LYN Is a Mediator of Epithelial-Mesenchymal Transition and a Target of Dasatinib in Breast Cancer

Yoon-La Choi1,4, Melanie Bocanegra4, Mi Jeong Kwon3, Young Kee Shin3, Seok Jin Nam2, Jung-Hyun Yang2, Jessica Kao4, Andrew K. Godwin5, and Jonathan R. Pollack4

Abstract

Epithelial-mesenchymal transition (EMT), a switch of polarized epithelial cells to a migratory, fibroblastoid phenotype, is considered a key process driving tumor cell invasiveness and metastasis. Using breast cancer cell lines as a model system, we sought to discover gene expression signatures of EMT with clinical and mechanistic relevance. A supervised comparison of epithelial and mesenchymal breast cancer lines defined a 200-gene EMT signature that was prognostic across multiple breast cancer cohorts. The immunostaining of LYN, a top-ranked EMT signature gene and Src-family tyrosine kinase, was associated with significantly shorter overall survival (P = 0.02) and correlated with the basal-like (“triple-negative”) phenotype. In mesenchymal breast cancer lines, RNAi-mediated knockdown of LYN inhibited cell migration and invasion, but not proliferation. Dasatinib, a dual-specificity tyrosine kinase inhibitor, also blocked invasion (but not proliferation) at nanomolar concentrations that inhibit LYN kinase activity, suggesting that LYN is a likely target and that invasion is a relevant end point for dasatinib therapy. Our findings define a prognostically relevant EMT signature in breast cancer and identify LYN as a mediator of invasion and a possible new therapeutic target (and theranostic marker for dasatinib response), with particular relevance to clinically aggressive basal-like breast cancer. Cancer Res; 70(6); 2296–306. ©2010 AACR.

Introduction

In breast cancer, mortality results not from tumor growth per se but from the tumor invading through normal tissue boundaries and metastasizing to distant sites. To invade and metastasize, breast cancer cells must first dissociate from one another and become motile. These events are reminiscent of epithelial-mesenchymal transition (EMT), a process that occurs during tissue patterning in normal embryonic development.

EMT is a coordinated cellular program whereby epithelial cells in layers reversibly or irreversibly convert to mesenchymal cells—fibroblast-like cells loosely embedded in extracellular matrix (1). During EMT, epithelial cells dissociate, acquire a spindly or stellate morphology, and increase motility to carry out orchestrated migrations. EMT is required for normal gastrulation and the formation of the three-layered embryo, and later for the formation of normal tissues and organs, including the heart, musculoskeletal system, and peripheral nervous system (2).

Increasing evidence suggests that in breast cancer, malignant cells co-opt the EMT program (3). EMT provides a pathway by which cancerous layers of epithelial cells (carcinoma in situ) can dissociate and become motile, leading to invasion through the basement membrane into blood vessels or lymphatics, and metastatic spread. As such, targeting EMT represents an important new therapeutic strategy for the prevention or treatment of breast cancer. A framework of molecular and cellular events underlying EMT has been elucidated (4, 5). In different cell contexts, ligands such as hepatocyte growth factor (scatter factor), epidermal growth factor (and related growth factors), and transforming growth factor β can stimulate EMT, acting through signal transduction pathways (including SRC, RAS, and phosphoinositide 3-kinase) to alter cell adhesion (through adherens junctions and desmosomes) and cell motility (through cytoskeletal reorganization). Downstream transcriptional regulators, such as Snail, Slug, and Twist, repress the expression of E-cadherin (a key mediator of epithelial cell-cell adhesion), while activating expression of mesenchymal markers, e.g., vimentin, N-cadherin, and smooth muscle actin. However, the pace of recent advances in understanding EMT suggests that much yet remains unknown.

Authors’ Affiliations: 1Department of Pathology and 2Division of Breast and Endocrine Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine; 3Laboratory of Molecular Pathology, College of Pharmacy, Seoul National University, Seoul, Korea; 4Department of Pathology, Stanford University, Stanford, California; and 5Women’s Cancer Program, Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Y-L. Choi and M. Bocanegra contributed equally to this work.

Corresponding Author: Jonathan R. Pollack, Department of Pathology, Stanford University, 269 Campus Drive, CCSR-3245A, Stanford, CA 94305-5176. Phone: 650-736-1987; Fax: 650-736-0073; E-mail: pollack1@stanford.edu.

doi: 10.1158/0008-5472.CAN-09-3141

©2010 American Association for Cancer Research.
The molecular pathways of EMT have been studied largely in the context of embryogenesis in model organisms, such as Drosophila, Xenopus, and mice, and in mammalian cell culture systems (1). The latter include Madin-Darby canine kidney cells, NBII rat bladder carcinoma cells, and NMuMG mouse mammary cancer cells, each of which can be stimulated in culture to undergo EMT. However, these canine and rodent cell culture model systems may not faithfully replicate EMT events in human breast cancer.

Breast cancer cell lines display varied morphologies when grown in culture. Some appear epithelial-like, forming cell clusters, whereas others appear more fibroblast-like (mesenchymal), with dispersed and spindle-shaped cells. The latter cell lines tend to express vimentin and to be more invasive in vitro and metastatic in vivo (6, 7), suggesting that they have undergone stable EMT conversion. These cell lines provide a useful model for studying the underpinnings of EMT in breast cancer. Here, we set out to explore gene expression patterns associated with EMT in breast cancer cells in culture and, in particular, to discover molecular signatures and biomarkers of EMT with possible prognostic, mechanistic, and therapeutic relevance.

Materials and Methods

Specimens. Breast cancer cell lines were obtained directly from the American Type Culture Collection or DSMZ, and grown in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Early-passage primary breast fibroblasts were prepared from reduction mammoplasties or prophylactic mastectomies. In brief, surgical breast tissue specimens were obtained following informed consent by the Biosample Repository staff and surgery at the Fox Chase Cancer Center. Deidentified tissue specimens were finely minced and incubated in a collagenase solution (DMEM/medium 199, 10% horse serum + collagenase, hyaluronidase, antibiotic/antimycotic, insulin, and hydrocortisone) overnight at 37°C in a rotating water bath and then centrifuged at 2,500 rpm for 10 min. The supernatant was decanted to a sterile tube and the residual tissue was rinsed several times, resuspended in culture medium, combined with the supernatant, and centrifuged as before. The resulting tissue pellet was resuspended in fibroblast medium (DMEM, 15% FBS, penicillin/streptomycin, Cipro, fungizone, and gentamicin) and was plated in a swine skin gelatin and FBS–coated flask. The tissue was permitted to attach to flasks for 24 to 48 h. Once this occurred, cells were fed twice weekly, increasing medium amounts incrementally. Cells were passaged (at a 1:2 split ratio) until characterized. A tissue microarray (TMA) was constructed from 970 clinically annotated breast cancer cases (each represented by duplicate 2-mm cores) archived at the Samsung Medical Center (Seoul, Korea). All research was conducted with institutional review board approval.

Expression profiling. Gene expression profiling was done using human exonic evidence-based oligonucleotide arrays obtained from the Stanford Functional Genomics Facility and representing 24,207 human genes. Briefly, 40 μg of sample RNA and 40 μg of "universal" reference RNA were differentially labeled with Cy5 and Cy3, respectively, then cohybridized onto the microarray in a high-volume mixing hybridization at 65°C for 40 h. Details of the array processing and sample labeling and hybridization methods have been described in ref (8). The complete microarray data are available at the Stanford Microarray Database (9) and Gene Expression Omnibus (accession GSE13915). Some of these microarray data were included in a recent study integrating genomic and transcriptional profiles of breast cancer lines (10).

Microarray data analysis. Background-subtracted fluorescence log 2 ratios were globally normalized for each array, and then mean centered for each gene (i.e., reporting relative to the average log 2 ratio across all samples). Subsequent analysis included only the 6,947 well-measured and variably expressed genes, defined as those with intensities in the Cy5 or Cy3 channel at least 1.5-fold above background in at least 80% of samples, and with at least 3-fold ratio variation from the mean in at least three samples. Differentially expressed genes were identified by two-class significance analysis of microarrays (false discovery rate <5%; ref. 11). An EMT signature was defined by combining the top-ranked 100 genes overexpressed in mesenchymal breast cancer lines compared with both epithelial breast cancer lines and normal breast fibroblasts, and in epithelial breast cancer lines compared with both mesenchymal breast cancer lines and normal breast fibroblasts. Gene Ontology (GO) term enrichment was done using FatiGO (12). The clinical relevance of the EMT signature was evaluated using publicly available microarray data for primary breast tumor cohorts (13–15). Data sets were mean centered and log transformed, and the corresponding EMT signature genes were identified by Entrez Gene ID. Breast tumors were then clustered in the space of the EMT signature genes, and the resultant two main sample branches were evaluated by Kaplan-Meier analysis.

Western blot, immunohistochemistry, and DNA sequencing. Cells were lysed in 1× radioimmunoprecipitation assay buffer. Forty micrograms of total protein lysate were electrophoresed on a 4% to 15% polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and blocked in TBST-T with 5% dry milk. Anti-LYN, p-LYN (Tyr507), SRC, p-SRC (Tyr527), and p-p130cas (Tyr410) antibodies (Cell Signaling Technology) were used at 1:1,000 dilution. After incubation with a horseradish peroxidase–conjugated secondary antibody, detection was done using an enhanced chemiluminescence kit (GE Healthcare). Band intensities were quantified by densitometry using ImageJ software (16). Immunohistochemistry was done using 4-μm sections of the breast cancer TMA. Following heat-induced antigen retrieval, anti-LYN antibody (Santa Cruz Biotechnology) was used at 1:20 dilution, with chromogenic detection by peroxidase–conjugated secondary antibody and 3,3′-diaminobenzidine reagents (Envision detection kit; DAKO). Parallel
TMA sections were stained with anti-CK5/6 (1:100; D5/16 B4, DAKO), epidermal growth factor receptor (EGFR; 1:30; E30, DAKO), HER2 (1:200; CB11, DAKO), estrogen receptor (ER; 1:100; 6F11; Novocastra), and progesterone receptor (PR; 1:50; 1A6; Novocastra) antibodies. DNA sequencing of LYN (exons 8–13, including intron-exon junctions) was done from PCR-amplified genomic DNAs (PCR primers and conditions in Supplementary Table S1), with Sanger sequencing by Geneway Research.

Small inhibitory RNA knockdown and dasatinib treatment. Synthetic 21-nucleotide small inhibitory RNAs (siRNA) directed against LYN and SRC, and a nontargeting SMART pool, were obtained from Dharmacon (sequences in Supplementary Table S2). Briefly, 200,000 cells were seeded in triplicate in six-well plates and transfected with a final concentration of 50 nmol siRNA for 16 h using Lipofectamine 2000 (Invitrogen). Dasatinib (LC Laboratories) was reconstituted in DMSO at 200 nmol/L and used at the indicated concentrations.

Cell proliferation, migration, and invasion assays. The effect of gene knockdown or drug treatment on cell proliferation was measured by quantifying the metabolic cleavage of the tetrazolium salt WST-1 (Roche Applied Science) in viable cells. Motility and invasion were quantified by the Boyden chamber assay (BD Biosciences). Briefly, 10,000 (migration) or 20,000 (invasion) cells were plated into 24-well inserts using a 0.5% to 5% FBS gradient. Cells were fixed and stained with crystal violet; cells traversing the membrane were counted. All assays were performed in triplicate, and mean values and SDs were reported. IC50 values were determined using a 0.5% to 5% FBS gradient. Cells were fixed and stained with crystal violet; cells traversing the membrane were counted. All assays were performed in triplicate, and mean values and SDs were reported. IC50 values were determined by fitting sigmoidal (four-parameter logistic) curves with Prism 4.0 software (GraphPad).

Results

Prognostic EMT signature from breast cancer cell lines. Breast cancer cell lines grown in culture display distinct morphologies, with a small subset of lines appearing more “fibroblast-like” (mesenchymal) and being more invasive (refs. 6, 7 and our observations), features suggestive of having undergone EMT. To explore the molecular variation associated with this phenotype, we used whole-genome oligonucleotide microarrays to profile the gene expression of 5 epithelial-like breast cancer cell lines (BT20, MDA157, MDA231, and MDA436), compared with a diverse set of 10 epithelial-like breast cancer cell lines (BT20, BT474, EFM19, MCF7, MDA-361, MDA-453, MDA-468, SKBR3, T47D, and UACC893) representing both luminal and basal-like subtypes (10, 17). Molecular pathologic features of cell lines and representative morphologies are shown in Fig. 1A. We also profiled five early-passage primary breast fibroblast cultures to help identify characteristic profiles of EMT distinct from fibroblasts.

Unsupervised cluster analysis of gene expression readily differentiated the three groups (epithelial, mesenchymal, normal fibroblasts; Supplementary Fig. S1), indicating robust expression differences. We therefore sought to build a signature that distinguished epithelial from mesenchymal breast cancer cells, distinct from fibroblasts (anticipating stromal “contamination” in subsequently evaluated clinical samples). We defined a 200-gene “EMT signature” by combining the top 100 genes significantly overexpressed (false discovery rate <0.05) in mesenchymal breast cancer lines compared with both epithelial breast cancer lines and fibroblasts, and the top 100 genes significantly overexpressed in epithelial breast cancer lines compared with both mesenchymal breast cancer lines and fibroblasts (Supplementary Fig. S2; Fig. 1A). Assurignly, the top 100 genes overexpressed in epithelial lines were enriched for the GO term “intercellular junction” (disrupted in EMT; including genes CLDN3, CLDN4, CLDN7, EVPL, MARVELD2, OCLN, and PKP3; corrected P = 0.001), and related GO terms, although no GO term enrichment was identified among the top 100 genes overexpressed in mesenchymal lines.

EMT is thought to underlie aggressive tumor behavior (1). To determine a possible clinical relevance of our EMT signature, we evaluated the signature genes in three different primary breast cancer cohorts using publicly available microarray data. The first cohort, Sotiriou and colleagues (13), from the John Radcliffe Hospital (United Kingdom) and Uppsala University Hospital (Sweden), comprised 169 cases of invasive ductal carcinoma, profiled with the Affymetrix U133A GeneChips. To evaluate the EMT signature, we clustered those samples in the space of the EMT signature genes (Supplementary Fig. S3) and compared clinical outcomes between the two major sample clusters by Kaplan-Meier analysis. Notably, the sample cluster associated with EMT genes overexpressed in mesenchymal lines (compared with the converse pattern of genes overexpressed in epithelial lines) showed a strong trend toward decreased relapse-free survival (P = 0.058) and significantly decreased distant metastasis-free survival (P = 0.014; Fig. 1B). A similar analysis of a second cohort, van de Vijver and colleagues (295 cases from the Netherlands Cancer Institute, profiled with Agilent oligonucleotide microarrays; ref. 14), revealed significant association with both metastasis-free (P < 0.001) and overall survival (P = 0.017; Supplementary Fig. S3). Likewise, analysis of a third cohort, Bild and colleagues (171 cases profiled with the Affymetrix U133 2.0 GeneChips; ref. 15), showed a significant association with overall survival (P = 0.015; Supplementary Fig. S3).

Where sufficient clinical annotations were available, we also evaluated the EMT signature in multivariate analysis. In the Sotiriou and colleagues cohort (13), the EMT signature was a significant independent predictor of relapse-free and distant metastasis-free survival (Table 1). In the Netherlands Cancer Institute data set, the signature was significant only when ER status was omitted from the model (data not shown).

Signature gene LYN functions in invasion. LYN is among the highly ranked EMT signature genes overexpressed in mesenchymal lines (Fig. 1A). LYN is a member of the Src-family kinases, a family of nonreceptor tyrosine kinases with roles in signal transduction, often deregulated in cancers and linked to neoplastic transformation (18). LYN was of particular interest because, as a kinase, it is “druggable” and might provide a therapeutic opportunity for targeting EMT. We first
Cell line–derived EMT signature shows prognostic relevance. A, expression profiling of morphologically distinct breast cancer cell lines defines signature of EMT. Expression profiles were compared among breast cancer cell lines exhibiting epithelial-like and mesenchymal morphologies, and normal breast fibroblasts. Cell line characteristics (7, 10) are indicated (black box means yes), along with representative photos showing cell culture morphologies (equal cell numbers plated). The EMT signature (heat map shown) comprises the top-ranked 100 genes overexpressed in mesenchymal breast cancer cells compared with epithelial breast cancer cells and normal breast fibroblasts, and in epithelial breast cancer cells compared with mesenchymal breast cancer cells and normal breast fibroblasts. The top 20 genes, including the LYN tyrosine kinase (arrow), are shown. Expression ratio (log 2) scale is shown. B, EMT signature is predictive of clinical outcome. Primary breast tumors from a publicly available microarray data set (Sotiriou and colleagues; ref. 13) were clustered in the space of the EMT signature genes, and the two major sample clusters were then compared by Kaplan-Meier analysis ($P$ values are shown). The “EMT+” group, associated with EMT genes overexpressed in mesenchymal lines (compared with the converse pattern of genes overexpressed in epithelial lines), showed increased risk of relapse (top) and distant metastasis (below); $P$ values are shown.
LYN expression was associated with the triple-negative phenotype of other clinical variables (Table 1). Notably, LYN expression was a significant prognostic factor independent of distant metastasis-free survival, most evident between 2 and 6 years after surgery (Fig. 2B). In multivariate analysis, LYN expression was associated with shorter overall survival (P = 0.02; with a trend for relapse-free survival), most evident between 2 and 6 years after surgery (Fig. 2B). In multivariate analysis, LYN expression was associated with shorter overall survival (P = 0.02; with a trend for relapse-free survival), most evident between 2 and 6 years after surgery (Fig. 2B).

To evaluate functional connections between LYN and EMT, we first assayed protein levels in breast cancer cell lines by Western blot. Both total LYN and phospho-LYN (Tyr507; indicative of LYN activation; ref. 19) were elevated in mesenchymal compared with epithelial breast cancer lines (Fig. 3A). We next used a siRNA pool to knock down LYN expression in two mesenchymal breast cancer lines (BT549 and Hs578T). Knockdown of LYN (and p-LYN), confirmed by Western blot (75–90% knockdown), led to the decreased expression of the mesenchymal markers vimentin (65–75% reduction) and N-cadherin (50–70% reduction), but not to increased E-cadherin (Fig. 3B) nor to observed morphologic changes (Supplementary Fig. S4). In cell lines, knockdown of LYN did not alter cell proliferation levels but led to significantly decreased cell migration and invasion (Fig. 3C). Transfection of individual siRNAs from the pool similarly inhibited invasion (Supplementary Fig. S5), effectively excluding possible RNAi off-target effects.

To determine whether LYN overexpression/activation might be driven by DNA amplification, we analyzed our array-based comparative genomic hybridization data for 49 breast cancer cell lines (10) and 172 breast tumors (8). LYN (residing at cytoband 8q12.1) was not found to be focally amplified, although it resided within broad gains spanning some or all of 8q in 20 of 49 (41%) breast cancer lines (including BT549 and Hs578T) and in 51 of 172 (30%) breast tumors. To determine whether LYN activity might be associated with activating mutations, we sequenced exons 8 to 13 (corresponding to the P-loop and activation segment of the kinase domain) of LYN from PCR-amplified genomic DNA of the five mesenchymal breast cancer cell lines and 50 breast tumors (LYN positive from the TMA). No mutations were identified.

**LYN is a likely target of dasatinib.** We also sought to determine whether we could inhibit LYN pharmacologically. Dasatinib is a dual-specificity tyrosine kinase inhibitor, active against both ABL and the Src-family tyrosine kinases (of which LYN is a member). Of note, dasatinib was also recently reported to show selective growth inhibition of basal-like breast cancer cell lines (20, 21). In our studies, dasatinib treatment of the mesenchymal (and also basal like) breast cancer lines BT549 and Hs578T resulted in decreased cell

### Table 1. Multivariate analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sotiriou and colleagues (13), relapse-free survival</td>
<td>EMT signature</td>
<td>2.02 (1.07–3.81)</td>
</tr>
<tr>
<td></td>
<td>ER negativity</td>
<td>1.62 (0.77–3.39)</td>
</tr>
<tr>
<td></td>
<td>Grade (2)</td>
<td>2.00 (0.92–4.36)</td>
</tr>
<tr>
<td></td>
<td>Grade (3)</td>
<td>1.13 (0.53–2.42)</td>
</tr>
<tr>
<td></td>
<td>Tumor size (2–5 cm)</td>
<td>2.66 (1.38–5.13)</td>
</tr>
<tr>
<td></td>
<td>Tumor size (&gt;5 cm)</td>
<td>5.69 (1.59–20.4)</td>
</tr>
<tr>
<td></td>
<td>Lymph node positivity</td>
<td>0.73 (0.30–1.78)</td>
</tr>
<tr>
<td>Sotiriou and colleagues (13), distant metastasis-free survival</td>
<td>EMT signature</td>
<td>2.72 (1.16–6.34)</td>
</tr>
<tr>
<td></td>
<td>ER negativity</td>
<td>1.48 (0.55–3.98)</td>
</tr>
<tr>
<td></td>
<td>Histologic grade (2)</td>
<td>2.20 (0.79–6.11)</td>
</tr>
<tr>
<td></td>
<td>Histologic grade (3)</td>
<td>1.24 (0.49–3.14)</td>
</tr>
<tr>
<td></td>
<td>Tumor size (2–5 cm)</td>
<td>5.13 (1.90–13.8)</td>
</tr>
<tr>
<td></td>
<td>Tumor size (&gt;5 cm)</td>
<td>5.91 (0.98–35.8)</td>
</tr>
<tr>
<td></td>
<td>Lymph node positivity</td>
<td>1.09 (0.40–2.94)</td>
</tr>
<tr>
<td>TMA overall survival</td>
<td>LYN positivity</td>
<td>2.29 (1.18–4.42)</td>
</tr>
<tr>
<td></td>
<td>ER negativity</td>
<td>1.28 (0.65–2.49)</td>
</tr>
<tr>
<td></td>
<td>Tumor size (2–5 cm)</td>
<td>2.36 (0.83–6.72)</td>
</tr>
<tr>
<td></td>
<td>Tumor size (&gt;5 cm)</td>
<td>7.45 (2.47–22.5)</td>
</tr>
<tr>
<td></td>
<td>Lymph node positivity</td>
<td>2.06 (1.12–3.80)</td>
</tr>
</tbody>
</table>
growth/viability (Fig. 4A), with an IC\textsubscript{50} of 1.6 and 0.30 \(\mu\)mol/L, respectively. Notably, these growth-inhibitory concentrations were, respectively, 188- and 35-fold higher than the reported dasatinib IC\textsubscript{50} of LYN tyrosine kinase activity \textit{in vitro} (8.5 nmol/L; ref. 22), suggesting that the effect on growth was likely not mediated through LYN. We also measured the effect of dasatinib on cell invasion. In BT549 and Hs578T cells, the IC\textsubscript{50} for invasion was 0.028 and 0.026 \(\mu\)mol/L (Fig. 4B), or respectively, 57- and 12-fold lower than the IC\textsubscript{50} for cell growth, and more comparable (only 3-fold higher) to the reported IC\textsubscript{50} for LYN kinase activity. Western blot confirmed dasatinib inhibition of LYN activity (i.e., p-LYN) at nanomolar concentrations (Fig. 4C).

These data are consistent with dasatinib targeting LYN to inhibit cell invasion. However, it remained possible that dasatinib was acting instead through a different Src-family kinase, in particular SRC itself, which has been linked previously to cell invasion (23). Several additional findings argue against this possibility. First, in examining transcript levels of all Src-family kinases, we found that LYN but not SRC was relatively overexpressed in invasive, mesenchymal breast cancer lines (\(P = 0.006\); Mann-Whitney \(U\) test; Fig. 5A) and, more broadly, in basal-like breast cancer cell lines (\(P < 0.001\); Supplementary Fig. S6). Consistent with this finding, SRC was also not expressed at higher protein levels (by Western blot) in mesenchymal lines (Fig. 5B). Second, siRNA-mediated knockdown of SRC, confirmed by Western blot (Fig. 5C), did not inhibit BT549 and Hs578 cell invasion (Fig. 5D), suggesting that LYN and not SRC mediates invasiveness. Last, siRNA-mediated LYN knockdown and dasatinib treatment did not show additive effects in inhibiting BT549 and Hs578 cell invasion (Fig. 5D), suggesting that LYN siRNA and dasatinib are acting through the same target (i.e., LYN).

The finding that LYN but not SRC promotes invasion in mesenchymal breast cancer lines suggests the possibility that LYN and SRC tyrosine kinases are phosphorylating distinct target proteins. Although there are many known targets of Src-family kinases (24), as a starting point, we chose to focus on p130\textsuperscript{CAS} (Crk-associated substrate), previously linked to cell motility/invasion (25, 26). In BT549 (mesenchymal) cells, knockdown of LYN but not SRC led to decreased phospho-p130\textsuperscript{CAS} (Tyr410) levels (65% reduction; Fig. 5F). Although a similar reduction was not observed in Hs578T cells (Fig. 5F), this finding nevertheless supports a likelihood that the differential effects of LYN and SRC on cell invasion are manifested through the phosphorylation/activation of distinct downstream targets. Future studies should clarify the mechanisms linking LYN to EMT phenotypes.

**Discussion**

The broad goal of our study is to explore expression patterns of EMT, using breast cancer cell lines as a model system. Comparing mesenchymal and epithelial breast cancer lines (and distinct from normal breast fibroblasts), we defined a 200-gene EMT signature that was robustly prognostic across three breast cancer microarray data sets representing independent cohorts and different microarray platforms. The
EMT signature was a significant predictor independent of clinically used prognostic factors (tumor size, grade, lymph node, and ER status) in the Sotiriou and colleagues cohort, although not in the Netherlands Cancer Institute data set, in which there was some relation with ER status. Nonetheless, the EMT signature does not seem to represent only an ER-negative/ER-positive or basal/luminal signature (despite having fewer basal-like lines in the epithelial group due to repository availability at the start of our study). Notably absent among EMT signature genes was \textit{ESR1} (ER) itself, as well as any of 89 empirically defined ER target genes (27) or key basal-luminal discriminatory genes (\textit{CAV1, CD44, EGFR, MET, ETS1, GATA3, KRT19, MME, and MSN}; ref. 28).

Although the EMT signature was prognostic, eventual clinical utility is less certain. The "prognostic space" for breast cancer is becoming increasingly crowded. Microarray-derived prognosticators include a 70-gene outcome signature ("Mammprint"; ref. 29), a 21-gene "OncotypeDx" signature (30), "intrinsic" subtypes (31), wound signature (32), hypoxia signature (33), stem cell (CD44+/CD24−/low) signature (34), and stroma signature (35), among several others. Many of these signatures identify the same poor outcome cases and are likely capturing the same underlying biology (36). Indeed, recent studies support a connection between breast cancer stem cells, the basal-like phenotype, and EMT (37, 38). Irrespective of clinical utility, our finding that the EMT signature identifies aggressive tumors supports a clinical relevance of EMT.

Among the top signature genes, we carried out additional studies of \textit{LYN}, a Src-family kinase. Members of this family of nonreceptor tyrosine kinases function in signal transduction, regulating diverse cellular activities, including growth, survival, motility, and invasion (23). Src-family kinases, foremost SRC, are also frequently deregulated in cancer, in which they

![Figure 3](https://example.com/figure3.png)

**Figure 3.** LYN overexpression contributes to invasiveness. A, LYN exhibits relative overexpression and activation in mesenchymal breast cancer lines. Western blot probed with anti-LYN, phosphorylated (activated) LYN (Tyr507), vimentin and N-cadherin (markers of EMT), E-cadherin (a marker of epithelial morphology; overnight exposure; positive control was not shown), and GAPDH (loading control). B, validation of LYN knockdown by siRNA, and effect on vimentin, N-cadherin, and E-cadherin levels. Transfected siRNA pools (LYN or control nontargeting siRNA) are indicated. C, LYN knockdown (compared with nontargeting control) does not alter cell proliferation (left; measured by WST-1 assay) but leads to significantly decreased cell migration (center) and invasion (right; measured by Boyden chamber assay). Columns, mean; bars, SD. *, \(P < 0.05\); **, \(P < 0.01\); Student’s \(t\) test. OD, absorbance.
have been linked to tumor development and progression (18). LYN itself has been studied mainly in hematopoietic cells but was recently linked to prostate cancer (in which expression was associated with growth, invasion, and metastasis; refs. 22, 39, 40), glioblastoma (41), and Ewing's sarcoma (42). In our studies, we found LYN to be expressed in 14% of breast cancers, in which immunostaining was prognostic, and associated with (although not equivalent to) the triple-negative/basal-like subtype (about half of triple-negatives were LYN positive). Of note, in breast cancer cases with positive immunostaining, most (or all) cancer cells expressed LYN rather than only those cells at the leading edge of invasion. This finding is consistent with the idea that tumor phenotypes, like EMT and metastatic potential, might be encoded in the bulk tumor (rather than in a select subpopulation; ref. 43).

In examining cell lines, we confirmed LYN overexpression and increased activity (p-LYN) in mesenchymal breast cancer lines. Knockdown experiments revealed a function of LYN in cell motility and invasion but not in cell proliferation (i.e., growth, survival). The knockdown of LYN also led to reduced vimentin and N-cadherin (EMT markers) but not increased E-cadherin, suggesting that LYN directs only a portion of the mesenchymal phenotype. Although a single potential LYN activating mutation (D385Y, in activation segment) was reported in a breast cancer case (among 80 samples screened; ref. 44), we did not identify any mutations in 55 samples. We also did not find focal DNA amplification in breast cancer lines or tumors. Therefore, LYN overexpression/activity is more likely controlled mainly by upstream regulators that remain to be defined.

Dasatinib is a dual-specificity tyrosine kinase inhibitor, with activity against both ABL and the Src-family tyrosine kinases (45). It is currently used as a second-line therapy for imatinib (Gleevec)–resistant chronic myeloid leukemia, and efficacy is being explored in solid tumors (46). Indeed, recent studies indicate the selective growth inhibition of basal-like breast cancer lines (20, 21), and clinical trials are under way (47). In our study, we found that dasatinib treatment also inhibited cell invasion and at levels comparable with...
LYN kinase inhibition and up to ~60-fold lower than required to inhibit cell growth. These findings suggest that LYN is a target of dasatinib and that invasion is a relevant end point for measuring drug response.

Although dasatinib is active against other Src-family kinases, in particular SRC, multiple lines of evidence support LYN as the presumptive target. First, LYN (but not SRC) was expressed at higher levels in the invasive, mesenchymal breast cancer lines and indeed generally at higher levels in basal-like breast cancer lines in which dasatinib was previously shown to be selectively inhibitory (21). Second, knockdown of LYN (but not SRC) in mesenchymal breast cancer lines inhibited invasion (which was also inhibited by dasatinib). Third, the effects of LYN knockdown and dasatinib

Figure 5. Relevant dasatinib target is likely LYN and not SRC. A, heat map showing microarray expression levels (mean-centered log 2 ratios; fold change indicated) of ABL and Src-family kinase genes. LYN (but not SRC) is significantly overexpressed in mesenchymal (compared with epithelial) breast cancer lines (P = 0.006; Mann-Whitney U test). Note, however, that LYN seems to be expressed in the epithelial-like (and not highly invasive) lines BT20 and MCF7, suggesting that its role in EMT may be context specific. B, Western blot shows that SRC is not relatively overexpressed or phosphorylated (Tyr527) in mesenchymal breast cancer cell lines. GAPDH serves as loading control. C, validation of SRC knockdown by siRNA. Transfected siRNA pools (SRC, LYN, or control nontargeting siRNA) are indicated; SRC levels assayed by Western blot (GAPDH serves as loading control). D, knockdown of SRC (in contrast to LYN) does not inhibit cell invasion in mesenchymal lines BT549 and Hs578T. Note that the apparent augmentation of invasion observed with SRC knockdown in Hs578T cells is not reproducible in replicate experiments. E, LYN knockdown and dasatinib treatment each significantly inhibits invasion in mesenchymal lines BT549 and Hs578T; however, the effect is not additive. Dasatinib was used at 0.03 and 0.015 μmol/L for BT549 and Hs578T, respectively. *, P < 0.05; **, P < 0.01; n.s., not significant; Student’s t test. F, knockdown of LYN (but not SRC) leads to reduced phospho-p130CAS (Tyr410) levels in BT549 cells, assayed by Western blot (GAPDH serves as loading control).
treatment were not additive, consistent with their sharing the same target. Taken together, our data support LYN as a relevant target of dasatinib in invasive breast cancer cells. Nonetheless, SRC may still function in other aspects of breast cancer pathogenesis. Indeed, SRC was recently shown to support survival of breast cancer cells in the bone marrow (48).

Our studies of LYN have important clinical implications. Foremost, our findings identify LYN as a novel target for therapy in breast cancer, with particular relevance to clinically aggressive basal-like breast cancers. These typically triple-negative tumors are not treatable by standard therapies such as ER modulators or HER2 antagonists. Recent studies suggest a promise of poly(ADP-ribose) polymerase inhibitors (49), leveraging probable tumor defects in DNA repair. Dasatinib, targeting Src-family kinases, represents an additional possible treatment (47). Importantly, our findings suggest that LYN immunostaining might be a good “theranostic” biomarker for dasatinib (or similar inhibitor) response, and incorporating LYN immunostaining into clinical trials is worthwhile. Given its role in invasion (which precedes metastasis), the inhibitory effects on LYN might be best observed in adjuvant studies, which can be lengthy and challenging.

Finally, our studies also underscore the relevance of EMT/invasion (rather than or in addition to cell growth) as a meaningful biological and clinical end point. Currently, most drug screening programs rely on assaying cell growth/cytotoxicity. Indeed, BT549 cells were thus classified as “resistant” to dasatinib (21), although clearly dasatinib inhibits BT549 cell invasion. However, tumor invasion and metastasis, not growth per se, are the real drivers of cancer mortality. Inhibiting tumor cell invasion and metastasis may represent a distinct approach to control (rather than cure) cancer. Our findings highlight the importance of incorporating phenotypes, like cell invasion, into drug screening, and designing clinical trials that use such drugs earlier, to block tumor metastasis, thereby effectively managing cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the Stanford Functional Genomics Facility for the microarray, SMD for the database support, and the members of the Pollack Laboratory (in particular Keyan Salari) for the helpful discussion.

Grant Support

NIH grants CA97139 (J.R. Pollack), CA113916 (A.K. Godwin), CA09302 (M. Bocanegra), and CA130172 (M. Bocanegra); NIH contract N01-CN-43309 (A.K. Godwin); Department of Defense (BC073467); California Breast Cancer Research Program, SKB-0135 (J.R. Pollack); and Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2009-0071010; Y-L. Cho).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 08/25/2009; revised 12/01/2009; accepted 12/21/2009; published OnlineFirst 03/09/2010.

References

LYN Is a Mediator of Epithelial-Mesenchymal Transition and a Target of Dasatinib in Breast Cancer

Yoon-La Choi, Melanie Bocanegra, Mi Jeong Kwon, et al.

Cancer Res 2010;70:2296-2306. Published OnlineFirst March 9, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-3141

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/03/08/0008-5472.CAN-09-3141.DC1

Cited articles
This article cites 47 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/6/2296.full#ref-list-1

Citing articles
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/70/6/2296.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/70/6/2296.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.