|
| **PTK2B**   | YIREDYyKAVSRTRLPK       | focal adhesion kinase 2 (FAK2) | Y580 | 25.74     |
|             | RHsMREDIQFQSSR          |              | S778 | 8.91      |
| **PTK2**    | YMEDSTYyKASK            | focal adhesion kinase 1 (FAK1) | Y662 | 2.20      |
|             | GSIDREDGSLQGPNQHlyQPVGKPDAPPK |              | Y946 | 2.74      |
| **SRC**     |                          |              | Y439 |           |
| **FY**      | WTAPEAALyGR             | v-src viral oncogene | Y440 | 4.21      |
| **YES**     |                          |              | Y446 |           |
| **BRK**     | sPQKPIVR                | v-raf viral oncogene homolog B | S151 | 0.45      |
| **BCAR1**   | RPPGPTyDVPR             | breast cancer anti-estrogen resistance 1 | Y433 | 7.41      |
| **PXN**     | VGESEHYySFPNKK          | paxillin     | Y174 | 8.35      |
|             | FIHQQPOSSyPVGSAK         |              | Y124 | 3.42      |
| **TLN1**    | STVLQOyNR               | talin 1      | Y436 | 4.61      |
|             | TMQEPTSTMVyDACR          |              | Y24  | 5.94      |
| **FY**      | KLDNGGyYITTR            | FYN oncogene | Y213 | 2.80      |
| **GAY**     | GALSIR                  |              | Y185 | 4.14      |
| **CRK**     | YRPAsAVSALIGGR          | v-crk avian sarcoma virus CT10 oncogene | S194 | 5.89      |
| **JUN**     | LAyPLEL                  | jun proto-oncogene | S73  | 3.68      |
| **MAPK8**   | TAGTSFMMPVyVVTR         | mitogen-activated protein kinase 8, 10 | Y185 | 0.37      |
| **ACTG1**   | EITALyPSMK              | actin, gamma 1 | S323 | 3.18      |
| **ACTB**    |                          | actin, beta  | Y226 |           |
| **ACTN1**   | HRPRLyDK                | actinin, alpha 1 | Y193 | 4.81      |
| **ARHGA5**  | GGIQNPAlSDELDK          | Rho GTPase activating protein 5 | S1218 | 4.41      |
|             | RTTSdASDEFTGTSK         |              | S1173, S1176 | 3.91      |
| **BAD**     | RMasyDEFDSFKK            | BCL2-associated agonist of cell death | S118 | 4.99      |
| **FLNA**    | CSGPGPsFGMV              | filamin A, alpha | S1459 | 5.06      |
|             |                         |              | S2152 | 4.92      |
| **ILK**     | NGILNKHSGIDFK            | integrin-linked kinase | T181 | 2.25      |
| **PAK2**    | FYDSNTVK                | p21 protein (Cdc42/Rac)-activated kinase 2 | S132 | 2.79      |
|             |                         |              | S152  | 2.04      |
| **PARVA**   | SPSVPKsPTPPSSR          | parvin, alpha | S54  | 12.72     |
| **PPP1CA**  | yGQGFLPnpgPPRIPPPR      | protein phosphatase 1, catalytic subunit, alpha isoyme | Y317 | 3.53      |
| **PPP1CB**  | YQQGGLNSGtWPPR          | protein phosphatase 1, catalytic subunit, beta isoyme | T316 | 2.49      |
| **PPP1R12A**| LAtSTISEEEM             | protein phosphatase 1, regulatory subunit 12A | S507 | 5.55      |
|             |                         |              | S422  | 6.37      |
| **VAP**     | VQYIYNPANSFR            | vasodilator-stimulated phosphoprotein | T43  | 2.13      |
| **VAV2**    | AsyRyPFPYPR             | vav 2 guanine nucleotide exchange factor | S769, S771 | 0.50      |
| **VCL**     | ILLRNPGQGAayEHETFMTK    | vinculin     | Y693  | 3.48      |
|             | DPSAsPGOSQAEQAIR         |              | S291  | 28.40     |
| **ZYX**     | FsPGAPGSQGSPNQK         | zyxin        | S431  | 4.23      |
|             | sPGAPGPLTLK             |              | S404  | 7.30      |
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**Fig. 4.** Activation of focal adhesion pathways in tamoxifen resistance. A, Hyperphosphorylated and hypophosphorylated (MCF7-TamR versus MCF7-CTRL) proteins identified in the MCF7-TamR cells that are involved in the focal adhesion pathways. Protein phosphorylation sites and phospho-regulation patterns were specified with color-coded circles. Diagram adapted from Pathvisio, a pathway analysis tool. B–E, Representative MS spectra of hyperphosphorylated peptides of FAK2, FAK1, PXN and BCAR1.
tification of these focal adhesions revealed an increase of fivefold in MCF7-TamR cells than in MCF7-CTRL cells (p < 0.001) (Fig. 5E). Taken together, this suggests that transcriptional up-regulation and overexpression of FAK2 could play a critical role in the development of tamoxifen resistance, and suppression of FAK2 could potentially reverse the resistance to tamoxifen.

**Targeting FAK2 to Suppress Proliferation of Cells with Tamoxifen Resistance**—In order to evaluate the role of FAK2 in the development of tamoxifen resistance, we performed siRNA-mediated knockdown to specifically suppress FAK2 expression. Fig. 6A shows that FAK2 knockdown effectively inhibited FAK2 expression in both MCF7-CTRL and MCF7-TamR cells. We found that knockdown of FAK2 significantly reduced the proliferation of MCF7-TamR cells (Fig. 6B). Interestingly, knockdown of FAK2 also suppressed the growth of MCF7-CTRL cells and sensitized these cells to tamoxifen even further. We next examined the effect of suppressing FAK2 on signaling molecules downstream of the focal adhesion pathway. Remarkably, knockdown of FAK2 with two different sets of siRNAs reduced the phosphorylation of FAK1 and Paxillin but not SRC (Fig. 6C), suggesting that overexpression of FAK2 is pivotal in the activation of focal adhesion signaling pathway in MCF7-TamR cells.

In order to determine the therapeutic potential of targeting focal adhesion pathway in tamoxifen resistant breast cancer, we employed a potent pan-FAK selective pharmacological inhibitor, PF562271. PF562271 has been selected for clinical trials in patients with pancreatic, head and neck, and prostatic neoplasms (50). We performed proliferation assays to assess the IC50 of PF562271 in MCF7-CTRL or MCF7-TamR cells and found that MCF7-TamR cells were four times more sensitive to PF562271 than MCF7-CTRL cells (Fig. 6D). This suggests that activation of focal adhesion pathway significantly contributes to
the resistance to tamoxifen treatment. We further validated the therapeutic potential of PF562271 in suppression of tamoxifen resistant tumor growth in a xenograft tumor model. MCF7-CTRL or MCF7-TamR cells were orthotopically transplanted into the mammary gland fat pad of immunocompromised mice and treated with DMSO or PF562271. As demonstrated in Fig. 6E, the treatment with PF562271 significantly reduced the tumor formation of MCF7-TamR cells.

Fig. 6. FAK2 is a novel therapeutic target in tamoxifen-resistant breast cancer. A, Western blot analysis of siRNA knockdown of FAK2 (siFAK2) or control (siCTRL). β-actin serves as a loading control. B, Proliferation assay following siRNA knockdown of FAK2 (siFAK2) or control siRNA (siCTRL) with tamoxifen or vehicle. Student’s t test was performed for statistical analysis. C, Western blot analysis of MCF7-CTRL and MCF7-TamR cells treated with two different siRNA against FAK2. Protein expression and phosphorylation levels of the proteins involved in the focal adhesion pathway were examined. D, The inhibitory effect of PF562271 on cell growth of MCF7-CTRL and MCF7-TamR cell. The IC_{50} curve was plotted using a nonlinear regression dose-response (variable slope) curve fit. E, Tumor volumes of mice xenograft grown with either MCF7-TamR or MCF7-CTRL cells with the treatment of PF562271 or DMSO control. Two-way Anova was performed for statistical analysis. F, Kaplan-Meier curve of metastasis-free survival of ER+ patients treated with tamoxifen. The patients were stratified based on expression of FAK2.
Finally, we sought to examine whether FAK2 expression correlated with clinical outcomes in patients with ER+ breast cancers. A publicly available gene expression database (51) of tumors from breast cancer patients was used for this survival analysis. We focused our analysis on 657 ER+ tumors treated with tamoxifen-based therapy. We found that high expression level of FAK2 is significantly associated (p = 0.021) with poorer outcomes of these patients (Fig. 6F). These clinical data suggest that FAK2 is a potential target of relevance to aggressive clinical behavior and hormone resistance in breast cancer.

**DISCUSSION**

Tamoxifen resistance remains a major challenge in current breast cancer management. Unveiling druggable protein targets that are critical for ER+ breast cancer cells to evade the inhibitory effects of endocrine therapy is an urgent and unmet need for developing novel therapeutic options to benefit patients with hormone refractory breast cancer. In this study, we employed SILAC labeling based LC-MS/MS proteomic approaches to comprehensively profile the phosphoproteome of MCF7 cells with tamoxifen resistance. We applied two phosphopeptide enrichment methods to identify and quantify alterations in serine, threonine and tyrosine phosphorylated peptides. This strategy allowed us to decipher signaling pathway changes at a much greater depth compared with other published studies (21, 22). With this comprehensive phosphoproteome profiling, we discovered for the first time, substantial and global elevation of protein phosphorylation in MCF7 tamoxifen resistant cells, strongly implicating that a number of kinases are activated in these cells. Our pathway analysis identified multiple crucial kinase-mediated signaling pathways to be hyperactive in tamoxifen resistant cells, including MAPK, ERBB, insulin and FAK signaling pathways. Our findings support previous reports and offer a more global and complete view of these activated pathways. For instance, in addition to BRAF and multiple MAP kinases, we also found 29 additional important signaling molecules in the MAPK signaling network to be regulated in tamoxifen resistant cells.

Previous studies have shown that the SRC and FAK signaling pathway were activated during the progression of hormone dependent breast cancer (33, 34). However, the mechanism of activation of SRC and FAK signaling has not been fully elucidated. In our study, both SRC and FAK1 were identified to be hyperphosphorylated in tamoxifen resistant cells. Moreover, several SRC and FAK substrates including SHC, Paxillin and BCAR1 and many downstream proteins were also found to be hyperphosphorylated in these cells. Most importantly, for the first time, we showed that FAK2 was transcriptionally upregulated and hyperphosphorylated in tamoxifen resistant cells. Inhibition of FAK2 with small molecule inhibitor or specific siRNA significantly suppressed cell growth and tumor formation of resistant cells. We also demonstrated that specific siRNA targeting FAK2 substantially reduced the protein phosphorylation level of FAK1 and Paxillin, suggesting that FAK2 is a key kinase modulating the focal adhesion pathway. Our patient survival analysis using breast cancer expression database showed that high expression of FAK2 is associated with a significant decrease in the survival of ER+ patients on tamoxifen treatment, suggesting the clinical importance of FAK2 in this disease.

In summary, we show through our phosphoproteomic approaches that multiple kinase-mediated signaling pathways are activated in tamoxifen resistant cells. Our *in vitro* and *in vivo* functional studies demonstrated that the tyrosine kinase FAK2 plays a pivotal role in the development of resistance to tamoxifen and could potentially be a novel therapeutic target for this disease. However, given the complexity of signaling pathways and tumor heterogeneity, these discoveries require further testing and validation in larger cohorts of patients. Because our study was performed on one cell line, further investigation is needed to support the broader applicability of our findings. In particular, the exact mechanisms underlying FAK2 up-regulation and activation in tamoxifen resistant tumors and the efficacy of inhibition of FAK2 in pre-clinical and clinical settings need to be further investigated.

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[§] This article contains supplemental Fig. S1 and Tables S1 to S3.

**Conflict of Interest Statement:** All authors declare no conflict of interests.

**Data and Materials Availability:** The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium ([http://proteomecentral.proteomexchange.org](http://proteomecentral.proteomexchange.org)) via the PRIDE partner repository with the data set identifier PXD001812. PX reviewer account: Username: reviewer00970@ebi.ac.uk Password: wiEhx0MA.

**REFERENCES**

1. Yamashita, H. (2008) Current research topics in endocrine therapy for breast cancer. *Int. J. Clin. Oncol.* 13, 380–383
2. Ali, S., and Coombes, R. C. (2002) Endocrine-responsive breast cancer and strategies for combating resistance. *Nat. Rev. Cancer* 2, 101–112

2898

Molecular & Cellular Proteomics 14.11
3. Osborne, C. K., Zhao, H., and Fuqua, S. A. (2000) Selective estrogen receptor modulators: structure, function, and clinical use. J. Clin. Oncol. 18, 3172–3186

4. Osborne, C. K., and Schiff, R. (2005) Estrogen-receptor biology: continuing progress and therapeutic implications. J. Clin. Oncol. 23, 1616–1622

5. Burstein, H. J., Temin, S., Anderson, H., Buchholtz, T. A., Davidson, N. E., Gelmon, K. E., Giordano, S. H., Hudis, C. A., Rowden, D., Solk, A. J., Stearns, V., Winer, E. P., and Griggs, J. J. (2014) Adjuvant endocrine therapy for women with hormone receptor-positive breast cancer: американской society of clinical oncology clinical practice guideline focused update. J. Clin. Oncol. 32, 2255–2269

6. Visvanathan, K., Hurley, P., Bantu, E., Brown, P., Col, N. F., Cuzick, J., Davidson, N. E., Decensi, A., Fabian, C., Ford, L., Garber, J., Katapodi, M., Kramer, B., Morrow, M., Parker, B., Runowicz, C., Vogel, V. G., 3rd, Wistow, G., and Winter, L. (2006) Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet 378, 771–784

7. Ring, A., and Dowssett, M. (2004) Mechanisms of tamoxifen resistance. Endocr. Relat. Cancer 11, 643–658

8. Early Breast Cancer Trialists’ Collaborative Group, Davies, C., Godwin, J., Gray, R., Clarke, M., Cuttler, D., Darby, S., McGale, P., Pan, H. C., Taylor, C., Peto, J., British National Confidential Enquiry into Perioperative Deaths. (2005) Long-term effects of chemotherapy and hormonal therapy for early breast cancer on overall survival, and on the incidence of secondary primary cancers: cumulative meta-analysis of individual patient data for 10,801 women in 17 randomised trials. Lancet 366, 1687–1696

9. Osborne, C. K., Bardou, V., Hopp, T. A., Chamness, G. C., Hilsenbeck, S. G., Fuqua, S. A., Wong, J., Allred, D. C., Clark, G. M., and Schiff, R. (2003) Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2 in tamoxifen resistance in breast cancer. J. Natl. Cancer Inst. 95, 353–361

10. Osborne, C. K., and Schiff, R. (2003) Growth factor receptor cross-talk with estrogen receptor as a mechanism for tamoxifen resistance in breast cancer. Breast 12, 362–367

11. Knowlden, J. M., Hutcheson, I. R., Jones, H. E., Madden, T., Gee, J. M., Harper, M. E., Barrow, D., Wakeling, A. E., and Nicholson, R. I. (2003) Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. Endocrinology 144, 1032–1044

12. McClelland, R. A., Barrow, D., Madden, T. A., Dutkowski, C. M., Pamment, J., Knowlden, J. M., Gee, J. M., and Nicholson, R. I. (2001) Enhanced epidermal growth factor receptor signaling in MCF7 breast cancer cells after long-term culture in the presence of the pure antiestrogen ICI 182,780 (Faslodex). Endocrinology 142, 2776–2785

13. Hawthorne, V. S., Huang, W. C., Neal, C. L., Tseng, L. M., Hung, M. C., and Menter, R. J. (2001) Activation of AKT/PKB in breast cancer predicts a worse outcome with poor response to anti-hormonal therapy and decreased patient survival in clinical breast cancer. Clin. Exp. Metastasis 18, 2469–2476

14. Kirkegaard, T., Witton, C. J., McGlynn, L. M., Tovey, S. M., Dunne, B., Lyon, A., and Bartlett, J. M. (2006) AKT activation predicts outcome in breast cancer patients treated with tamoxifen. J. Pathol. 207, 139–146

15. Leary, A. F., Sirohi, B., and Johnston, S. R. (2007) Clinical trials update: endocrine and biological therapy combinations in the treatment of breast cancer. Breast Cancer Res. 9, 112

16. Moy, B., and Goss, P. E. (2006) Estrogen receptor pathway: resistance to endocrine therapy and new therapeutic approaches. Clin. Cancer Res. 12, 4790–4793

17. Browne, B. C., Hochgräfe, F., Wu, J., Miller, E. K., Barracough, J., Stone, A., McCoy, R. A., Lee, C. S., Roberts, C., Ali, N. A., Bouglhourian, A., Schmich, F., Linding, R., Farrow, L., Gee, J. M., Nicholson, R. I., O’Toole, S. A., Sutherland, R. L., Musgrove, E. A., Butt, A. J., and Daly, R. J. (2013) Global characterization of signalling networks associated with tamoxifen resistance in breast cancer. FEBS J 280, 5237–5257

18. Oyama, M., Nagajima, T., Suzuki, T., Kozuoka-Hata, H., Yumoto, N., Shiraiha, Y., Ikeda, K., Kuroki, Y., Gotoh, N., Ishida, T., Inoue, S., Kitano, H., and Okada-Hatakeyama, M. (2011) Integrated quantitative analysis of the proteosome and transcription in tamoxifen-resistant breast cancer. J. Biol. Chem. 286, 818–829

19. Zhou, C., Zhong, Q., Rhodes, L. V., Townley, I., Bratrton, M. R., Zhong, Q., Martin, E. C., Elliott, S., Collins-Burrow, B. M., Burrow, M. E., and Wang, G. (2006) Proteomics of acquired tamoxifen resistance in MCF-7 cells reveals expression signatures associated with enhanced migration. Breast Cancer Res. 14, R45

20. Hengel, S. M., Murray, E., Langdon, S., Hayward, L., O’Donoghue, J., Pash, A., and Goodlett, D. R. (2011) Data-independent proteome screen identifies novel tamoxifen agonist that mediates drug resistance. J. Proteome Res 10, 4567–4578

21. Umar, A., Kang, H., Petri, R. (2003) Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2 in tamoxifen resistance in breast cancer. Mol. Cell. Proteomics 3, 873–886

22. Besada, V., Diaz, M., Becker, M., Ramos, Y., Castellanos-Serra, L., and Fichtner, I. (2006) Proteomics of xenografted human breast cancer indicates novel targets related to tamoxifen resistance. Proteomics 6, 1038–1048

23. Beausoleil, S. A., Jedrychowski, M., Schwartz, D., Elias, J. E., Villen, J., Li, J., Cohn, M. A., Cantley, L. C., and Gygi, S. P. (2004) Large-scale characterization of HeLa cell nuclear phosphoproteins. Proc. Natl. Acad. Sci. U. S. A. 101, 12130–12135

24. Larsen, M. R., Thingholm, T. E., Jensen, O. N., Roepstorff, P., and Jorgensen, T. J. (2005) Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. Mol. Cell. Proteomics 4, 878–883

25. Spivak, M., Weston, J., Bottou, L., Käll, L., and Noble, W. S. (2009) Improvements to the percolator algorithm for Peptide identification from shotgun proteomics data sets. J. Proteome Res. 8, 3737–3745

26. Wu, X., Chen, H., Parker, B., Rubini, E., Zhu, T., Lee, J. S., Argani, P., and Sukumar, S. (2006) HOX8/B7, a homeodomain protein, is overexpressed in breast cancer and confers epithelial-mesenchymal transition. Cancer Res. 66, 9527–9534

27. Blume-Jensen, P., and Hunter, T. (2001) Oncogenic kinase signalling. Nature 411, 355–365

28. Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A., and Mann, M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression profiling. Mol. Cell. Proteomics 1, 376–386

29. Hiscox, S., Jordan, N. J., Morgan, L., Green, T. P., and Nicholson, R. I. (2007) Snc kinase promotes adhesion-independent activation of FAK and enhances cellular migration in tamoxifen-resistant breast cancer cells. Clin. Exp. Metastasis 24, 157–167

30. Planas-Silva, M. D., Bruggeman, S. D., Grenko, R. T., and Stanley, J. (2006) Role of c-Src and focal adhesion kinase in progression and metastasis of estrogen receptor-negative breast cancer. Biochem. Biophys. Res. Commun. 341, 73–81

31. Gutierrez, M. C., Detre, S., Johnston, S., Mohsin, S. K., Shou, J., Allred, D. C., Schiff, R., Osborne, C. K., and Dowssett, M. (2005) Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. J. Clin. Oncol. 23, 2469–2476

32. Donovan, J. C., Milic, A., and Slingerland, J. M. (2001) Constitutive MEK/ERK activation leads to p73(Kip1) deregulation and autotestrogen resistance in human breast cancer cells. J. Biol. Chem. 276, 40888–40895

33. Kurokawa, H., Lenferink, A. E., Simpson, J. F., Pisacane, P. I., Sliwkowski, M. X., Forbes, J. T., and Arteaga, C. L. (2000) Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer.
Phosphoproteomic Analysis of Tamoxifen Resistant Breast Cancer

38. Hutcheson, I. R., Knowlden, J. M., Madden, T. A., Barrow, D., Gee, J. M., Wakeling, A. E., and Nicholson, R. I. (2003) Oestrogen receptor-mediated modulation of the EGFR/MAPK pathway in tamoxifen-resistant MCF-7 cells. *Breast Cancer Res. Treat.* **81**, 81–93.

39. Zhang, Y., Moerkens, M., Ramaiahgari, S., de Bont, H., Price, L., Meerman, J., and van de Water, B. (2011) Elevated insulin-like growth factor 1 receptor signaling induces antiestrogen resistance through the MAPK/ERK and PI3K/Akt signaling routes. *Breast Cancer Res. Treat.* **13**, R52.

40. van der Flier, S., Brinkman, A., Kok, E. M., Meijer-van Gelder, M. E., Klijn, J. G., Dorssers, L. C., and Foekens, J. A. (2000) Bcar1/p130Cas protein and primary breast cancer: prognosis and response to tamoxifen treatment. *J. Nat. Cancer Inst.* **92**, 120–127.

41. Brinkman, A., van der Flier, S., Kok, E. M., and Dorssers, L. C. (2000) BCAR1, a human homologue of the adapter protein p130Cas, and anti-estrogen resistance in breast cancer cells. *J. Nat. Cancer Inst.* **92**, 112–120.

42. Barrett, A., Pellet-Many, C., Zachary, I. C., Evans, I. M., and Frankel, P. (2013) p130Cas: a key signalling node in health and disease. *Cell. Signalling* **25**, 766–777.

43. Riggins, R. B., Quilliam, L. A., and Bouton, A. H. (2003) Synergistic promotion of c-Src activation and cell migration by Cas and AND-34/BCAR3. *J. Biol. Chem.* **278**, 28264–28273.

44. Brábek, J., Constancio, S. S., Shin, N. Y., Pozzi, A., Weaver, A. M., and Hanks, S. K. (2004) CAS promotes invasiveness of Src-transformed cells. *Oncogene* **23**, 7406–7415.

45. Ruest, P. J., Shin, N. Y., Polte, T. R., Zhang, X., and Hanks, S. K. (2001) Mechanisms of CAS substrate domain tyrosine phosphorylation by FAK and Src. *Mol. Cell. Biol.* **21**, 7641–7652.

46. Chodniewicz, D., and Klemke, R. L. (2004) Regulation of integrin-mediated cellular responses through assembly of a CAS/Crk scaffold. *Biochim. Biophys. Acta* **1692**, 63–76.

47. Cowell, L. N., Graham, J. D., Bouton, A. H., Clarke, C. L., and O’Neill, G. M. (2006) Tamoxifen treatment promotes phosphorylation of the adhesion molecules, p130Cas/BCAR1, FAK and Src, via an adhesion-dependent pathway. *Oncogene* **25**, 7597–7607.

48. Sun, C. K., Ng, K. T., Sun, B. S., Ho, J. W., Lee, T. K., Ng, I., Poon, R. T., Lo, C. M., Liu, C. L., Man, K., and Fan, S. T. (2007) The significance of proline-rich tyrosine kinase2 (Pyk2) on hepatocellular carcinoma progression and recurrence. *Br. J. Cancer* **97**, 50–57.

49. Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. (2005) Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Biol.* **6**, 56–68.

50. (2013) Study Of PF-00562271, Including Patients With Pancreatic, Head And Neck, Prostatic Neoplasms.

51. Györfy, B., Surowiak, P., Budczies, J., and Lánzky, A. (2013) Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. *PloS one* **8**, e82241.