

Tyrosine Phosphorylation Profiling Reveals the Signaling Network Characteristics of Basal Breast Cancer Cells

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Abstract

To identify therapeutic targets and prognostic markers for basal breast cancers, breast cancer cell lines were subjected to mass spectrometry-based profiling of protein tyrosine phosphorylation events. This revealed that luminal and basal breast cancer cells exhibit distinct tyrosine phosphorylation signatures that depend on pathway activation as well as protein expression. Basal breast cancer cells are characterized by elevated tyrosine phosphorylation of Met, Lyn, EphA2, epidermal growth factor receptor (EGFR), and FAK, and Src family kinase (SFK) substrates such as p130Cas. SFKs exert a prominent role in these cells, phosphorylating key regulators of adhesion and migration and promoting tyrosine phosphorylation of the receptor tyrosine kinases EGFR and Met. Consistent with these observations, SFK inhibition attenuated cellular proliferation, survival, and motility. Basal breast cancer cell lines exhibited differential responsiveness to small molecule inhibitors of EGFR and Met that correlated with the degree of target phosphorylation, and reflecting kinase coactivation, inhibiting two types of activated network kinase (e.g., EGFR and SFKs) was more effective than single agent approaches. FAK signaling enhanced both proliferation and invasion, and Lyn was identified as a proinvasive component of the network that is associated with a basal phenotype and poor prognosis in patients with breast cancer. These studies highlight multiple kinases and substrates for further evaluation as therapeutic targets and biomarkers. However, they also indicate that patient stratification based on expression/activation of drug targets, coupled with use of multi-kinase inhibitors or combination therapies, may be required for effective treatment of this breast cancer subgroup. *Cancer Res*; 70(22); 9391–401. ©2010 AACR.

Introduction

Gene expression profiling has revealed five breast cancer subtypes that differ in their prognosis and therapeutic responsiveness: luminal A and B [both estrogen receptor (ER)-positive], erbB2 (also known as HER2), basal and normal-like (ER-negative; ref. 1) subtypes. Of these, the erbB2 and basal subsets exhibit the shortest overall and relapse-free survival. The basal subtype accounts for 10% to 27% of breast cancer cases, depending on the patient population (2, 3). The common absence of ER, progesterone receptor (PR) and

erbB2 in this subgroup, or “triple-negative” phenotype, leads to clinical resistance to endocrine and trastuzumab-based therapies (2). In addition, basal breast cancers exhibit heterogeneity in terms of prognosis, but markers that accurately stratify patients at primary diagnosis are currently unavailable (2). Consequently, critical issues facing the clinician are the lack of targeted treatment strategies and prognostic markers for this patient subset.

Approximately half of the tyrosine kinase complement of the human “kinome” is implicated in human cancers (4), and provides important targets for cancer treatment, as well as biomarkers for patient stratification. Characterization of tyrosine kinase signaling networks has been greatly facilitated by recent advances in mass spectrometry-based phosphoproteomics (5). In the current manuscript, we have used mass spectrometry-based phosphoproteomic profiling to characterize the tyrosine kinase signaling networks associated with different breast cancer subgroups. This has revealed a tyrosine phosphorylation signature that characterizes the basal phenotype, and identified a prominent Src family kinase (SFK) signaling network in basal breast cancer cells. These findings provide important insights into the biology of basal breast cancers and have significant implications for the development of therapeutic strategies that target this disease subtype.

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Materials and Methods

Cell culture

All cell lines were obtained from the American Type Culture Collection, except for MDA-MB-231 and T-47D (EG&G Mason Research Institute) and MCF-7 (Michigan Cancer Foundation). Cell lines were authenticated by short tandem repeat polymorphism, single nucleotide polymorphism, and fingerprint analyses, passaged for less than 6 months, and cultured as previously described (6, 7).

Phosphoproteomic profiling

Phosphopeptide immunoprecipitation was performed as described (5). Immunoprecipitated peptides were dissolved in 1% (v/v) formic acid, 0.05% (v/v) heptafluorobutyric acid. For each mass spectrometry measurement, the samples were separated by nano-LC using an Ultimate 3000 HPLC and autosampler system (Dionex) and mass spectra were collected with an LTQ FT Ultra mass spectrometer (Thermo Electron). Peak lists were created from the raw data using the Mascot Distiller algorithm version 2.2.1.0 (Matrix Science). The Mascot Daemon tool version 2.2.0 (Matrix Science) was used to search the peak lists against an in-house sequence database extracted from <ftp://ftp.expasy.org/databases/uniprot/knowledgebase> (release date December 19, 2008), with *Homo sapiens* set for taxonomy (20,411 sequences). Final filtering criteria were applied to achieve a false positive rate for peptide identifications of <1%. Spectral counting was used to estimate the degree of phosphorylation for a particular protein or site. The average was calculated from replicates and data were normalized for the spectral counts of GSK3 α/β (5). Complete linkage hierarchical clustering and assembling of heat maps were carried out with an Euclidian distance matrix on In-transformed data using TIGR's MeV program version 4.1 (8). When comparing MCF-10A and MCF-10A/SrcY527F cells, phosphorylation sites exhibiting a difference in mean spectral counts of at least 1.5 in two independent experiments were considered as differentially phosphorylated.

Tyrosine kinase inhibitors

For phosphorylation assays, cells were treated with PP2 (Calbiochem; 30 $\mu\text{mol/L}$) or Src kinase inhibitor I (Src-I1; Calbiochem; 10 $\mu\text{mol/L}$) for 1 hour. For proliferation assays, PP2 was used at 10 $\mu\text{mol/L}$. AG1478 and SU11274 were obtained from Sigma and used at a final concentration of 10 and 1 $\mu\text{mol/L}$, respectively. PF-562271 was obtained from Symansis and used at 1 to 100 nmol/L. All drugs were dissolved in DMSO.

Short interfering RNA transfection

Lyn short interfering RNAs (siRNA) "no. 12" and "no. 13" were obtained from Qiagen (Hs_LYN_12_HP and Hs_LYN_13_HP validated siRNA). The sequence for Lyn siRNA "no. 2" was 5'-AAUGGUGGAAAGCAAAGUCCC-3', for FAK siRNA "no. 1" and "no. 2" 5'-GCUAGUGACGUAUGGAUGU-3' and 5'-ACACCAAUUCGAGUACUA-3', respectively. ON-TARGET $plus$ Non-Targeting Pool was obtained from

Dharmacon. Cell lines were transfected with 5 to 20 nmol/L of siRNA using LipofectAMINE (Invitrogen). Forty-eight hours later, cells were lysed or used for particular assays.

Antibodies and Western blot analysis

The following antibodies were used: v-Src/c-Src (Calbiochem); EphA2 (Millipore); FAK and Hck (BD Transduction Laboratories); Yes (Transduction Laboratories); β -actin (Sigma-Aldrich); Erk1/2, Met, Caveolin-1, pY419-Src family, pY705-Stat3, pY14-Caveolin-1, pY249-p130Cas, pT184/202/pY186/204 Erk1/2, pY1148-epidermal growth factor receptor (EGFR), and pY1173-EGFR (Cell Signaling Technology); Fyn, EGFR, Stat3, p130Cas, Lyn pY1234-MET, and pY-576 FAK (Santa Cruz Biotechnology); and pY397 FAK (Biosource). Western blotting was undertaken as previously described (7).

Cellular assays

Proliferation was assayed using a MTS assay (Promega). Soft agar assays were as described previously (9). For apoptosis assays, cells were treated with drugs for 24 hours and the sub-G₁ population was determined by flow cytometry. Significance was assessed by one-way ANOVA across treatments. Cell tracking and determination of cell invasion through Matrigel (BD BioCoat Growth Factor Reduced Matrigel Invasion Chamber, BD Biosciences) were undertaken as previously described (10, 11).

Immunofluorescent staining of mouse mammary tumors

tp53-null mammary tumors were generated by transplanting epithelium from *tp53*-null mice (Jackson Laboratory; FVB/N background; stock no.002899) into the cleared mammary fat pads of recipient wild-type FVB/N mice (12). PyMT tumors were derived from MMTV-PyMT transgenic mice on an FVB/N background (13). All animal work was approved by the Garvan/St. Vincent's Hospital Animal Ethics Committee. Immunofluorescence analysis of frozen tumor sections was as previously described (14).

Immunohistochemical staining of breast cancer specimens

The clinicopathologic characteristics of the cohort and its subclassification are described elsewhere (15). Ethics approval for the study was granted by the Human Research Ethics Committee of St. Vincent's Hospital, Sydney (H00 036). Immunohistochemistry used an anti-Lyn monoclonal antibody (Santa Cruz Biotechnology) and anti-EGFR pY1173 rabbit polyclonal antibody (Cell Signaling Technology). Scoring was undertaken by a breast pathology specialist (S. O'Toole). A histoscore was calculated by multiplying the percentage of positively stained cells and the category of staining intensity. For comparison of EGFR pY1173 in different subgroups, three cores from 10 cases of each subgroup were stained. ANOVA was used to examine differences in antigen expression between breast cancer subtypes. Kaplan-Meier survival curves and Cox proportional hazard ratios were estimated to obtain risks of breast cancer-specific death. Results were considered significant at the two-sided

$P < 0.05$ level. StatView version 5.0 (Abacus Systems) was used for the analysis.

Reverse phase protein arrays

The study population consisted of 107 patients with primary breast carcinomas treated in the Edinburgh Breast Unit (Supplementary Table S1). The study was approved by the Lothian Research Ethics Committee (08/S1101/41). Tumors were assessed for ER and PR positivity by immunohistochemistry and HER2 positivity by immunohistochemistry and fluo-

rescence *in situ* hybridization, as previously described (16). Molecular phenotype was defined as ER+ in tumors with ER Allred scores ≥ 3 , and triple-negative in tumors with ER Allred scores < 3 , PR < 3 , HER2-negative by immunohistochemistry or fluorescence *in situ* hybridization. Tumor samples were homogenized on ice in lysis buffer [50 mmol/L Tris (pH 7.5), 5 mmol/L EGTA (pH 8.5), 150 mmol/L NaCl] supplemented with protease inhibitors (Roche), phosphatase inhibitors (Sigma) and aprotinin (Sigma). Triton X-100 was added to 1% (v/v) and the lysates clarified by centrifugation. Reverse

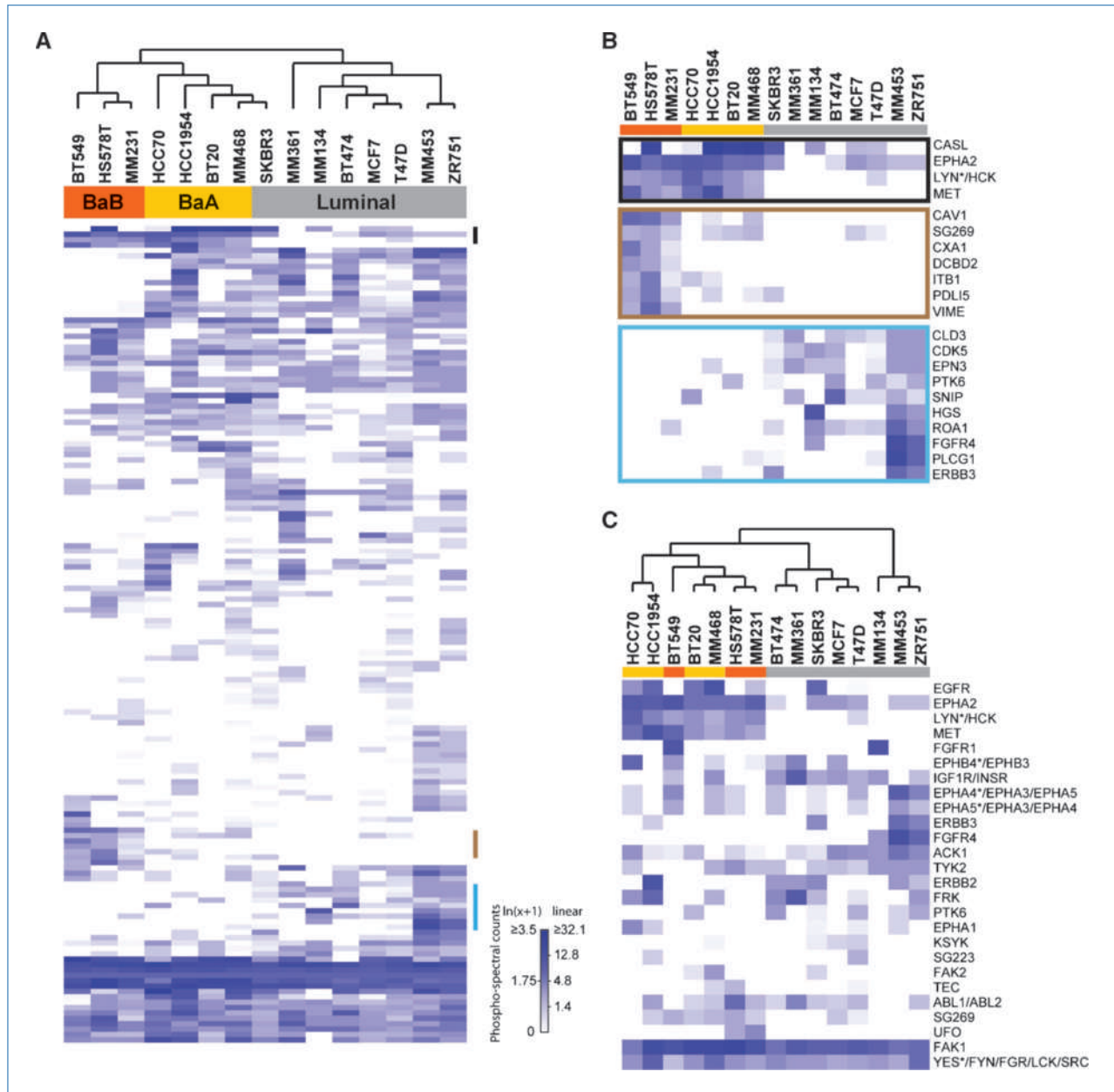


Figure 1. A, unsupervised hierarchical clustering of breast cancer cell lines based on all tyrosine-phosphorylated proteins. Classification of the cell lines based on transcript-profiling data is shown below the dendrogram. The vertical bars highlight differentially phosphorylated proteins, which are highlighted in B. Multiple possible assignments are indicated in a list separated by a forward slash (/). An asterisk indicates that unique peptides corresponding to that protein were also detected. C, hierarchical clustering based on tyrosine kinase phosphorylation.

phase protein array analysis was undertaken as previously described (17), using EGFR pY1173 and Met pY1234/1235 antibodies (Cell Signaling Technology).

Results

Phosphotyrosine profiling of human breast cancer cell lines

The PhosphoScan approach (5) was applied to a panel of 15 breast cancer cell lines. We identified 544 sites of tyrosine

phosphorylation in peptide sequences derived from 295 non-redundant proteins (Supplementary Tables S2 and S3). Thirty-one tyrosine phosphorylation sites were previously unidentified (Supplementary Table S4).

Upon unsupervised hierarchical clustering using data for all tyrosine-phosphorylated proteins, the cell lines largely clustered into two groups previously characterized as "basal" or "luminal" by transcript profiling (Fig. 1A; ref. 11). Coclustering of lines classified as basal A and basal B (stem cell-like), respectively, was also evident (11). A distinct erbB2 subgroup was not resolved, as observed in transcript

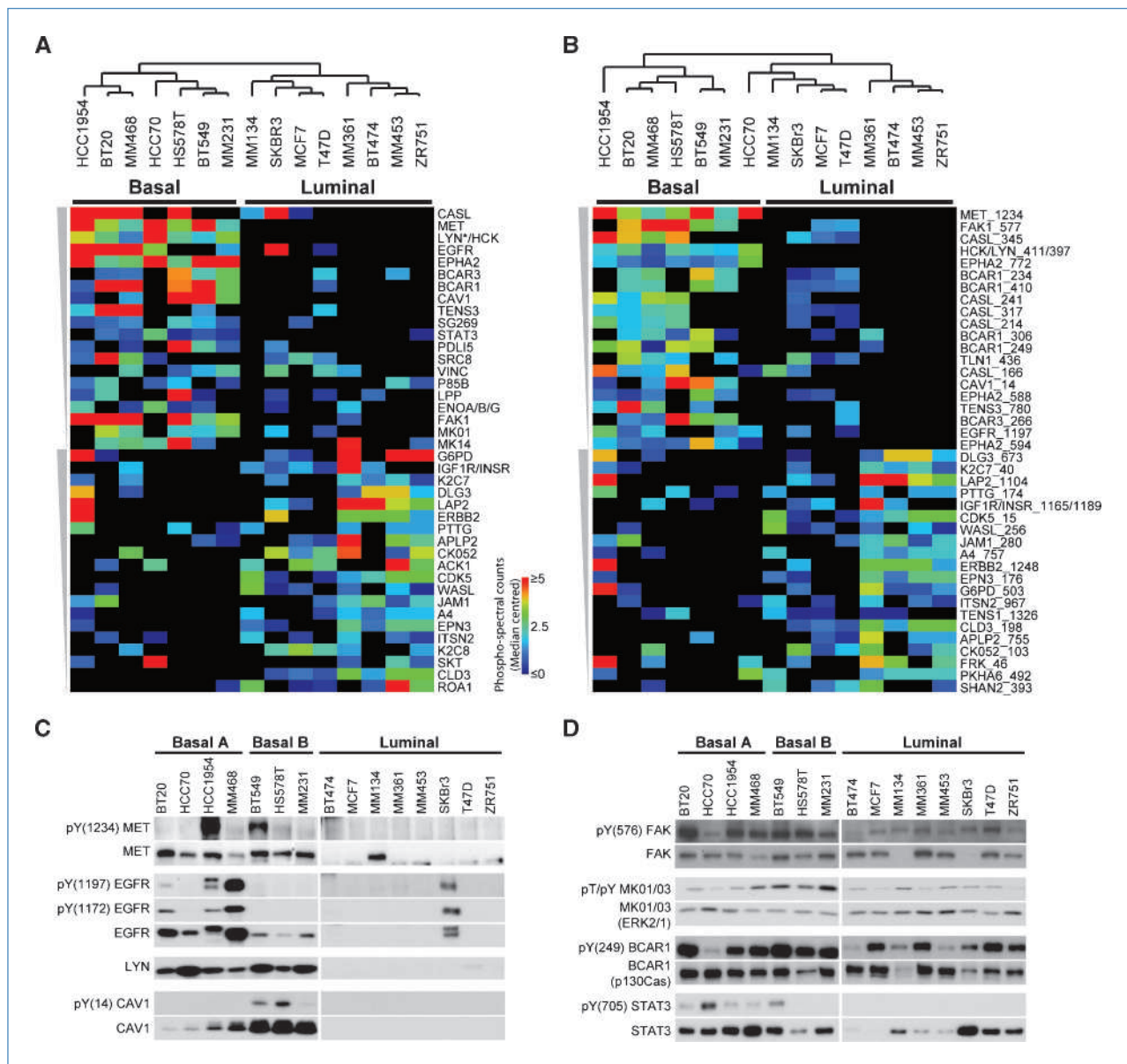


Figure 2. A, ranking of differentially phosphorylated proteins. Median-centered data for the top-ranked 20 proteins in the basal- or luminal subgroups. B, ranking of differentially phosphorylated sites. Minus the signal peptide, EGFR Y1197 is Y1173, and IGF1R/INSR Y1165/1189 are Y1135/Y1162.

C, proteins exhibiting increased total expression in the basal subset. EGFR Y1172 minus the signal peptide is Y1148. D, proteins exhibiting increased relative phosphorylation in the basal subset.

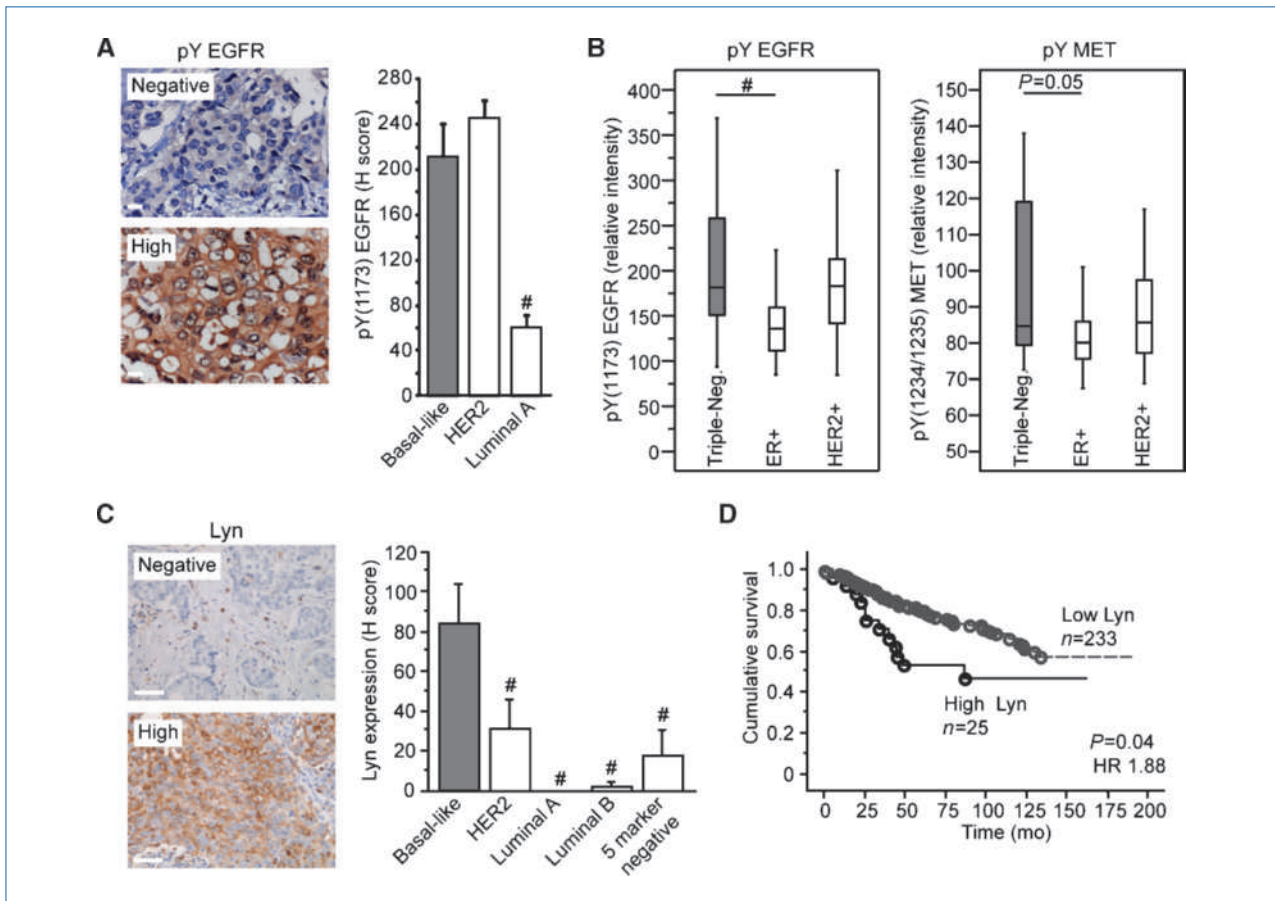


Figure 3. A, phosphorylation of EGFR Y1173 in human breast cancer specimens. Bars, SEM. B, reverse phase protein array analysis. Triple negative tumors, which are enriched for basal-like breast cancers, show significantly higher expression of EGFR pY1173 and Met pY1234/35 than ER+ (luminal) tumors by Kruskal-Wallis ANOVA. C, immunohistochemical detection of Lyn in human breast cancers. Bars, SEM. Lyn expression is significantly higher in the basal versus other subgroups. D, Kaplan-Meier curve for breast cancer–related death. #, $P < 0.0001$, for comparison of a particular breast cancer subgroup with basal/triple negative tumors.

profiling studies (11, 18). A subset of the phosphorylation events associated with basal and luminal cells are highlighted in Fig. 1B. Using data for tyrosine kinases only, the basal cell lines still clustered together (Fig. 1C). Subgroupings were also evident. For example, MDA-MB-231 and Hs578T cells formed a basal subgroup characterized by high phosphorylation of UFO (AXL), and MDA-MB-134, -453, and ZR-75-1 cells formed a luminal subgroup exhibiting enhanced phosphorylation of FGFR4.

In addition, proteins and individual phosphorylation sites were ranked according to the difference in median phosphorylation between the basal and luminal groups (Fig. 2A and B). Clustering of cell lines using these top-ranked proteins or sites led to the resolution of basal and luminal clusters that was not evident when the process was undertaken using randomly selected proteins or sites, validating the ranking process (Fig. 2; data not shown). Increased phosphorylation of several tyrosine kinases (Met, Lyn/Hck, EphA2, EGFR, and FAK) was characteristic of basal lines. In addition, we detected enhanced phosphorylation of the predicted pseudo-

kinase Sgk269, and increased abundance of a peptide corresponding to the Lyn/Hck activation loop. Western blotting revealed that although Lyn is expressed in all basal lines, Hck is undetectable (Supplementary Fig. S1), indicating that the Lyn/Hck phosphopeptide is derived from Lyn. In contrast, IGF1R/INSR, erbB2, and ACK1 exhibited increased phosphorylation in luminal breast cancer cells (Fig. 2). For all of the differentially phosphorylated kinases, increased phosphorylation was detected on sites that positively regulate kinase activity and downstream signaling. For example, Met Y1234, Lyn Y397, and FAK Y577 are activation loop sites (19), and phosphorylation of Y588 and Y594 in the juxtamembrane region of EphA2 is required for kinase activity (20). In the case of EGFR and erbB2, differential phosphorylation was predominantly on sites in the COOH-terminal tail that promote activation of the Ras/Erk pathway (21, 22).

A striking characteristic of basal breast cancer cells was increased tyrosine phosphorylation of numerous SFK substrates intimately involved in the regulation of cell migration. For example, this subgroup exhibited enhanced

phosphorylation of BCAR1 (p130Cas), its binding partner BCAR3 and other proteins previously linked to SFK signaling including Cav1, tensin-3, and Stat3 (Fig. 2; refs. 23–27). In the luminal cell lines, non-kinase tyrosine-phosphorylated proteins were more diverse in function, and involved in processes such as endocytic vesicle trafficking and the formation of cell-cell contacts.

Western blotting revealed that for Met, EGFR, Lyn, and Cav1, total protein expression was higher in the basal subgroup (Fig. 2C). In contrast, FAK, p130Cas, and Erk1/2 were expressed at similar levels in the two subgroups, but relative tyrosine phosphorylation was enhanced in basal breast cancer cells (Fig. 2D). In the case of Stat3, examples of both mechanisms were evident. These data highlight the ability of phosphoproteomic profiling to identify signaling network characteristics dependent on pathway activity, rather than gene expression.

Expression profile of candidate phosphoproteins in mouse mammary tumors and human breast cancers

To determine whether the subtype-specific signaling network characteristics of basal breast cancer cell lines *in vitro* reflect those of basal-type mammary tumors, we undertook several approaches. Elevated phosphorylation of EGFR, Met, and Lyn was characteristic of basal breast cancer cells (Fig. 2). Consistent with the profiling data, im-

munohistochemical staining of breast cancer specimens revealed that EGFR Y1173 (Y1197 including the signal peptide) phosphorylation is significantly higher in basal versus luminal A cancers (Fig. 3A), although phosphorylation was also high in the erbB2/HER2 subgroup. We were unable to detect Met pY1234/35 by immunohistochemistry on archival human breast cancer specimens. Instead, we undertook immunofluorescent staining for Met pY1234/35 on transplanted p53-null mouse mammary tumors, which exhibit a basal transcriptional signature (28), and MMTV-PyMT tumors, which are luminal. Only the former tumors exhibited positive staining (Supplementary Fig. S2). To complement these studies, we undertook reverse phase protein array analysis on an independent breast cancer cohort. This detected significantly higher EGFR Y1173 and Met Y1234/35 phosphorylation in triple-negative breast cancers, which are enriched for the basal phenotype, compared with luminal cancers (Fig. 3B). Phosphorylation on these sites was not significantly different between HER2 cancers and the other phenotypes.

We characterized the expression of Lyn over a large breast cancer patient cohort (15). High Lyn expression (> mean H score) was strongly associated with the basal subtype ($P < 0.0001$ by χ^2 analysis), but not the other subtypes, including HER2 (Fig. 3C). High Lyn expression was associated with reduced survival ($P = 0.04$, hazard ratios = 1.88, Fig. 3D), but it

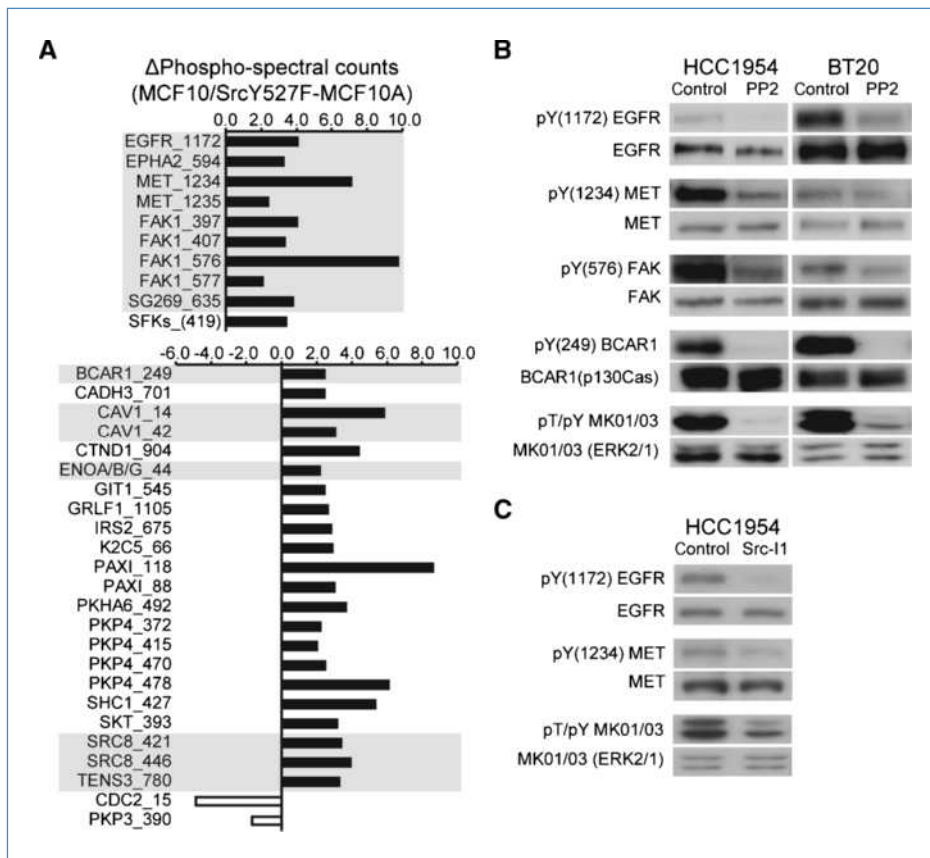
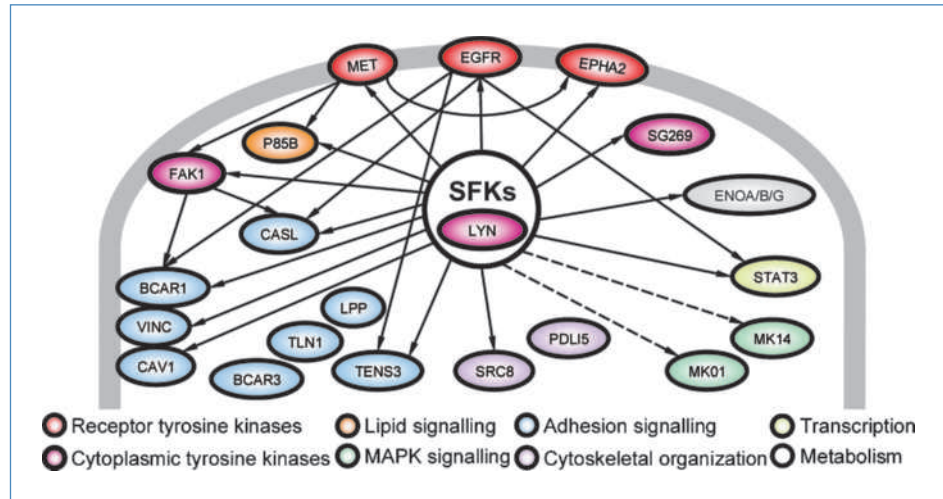


Figure 4. A, tyrosine phosphorylation changes associated with the expression of Src Y527F in MCF-10A cells. Shading: proteins with increased tyrosine phosphorylation in basal breast cancer cells relative to the luminal group (Fig. 2). B and C, phosphorylation of many basal-associated tyrosine kinases and signaling proteins is dependent on SFK activity. Cells were treated with the selective SFK inhibitors PP2 or Src-1, and cell lysates were Western blotted as indicated.

Figure 5. Basal breast cancer cells contain a prominent SFK signaling network. "Top-ranked" proteins are shown (Fig. 2) and are grouped and shaded according to function. Solid arrows indicate known phosphorylation events, as indicated by the literature or the databases PhosphoSite and Phospho.ELM. Dotted lines indicate indirect activation.



was not an independent prognostic marker in multivariate analysis with the final resolved model incorporating PR expression, *HER2* amplification, and lymph node status. These data confirm that our profiling strategy could identify tyrosine phosphorylation events characteristic of basal, versus luminal, breast cancers.

Characterization of a SFK signaling network in basal breast cancer cells

A prominent characteristic of basal breast cancer cells is enhanced phosphorylation of canonical SFK substrates (Fig. 2). To interrogate the role of SFK signaling in this cell type, we adopted two strategies. First, we used the immortalized basal mammary epithelial cell line MCF-10A (11). Expression of active Src (Src Y527F) in these cells leads to a transformed phenotype (7). Phosphoproteomic profiling was used to compare MCF-10A/SrcY527F cells with vector controls (Supplementary Tables S4 and S5, Fig. 4A). Comparison of proteins exhibiting enhanced phosphorylation in MCF-10A/SrcY527F cells and those with increased phosphorylation in basal breast cancer cells (Fig. 2) revealed a strong overlap. Notably, this overlap was not restricted to key sites on "downstream" SFK targets such as FAK and p130Cas, but also extended to the "upstream" receptor tyrosine kinases (RTK) EGFR, EphA2, and Met. Therefore, elevation of SFK activity is sufficient to enhance site-specific phosphorylation of these proteins in basal-type cells.

Second, to test whether SFK activity is required to maintain the phosphorylation of these sites in basal breast cancer cells, we used the small molecule SFK inhibitor PP2 (29). Treatment of HCC1954 and BT20 cells with this inhibitor led to a marked decrease in phosphorylation of FAK and p130Cas, as well as EGFR and Met. Furthermore, PP2 treatment almost completely abolished Erk activation (Fig. 4B). Treatment of cells with an unrelated Src inhibitor, Src-I1 (29), also inhibited EGFR, Met, and Erk activation, indicating that the effects of PP2 do not reflect off-target interactions (Fig. 4C). Overall, these data indicate

that SFK activity is a major contributor to the tyrosine phosphorylation signature characteristic of basal breast cancer cells (Fig. 5).

Sensitivity of basal breast cancer cell lines to EGFR, Met and SFK tyrosine kinase inhibitors

The identification of a prominent SFK signaling network led us to determine the biological effects of inhibiting SFK activity. Also, because basal breast cancer cells exhibit increased activation of EGFR and Met, we tested the efficacy of tyrosine kinase inhibitors (TKI) directed against these kinases. However, because the degree of activation of these RTKs in basal breast cancer cell lines varies (Fig. 2), we tested whether the sensitivity of the cell lines to corresponding TKIs depends on the target activation profile. In addition, coactivation of several tyrosine kinases in basal breast cancer cells may promote resistance to individual TKIs. Therefore, we determined whether particular combinations of inhibitors were more effective than single agents.

Three cell lines were chosen for this analysis: HCC1954 (which exhibits high phosphorylation of both EGFR and Met), BT20 (EGFR), and BT549 (Met). All three lines exhibit a phosphorylation signature characteristic of SFK signaling (Fig. 2). Proliferation of the HCC1954 and BT20 cell lines, but not BT549 cells, was significantly inhibited by the EGFR inhibitor AG1478 (Fig. 6A). When used alone, the Met inhibitor SU11274 had no effect on any line (Fig. 6A; data not shown). The SFK inhibitor PP2 inhibited the proliferation of HCC1954 and BT20 cells (Fig. 6A), and markedly increased apoptosis in the former cell line (Fig. 6B). Next, we tested combinations of TKIs. Coadministration of SU11274 and AG1478 to HCC1954 cells led to further growth inhibition compared with AG1478 alone. This effect was not evident in the other cell lines that do not exhibit high activation of both EGFR and Met (Fig. 6A; data not shown). Combining PP2 and SU11274 did not result in enhanced inhibition compared with PP2 alone for any cell line (data not shown). However, coadministration of PP2 and AG1478 to HCC1954 and

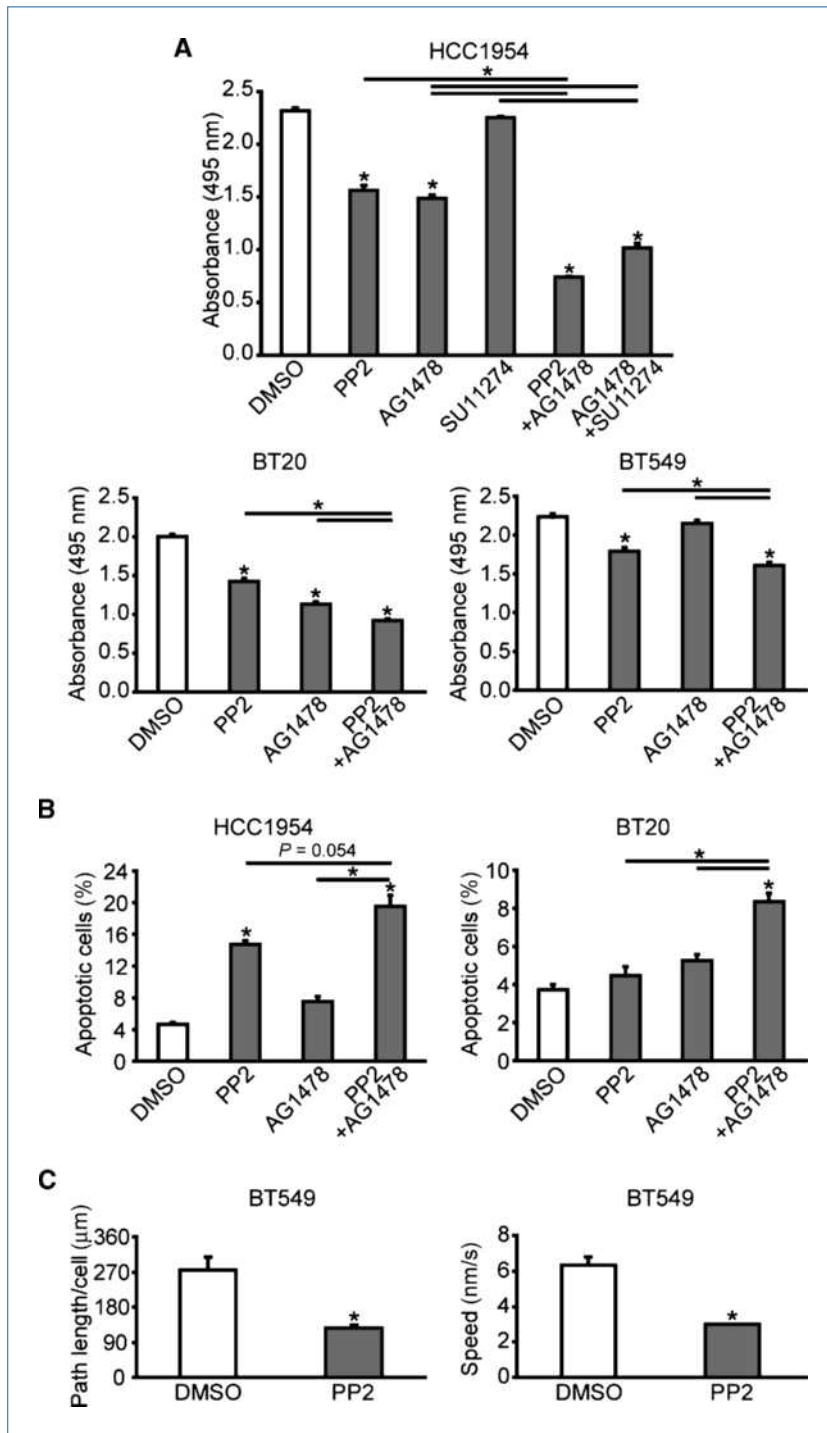


Figure 6. A, effect of particular TKIs, alone or in combination, on cell proliferation. B, effects on apoptosis. C, effect of SFK inhibition on random cell motility, as determined by live cell tracking. All data represent mean \pm SEM. *, $P < 0.05$, by ANOVA (A and B) or Student's *t* test (C). A and B, asterisk indicates significance relative to DMSO control, or as indicated.

BT20 cells led to further inhibition of proliferation compared with single agent treatments (Fig. 6A). Importantly, combining these drugs also led to increased cell death, particularly in BT20 cells (Fig. 6B). Throughout these experiments, we noted that inhibitory effects of particular TKIs on proliferation of BT549 cells, which exhibit a basal B transcriptional signature (11), were modest (Fig. 6A). However, PP2 markedly

inhibited the random motility of these cells, as indicated by a significant reduction in cell path length and speed in cell tracking experiments (Fig. 6C). Consequently, although some basal breast cancers may be refractory to growth inhibition by SFK inhibitors, these agents may still be of clinical benefit by attenuating processes linked to metastatic spread.

Overall, these data indicate that responses to TKIs in basal breast cancer cells are not uniform but are dependent on kinase activation profiles, and that combination-based approaches may be most effective. Specifically, they show that EGFR and SFKs represent attractive therapeutic targets, particularly in combination.

Characterization of the biological roles of FAK and Lyn in basal breast cancer cells

Two additional candidates identified by our profiling were subject to functional analysis: FAK, as it represents a key "node" of the basal breast cancer signaling network (Fig. 5); and Lyn, as we had confirmed the association of this SFK with basal breast cancer using our patient cohort (Fig. 3). Treatment of BT-549 and MDA-MB-231 cells with FAK TKI PF-562271 (30) led to marked inhibition of FAK phosphorylation on Y397 (Supplementary Fig. S3A). PF-562271 did not affect anchorage-dependent proliferation of these cells, or their invasion through Matrigel (data not shown). However, this TKI significantly inhibited colony growth of both cell lines in soft agar (Fig. 7A). In addition, we determined the effects of reducing FAK protein expression via siRNA-mediated knockdown (Supplementary Fig. S3B). This led to a significant reduction in anchorage-dependent cell proliferation, as well as a marked reduction in cell invasion (Fig. 7B). These data indicate that under anchorage-dependent conditions, basal breast cancer cells are more sensitive to attenuation of FAK expression than FAK kinase activity, but the latter is important for anchorage-independent growth.

Knockdown of Lyn (Supplementary Fig. S3C) did not significantly affect proliferation of HCC1954, BT-549, or MDA-MB-231 cells (data not shown). However, invasion of all three cell lines through Matrigel was markedly attenuated (Fig. 7C), indicating that Lyn is required to maintain the invasive phenotype of these cells. Overall, these data indicate that both FAK and Lyn may contribute to the aggressive nature of basal breast cancers.

Discussion

In this study, we have used a global phosphoproteomic approach to characterize phosphotyrosine-based signaling networks in breast cancer cells of contrasting phenotypes, providing a rich information resource that complements those describing genomic and transcriptomic alterations (11, 31). This approach has identified that SFKs govern a signaling network in basal breast cancer cells that extends not only downstream to canonical SFK substrates regulating cell adhesion and migration but also upstream to specific RTKs, and that Lyn is a key component of this network (Fig. 5). Subsequent functional analyses determined that SFKs transmit pro-proliferative, pro-survival and pro-motogenic signals in these cells, and that Lyn is an important regulator of cell invasion. In addition, SFKs promote tyrosine phosphorylation of specific RTKs in these cells, and this may attenuate cellular sensitivity to thera-

pies directed against these receptors. Consequently, our study strongly supports therapeutic targeting of SFKs in basal breast cancer, particularly as a component of combination therapies.

Met, EphA2, EGFR, and FAK also exhibited increased activation in basal breast cancer cells. Association of the

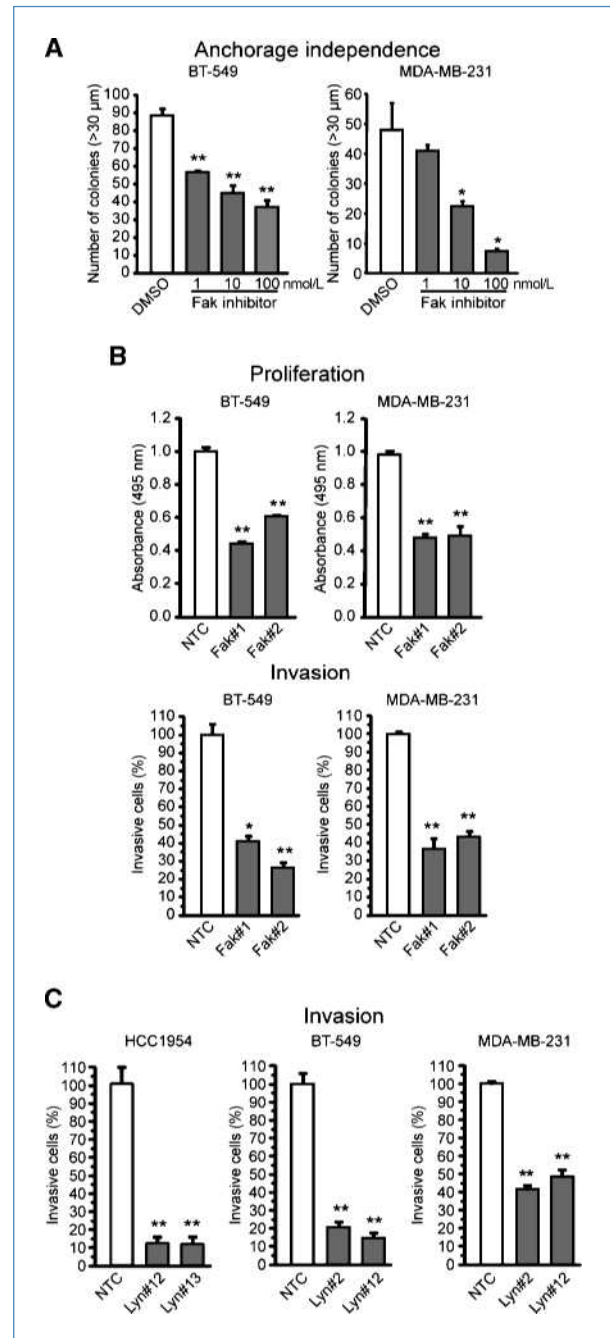


Figure 7. A, the FAK inhibitor PF-562271 inhibits anchorage-independent growth. B, FAK knockdown inhibits proliferation and invasion. The latter is expressed as a percentage of cells treated with the nontargeting control (NTC). C, Lyn knockdown attenuates cell invasion. *, $P < 0.05$ and **, $P < 0.005$ by Student's t test.

EGFR and Met with this disease subtype has been reported by other laboratories, lending further support to our approach (14, 32). However, our study highlights two important issues regarding the potential therapeutic targeting of such “basal-associated” kinases. First, phosphorylation of kinases such as Met and EGFR among the basal breast cancer cell lines was not uniform, and the cell lines exhibited differential responsiveness to anti-EGFR and anti-Met therapies that correlated with the degree of target activation (Fig. 6). Consequently, additional stratification of patients with basal breast cancer based on the expression of particular drug targets may be required to select the most appropriate therapy. Second, a striking characteristic of basal breast cancer cell lines is increased activation of multiple tyrosine kinases: all of the basal lines exhibited increased activation of Lyn, together with enhanced phosphorylation of one or more of the RTKs EGFR, Met, and EphA2 (Fig. 2). Reflecting this, basal breast cancer cells did not exhibit an obvious “addiction” to tyrosine kinases such as EGFR and Met, and combined inhibition of two types of kinase (e.g., the EGFR and SFKs) was more effective in inhibiting proliferation and survival than treatment with single agents. Therefore, basal breast cancers might exhibit a degree of resistance to individual TKIs, and combination therapies, or use of multikinase inhibitors, may prove most effective against this disease subtype. This hypothesis is supported by work from other laboratories. For example, effective growth inhibition of glioma cell lines requires combinatorial use of TKIs due to the presence of multiple activated RTKs (33).

We have interrogated publicly available data sets to determine whether any of the basal-associated phosphoproteins exhibit mutational activation or increases in gene copy number characteristic of “driver” oncogenes. Based on candidate-based studies (34–36), as well as the results of cancer genome sequencing initiatives (37, 38), somatic mutations in the “basal-associated” phosphoproteins identified by our study seem to be rare in breast cancer. In addition, somatic mutations in the basal-associated tyrosine kinases (Met, Lyn, EphA2, EGFR, and FAK) have not been detected in basal breast cancer cell lines (39). However, amplification of *EGFR* occurs in the basal breast cancer cell lines BT20 and MDA-MB-468, and the chromosomal region harboring *PTK2* (encoding FAK) is amplified in the basal breast cancer cell line MDA-MB-436 (18). Furthermore, amplification of *EGFR* occurs in primary basal breast cancers (40), and copy number gains in *PTK2* correlating with increased expression have been detected in triple-negative breast cancers (41). This suggests that screening for *EGFR* or *PTK2* copy number gains may aid the selection of basal breast cancer patients for treatment with corresponding targeted therapies. However, it should be noted that even if a basal-associated kinase does not exhibit mutations or copy number gains, this does not invalidate it as a potential therapeutic target. For example, Lyn does not exhibit common genetic aberration, but it is required for efficient basal breast cancer cell invasion, at least *in vitro*. Further analysis using appropriate animal models will reveal whether Lyn

can be targeted to prevent the multi-step process of metastatic spread.

Interestingly, whereas knockdown of FAK attenuated both monolayer proliferation and cellular invasion, FAK TKI PF-562271 did not affect either of these processes, presumably because key scaffolding functions are retained by FAK in the absence of kinase activity (42). However, PF-562271 significantly inhibited anchorage-independent growth of basal breast cancer cells. These data indicate that in the absence of cell-matrix contacts, the kinase activity of FAK is required to provide proliferative and/or survival signals that promote colony growth. Because this compound exhibits antitumor efficacy in several human cancer xenograft models (30), we now plan to test FAK inhibitors in mouse models of basal breast cancer.

Although most research on Lyn has centered on its role in hematopoietic cells, this kinase has an emerging role in solid malignancies including breast cancer, where it has recently been implicated in the regulation of epithelial-mesenchymal transition (36). Interestingly, Lyn is featured in a gene expression signature characteristic of luminal progenitor cells, which have been highlighted as candidate “cells-of-origin” for basal breast cancers (43). These data indicate that Lyn expression is not specific to malignant mammary epithelial cells and that this SFK also functions in normal development of the mammary gland. However, our finding that expression of this kinase is critical for the invasive potential of basal breast cancer cells suggests that its signaling function is “corrupted” in basal breast cancer cells, possibly due to the increased expression of RTKs such as EGFR, Met, or EphA2 that occurs in basal breast cancers. Further functional characterization of Lyn, and the associated SFK signaling network, in basal breast cancers cells is likely to yield important biological insights into the origin, initiation, and progression of basal breast cancers that can be exploited to improve patient treatment and management.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Tyrosine Phosphorylation Profiling Reveals the Signaling Network Characteristics of Basal Breast Cancer Cells

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