Tyrosine phosphorylation profiling reveals the signaling network characteristics of basal breast cancer cells

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Abstract

In order 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, 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 co-activation, inhibiting two types of activated network kinase (eg EGFR and SFKs) was more effective than single agent approaches. FAK signalling enhanced both proliferation and invasion, and Lyn was identified as a pro-invasive component of the network that in breast cancer patients is associated with a basal phenotype and poor prognosis. 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.
**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) (1). Of these, the erbB2 and basal subsets exhibit the shortest overall and relapse-free survival. The basal subtype accounts for 10-27% 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 (MS)-based phosphoproteomics (5). In the current manuscript we have utilized MS-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 development of therapeutic strategies that target this disease subtype.
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 and G Mason Research Institute) and MCF-7 (Michigan Cancer Foundation). Cell lines were authenticated by STR, SNP 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 MS 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 19.12.2008), with Homo sapiens set for taxonomy (20,411 sequences). Final filtering criteria were applied to achieve a false positive rate for peptide identifications <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 normalised for the spectral counts of GSK3alpha/beta (5). Complete linkage hierarchical clustering and assembling of heat maps were carried out with an Euclidian distance matrix upon 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 μM) or Src kinase inhibitor I (Src-I1) (Calbiochem) (10 μM) for 1 h. For proliferation assays, PP2 was used at 10 μM. AG1478 and SU11274 were obtained from Sigma and used at a final concentration of 10 μM and 1 μM, respectively. PF-562271 was obtained from Symansis and used at 1-100 nM. All drugs were dissolved in DMSO.

siRNA transfection

Lyn siRNAs “#12” and “#13” were obtained from Qiagen (Hs_LYN_12_HP and Hs_LYN_13_HP Validated siRNA). The sequence for Lyn siRNA “#2” was 5’-AAUGGUGGAAAGCAAAGUCCC-3’, for FAK siRNA “#1” and “#2” 5’-GCUAGUGACGUAUGGAUGU-3’ and 5’-ACACCAAAUUCGAGUACUA-3’, respectively. ON-TARGETplus Non-Targeting Pool (“NTC”) was obtained from Dharmacon. Cell lines were transfected with 5-20 nM 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, Hck (BD Transduction Laboratories); Yes (Transduction Laboratories); beta-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-EGFR, pY1173-EGFR (Cell Signaling Technology); Fyn, EGFR, Stat3, p130Cas, Lyn pY1234-MET, pY-576 FAK (Santa Cruz Biotechnology); 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 drug-treated for 24 h 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 tumours*

*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 wildtype FVB/N mice (12). PyMT tumours 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 tumour sections was as previously described (14).

*Immunohistochemical staining of breast cancer specimens*

The clinicopathological 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 (IHC) utilized an anti-Lyn mAb (Santa Cruz Biotechnology) and anti-EGFR pY1173 rabbit polyclonal antibody (Cell Signaling Technology). Scoring was undertaken by a specialist breast pathologist (SO'T). 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 (HR) 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 (RPPAs)

The study population consisted of 107 patients with primary breast carcinomas treated in the Edinburgh Breast Unit (Table S1). The study was approved by the Lothian Research Ethics Committee (08/S1101/41). Tumours were assessed for ER and PR positivity by IHC and HER2 positivity by IHC and FISH as previously described (16). Molecular phenotype was defined as ER+ in tumors with ER Allred scores $\geq 3$, and triple-negative in tumours with ER Allred scores $<3$, PR $<3$, HER2-negative by IHC or FISH. Tumor samples were homogenized on ice in lysis buffer (50 mM Tris pH 7.5, 5 mM EGTA pH 8.5, 150 mM 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. RPPA 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 (Tables S2 and S3). Thirty-one tyrosine phosphorylation sites were previously unidentified (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 (11) (Figure 1A). Co-clustering 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 profiling studies (11, 18). A subset of the phosphorylation events associated with basal and luminal cells are highlighted in Figure 1B. Using data for tyrosine kinases only, the basal cell lines still clustered together (Figure 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 (Figure 2A and B). Clustering of cell lines using these top-ranked proteins or sites led to 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 (Figure 2 and 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 pseudokinase SgK269, and
increased abundance of a peptide corresponding to the Lyn/Hck activation loop. Western blotting revealed that while Lyn is expressed in all basal lines, Hck is undetectable (Figure 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 (Figure 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 the EGFR and erbB2, differential phosphorylation was predominantly on sites in the C-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 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 (Figure 2) (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 (Figure 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 (Figure 2D). In the case of Stat3, examples of both mechanisms were evident. These data highlight the ability of phosphoproteomic profiling to identify signalling network characteristics dependent on pathway activity, rather than gene expression.
Expression profile of candidate phosphoproteins in mouse mammary tumours and human breast cancers

In order to determine whether the subtype-specific signaling network characteristics of basal breast cancer cell lines in vitro reflect those of basal-type mammary tumours, we undertook several approaches. Elevated phosphorylation of EGFR, Met and Lyn was characteristic of basal breast cancer cells (Figure 2). Consistent with the profiling data, immunohistochemical staining of breast cancer specimens revealed that EGFR Y1173 (Y1197 including the signal peptide) phosphorylation is significantly higher in basal versus luminal A cancers (Figure 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 tumours, which exhibit a basal transcriptional signature (28), and MMTV-PyMT tumours, which are luminal. Only the former tumours exhibited positive staining (Figure S2). To complement these studies, we undertook RPPA 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 (Figure 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 $\chi^2$ analysis), but not the other subtypes, including HER2 (Figure 3C). High Lyn expression was associated with reduced survival (p=0.04, HR = 1.88, Figure 3D), but it 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 can 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 (Figure 2). To interrogate the role of SFK signaling in this cell type, we adopted two strategies. First, we utilized 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 (Tables S4 and S5, Figure 4A). Comparison of proteins exhibiting enhanced phosphorylation in MCF-10A/SrcY527F cells and those with increased phosphorylation in basal breast cancer cells (Figure 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’ RTKs EGFR, EphA2 and Met. Therefore, elevation of SFK activity is sufficient to enhance site-specific phosphorylation of these proteins in basal-type cells.

Second, in order to test whether SFK activity is required to maintain 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 (Figure 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 (Figure 4C). Overall, these data indicate that SFK activity is a major contributor to the tyrosine phosphorylation signature characteristic of basal breast cancer cells (Figure 5).
Sensitivity of basal breast cancer cell lines to EGFR, Met and SFK tyrosine kinase inhibitors (TKIs)

The identification of a prominent SFK signaling network led us to determine the biological effects of inhibiting SFK activity. Also, since basal breast cancer cells exhibit increased activation of EGFR and Met, we tested the efficacy of TKIs directed against these kinases. However, since the degree of activation of these RTKs in basal breast cancer cell lines varies (Figure 2), we tested whether the sensitivity of the cell lines to corresponding TKIs depends upon the target activation profile. In addition, co-activation 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 exhibits high phosphorylation of both the EGFR and Met; BT20, the EGFR; and BT549, Met. All three lines exhibit a phosphorylation signature characteristic of SFK signaling (Figure 2). Proliferation of the HCC1954 and BT20 cell lines, but not BT549 cells, was significantly inhibited by the EGFR inhibitor AG1478 (Figure 6A). When used alone, the Met inhibitor SU11274 was without effect on any line (Figure 6A and data not shown). The SFK inhibitor PP2 inhibited proliferation of HCC1954 and BT20 cells (Figure 6A), and markedly increased apoptosis in the former cell line (Figure 6B). Next, we tested combinations of TKIs. Co-administration 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 (Figure 6A and data not shown). Combining PP2 and SU11274 did not result in enhanced inhibition compared to PP2 alone for any cell line (data not shown). However, co-administration of PP2 and AG1478 to HCC1954 and BT20 cells led to further inhibition of proliferation compared with single agent treatments (Figure 6A). Importantly, combining these drugs also led to increased cell death, particularly in BT20 cells (Figure 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 (Figure 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 (Figure 6C). Consequently, while 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 demonstrate that the 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 signalling network (Figure 5); and Lyn, as we had confirmed the association of this SFK with basal breast cancer using our patient cohort (Figure 3). Treatment of BT-549 and MDA-MB-231 cells with the FAK TKI PF-562271 (30) led to marked inhibition of FAK phosphorylation on Y397 (Figure 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 (Figure 7A). In addition, we determined the effects of reducing FAK protein expression via siRNA-mediated knockdown (Figure S3B). This led to a significant reduction in anchorage-dependent cell proliferation, as well as a marked reduction in cell invasion (Figure 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 (Figure S3C) did not significantly affect proliferation of HCC1954, BT-549 or MDA-MB-231 cells (data not shown). However, invasion of all 3 cell lines through Matrigel was markedly attenuated (Figure 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 (Figure 5). Subsequent functional analyses determined that SFKs transmit pro-proliferative, -survival and -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 therapies 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 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 amongst the basal breast cancer cell lines was not uniform, and the cell lines exhibited differential responsiveness to anti-EGFR and -Met therapies that correlated with the degree of target activation (Figure 6). Consequently, additional stratification of basal breast cancer patients based on expression of particular drug targets may be required in order 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 (Figure 2). Reflecting this, basal breast cancer cells did not exhibit obvious ‘addiction’ to tyrosine kinases such as the EGFR and Met, and combined inhibition of two types of kinase (eg the EGFR and SFKs) was more effective in inhibiting proliferation and survival than treatment with single agents. Therefore, basal breast cancers may 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 publically-available datasets in order 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 appear 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 harbouring 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 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
Further analysis using appropriate animal models will reveal whether Lyn can be targeted to prevent the multi-step process of metastatic spread.

Interestingly, while knockdown of FAK attenuated both monolayer proliferation and cellular invasion, the 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. Since this compound exhibits anti-tumour efficacy in several human cancer xenograft models (30), we now plan to test FAK inhibitors in mouse models of basal breast cancer.

While most research on Lyn has centred on its role in haematopoietic cells, this kinase has an emerging role in solid malignancies including breast cancer, where it has recently been implicated in regulation of epithelial-mesenchymal transition (36). Interestingly, Lyn features 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.
References

Figure Legends

Figure 1. A, Unsupervised hierarchical clustering of breast cancer cell lines based on all tyrosine-phosphorylated proteins. Classification of the cell lines based upon 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.

Figure 2. A, Ranking of differentially-phosphorylated proteins. Median-centred data for the top-ranked 20 proteins in the basal- or luminal subgroups are shown. 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. Error bars indicate SEM. B, RPPA analysis. Triple negative tumours, which are enriched for basal-like breast cancers, show significantly higher expression of EGFR pY1173 and Met pY1234/35 than ER+ (luminal) tumours by Kruskal-Wallis ANOVA. C, Immunohistochemical detection of Lyn in human breast cancers. Error bars indicate SEM. Lyn expression is significantly higher in the basal versus other subgroups. D, Kaplan-Meier curve for breast cancer-related death. For all panels, # indicates p<0.0001 for comparison of a particular breast cancer subgroup with basal/triple negative.
**Figure 4.** A, Tyrosine phosphorylation changes associated with expression of Src Y527F in MCF-10A cells. Shading: proteins with increased tyrosine phosphorylation in basal breast cancer cells relative to the luminal group (Figure 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-I1 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 (Figure 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.

**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 +/- SEM. * indicates p < 0.05 by ANOVA (A and B) or Students t-test (C). In A and B, * indicates significance relative to DMSO control, or as indicated.

**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 that for cells treated with the non-targetting control (NTC). C, Lyn knockdown attenuates cell invasion. For all graphs, * and ** indicate p<0.05 and p<0.005 by Student’s t-test.
Figure 4

A  \[\Delta\text{Phospho-spectral counts (MCF10/SrcY527F-MCF10A)}\]

-0.0  2.0  4.0  6.0  8.0  10.0

EGFR_1172
EPHA2_594
MET_1234
MET_1235
FAK1_397
FAK1_407
FAK1_576
FAK1_577
SG269_635
SFKs_(419)

-6.0  -4.0  -2.0  0.0  2.0  4.0  6.0  8.0  10.0

BCAR1_249
CADH3_701
CAV1_14
CAV1_42
CTND1_904
ENOAB/B/G_44
GIT1_545
GRLF1_1105
IRS2_675
K2C5_66
PAXI_118
PAXI_88
PKHA6_492
PKP4_372
PKP4_415
PKP4_470
PKP4_478
SHC1_427
SKT_393
SRC8_421
SRC8_446
TENSS3_780
CDC2_15
PKP3_390

B

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- pY(1172) EGFR
- EGFR
- pY(1234) MET
- MET
- pY(576) FAK
- FAK
- pY(249) BCAR1
- BCAR1(p130Cas)
- pT/pY MK01/03
- MK01/03 (ERK2/1)

C

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- pY(1172) EGFR
- EGFR
- pY(1234) MET
- MET
- pT/pY MK01/03
- MK01/03 (ERK2/1)
Figure 6

A

HCC1954

Absorbance (495 nm)

DMSO  PP2  AG1478  SU11274  PP2 + AG1478  AG1478 + SU11274

BT20

Absorbance (495 nm)

DMSO  PP2  AG1478  PP2 + AG1478

BT549

Absorbance (495 nm)

DMSO  PP2  AG1478  PP2 + AG1478

B

HCC1954

Apoptotic cells [%]

DMSO  PP2  AG1478  PP2 + AG1478

BT20

Apoptotic cells [%]

DMSO  PP2  AG1478  PP2 + AG1478

C

BT549

Path length/cell [μm]

DMSO  PP2

BT549

Speed [mm/s]

DMSO  PP2
Figure 7

A  
Anchorage independence

B  
Proliferation

C  
Invasion

Legend:
- NC: Normal cells
- L: Lung cancer cells
- L+5FU: Lung cancer cells treated with 5FU
- L+100: Lung cancer cells treated with 100 µM

** p < 0.01
* p < 0.05

Note: The figure shows the effect of different treatments on anchorage independence, proliferation, and invasion of normal and lung cancer cells.
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