Abstract

The ability of breast cancer cells to transiently transition between epithelial and mesenchymal states contributes to their metastatic potential. Therefore, driving tumor cells into a stable mesenchymal state, as opposed to complete tumor cell eradication, presents an opportunity to pharmacologically limit disease progression by promoting an asymptomatic state of dormancy. Here, we compare a reversible model of epithelial–mesenchymal transition (EMT) induced by TGFβ to a stable mesenchymal phenotype induced by chronic exposure to the ErbB kinase inhibitor lapatinib. Only cells capable of returning to an epithelial phenotype resulted in skeletal metastasis. Gene expression analyses of the two mesenchymal states indicated similar transition expression profiles. A potently downregulated gene in both datasets was spleen tyrosine kinase (SYK). In contrast to this similar diminution in mRNA, kinome analyses using a peptide array and DNA-conjugated peptide substrates showed a robust increase in SYK activity upon TGFβ-induced EMT only. SYK was present in cytoplasmic RNA processing depots known as P-bodies formed during the onset of EMT, and SYK activity was required for autophagy-mediated clearance of P-bodies during mesenchymal–epithelial transition (MET). Genetic knockout of autophagy-related 7 (ATG7) or pharmacologic inhibition of SYK activity with fostamatinib, a clinically approved inhibitor of SYK, prevented P-body clearance and MET, inhibiting metastatic tumor outgrowth. Overall, this study suggests assessment of SYK activity as a biomarker for metastatic disease and the use of fostamatinib as a means to stabilize the latency of disseminated tumor cells.

Significance: These findings present inhibition of spleen tyrosine kinase as a therapeutic option to limit breast cancer metastasis by promoting systemic tumor dormancy.

Graphical Abstract: http://cancerres.aacrjournals.org/content/canres/79/8/1831/F1.large.jpg.
See related commentary by Farrington and Narla, p. 1756

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

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However, recent studies indicate that autophagy is critical in tumorigenesis is dynamic and highly context-dependent. During mesenchymal P-bodies form during the onset of EMT and are removed and miRNA-mediated silencing. We recently established that the proteins involved in translation repression, mRNA degradation, foci that contain mRNAs, miRNAs, and mRNA-binding proteins. These included UPF1, LIMD1, EIF4ENIF1, and CNOT2, LARP1, HNRNPK, and DDX6. All of these proteins are known to localize in mRNA processing depots known as P-bodies (13, 14). P-bodies are dynamic cytoplasmic structures, whereby quantitative readouts of phosphorylation are achieved via qPCR. This method presents a highly sensitive and quantitative means to determine kinase activity within a sample (10, 11).

To establish the mechanisms by which SYK modulates EMT, we have previously utilized a mass spectrometry approach to establish a list of substrate proteins (12). Among the substrates uniquely phosphorylated by SYK were several RNA-binding proteins. These included UPF1, LIMD1, EIF4ENIF1, CNOT2, LARP1, HNRNPK, and DDX6. All of these proteins are known to localize in mRNA processing depots known as P-bodies (13, 14). P-bodies are dynamic cytoplasmic foci that contain mRNAs, miRNAs, and mRNA-binding proteins involved in translation repression, mRNA degradation, and miRNA-mediated silencing. We recently established that P-bodies form during the onset of EMT and are removed during mesenchymal–epithelial transition (MET) by the process of autophagy (15). Similar to EMT, the role of autophagy in tumorigenesis is dynamic and highly context-dependent. However, recent studies indicate that autophagy is critical for cancer cells to overcome the stresses associated with several processes of metastasis, including survival during dormancy (16, 17).

Overall, our results strongly support the conclusion that EMP facilitates metastasis. We present DNA-conjugated peptide substrate assays as a highly sensitive, robust means to identify aggressive breast cancers. Using this approach, we establish that SYK activity is required for autophagy-mediated clearance of P-bodies during MET. Finally, our data indicate that pharmacologic inhibition of SYK could serve as a unique therapeutic approach to limit the metastatic progression of breast cancer, not through tumor cell eradication, but maintenance of disseminated cells in an asymptomatic state of dormancy.

Materials and Methods

Cell lines and reagents

The HEK293 and 4T1 cells were obtained from the ATCC while the HMLE cells were a kind gift from Sendurai Mani (MD Anderson Cancer Center, Houston, TX). The 4T1 and HMLE cells were transformed to stably express firefly luciferase and HMLE cells were transformed via overexpression of HER2 (HME2). Construction of the HME2 cells and their mesenchymal variants via treatment with TGFβ or lapatinib were described previously (5, 18). Normal murine mammary gland (NMuMG) cells were purchased from the ATCC. All cells were validated for lack of Mycoplasma contamination using the IDEXX Cell check and Impact III testing on July 24, 2018.

Immunologic assays

For the coimmunoprecipitation assay, HEK293T cells stably expressing GST-SYK and EGFP-DCP1A were lysed as described above. EGFP-DCP1A was immunoprecipitated from the soluble fraction using GFP-Trap agarose beads (Chromotek). Bound immune complexes were washed with lysis buffer and subjected to immunoblotting using antibodies against GST and GFP. In separate experiments, whole-cell lysates were analyzed by immunoblot using the following antibodies: pSYK, SYK, AFG7, Zol1, Zeb1, Slug (Cell Signaling Technology), actin (Santa Cruz Biotechnologies), or β-tubulin (DSHB). Where indicated, cells were harvested by trypsinization and stained with antibodies specific for CD44 and CD24 (BioLegend). Differential staining for these markers was analyzed by flow cytometry.

RNA sequence analysis

Parental, HER2-transformed HMLE cells (HME2-parental) were treated every 3 days with TGFβ1 (5 ng/mL) or lapatinib (1 μmol/L) for a period of 4 weeks to generate lapatinib-resistant (LAPR) and post-TGFβ mesenchymal cell conditions (5). These cells were sorted for a CD44hi phenotype (BioLegend), and RNA was isolated using E.Z.N.A (Omega Bio-Tek). RNA sequencing was conducted using the Illumina HiSeq 2500 platform. These data have been deposited on the GEO database (GSE115255).

Kinomic analyses

Lysates, from HME2 cell conditions indicated above, were analyzed on tyrosine chip (PTK) and serine–threonine chip (STK) arrays using 15 μg (PTK) or 2 μg (STK) of input material as per...
standard protocol. Three replicates of chip-paired samples were used and phosphorylation data were collected over multiple computer-controlled kinetic pumping cycles, and exposure times (0, 10, 20, 50, 100, 200 ms) for each of the phosphorylatable substrates. Slopes of exposure values were calculated, log-transformed, and used for comparison. Raw image analysis was conducted using Evolve2, with comparative analysis done in BioNavigator v6.2 (PamGene).

qPCR-based kinase assays

A peptide substrate–oligonucleotide conjugate was prepared, as described previously (10). Briefly, a selective SYK peptide substrate derived from phage display selections (SYKtide, EDP-DYEWPSA; ref. 19) was purchased from GenScript on Rink amide poly styrene resin, and the N-terminus was acetylated with 5-hexynoic acid. Peptide was cleaved from resin by incubating in a cleavage cocktail (95:2.5:2.5 TFA:H2O:TIPS) for 4 hours followed by a precipitation in cold ether. The resulting precipitate was subsequently purified by semi-prep high-performance liquid chromatography (HPLC) at a flow rate of 5 mL/minute with H2O (0.1% TEA) and MeCN (0.1% TEA) as mobile phase. The peptide was characterized by MALDI/MS. m/z: [M+H]+ calcd. for C91H139N76O44 (fig. 8D); ref. 21). After 48 hours, cells were treated with 5 µg/mL puromycin for clonal selection. Genomic DNA and cell lysates from selected colonies were analyzed by the PAGE-genotyping method (22) and immunoblot analyses to screen for clones with SYK or ATG7 knockout.

Slopes of exposure values were calculated, log2-transformed, and used for comparison. Raw image analysis was conducted using Evolve2, with comparative analysis done in BioNavigator v6.2 (PamGene).

Gene knockout studies

The dimeric CRISPR RNA–guided FokI nuclease and Csy4-based multiplex gRNA expression system was used to generate the SYK and ATG7 knockout cell lines. Two annealed target-site oligoduplexes designed by Zifit Targeter (20) and a constant region oligoduplex were assembled with BsmBI-digested pSQT1313 in a single-step ligation. A total of 3 µg of the ligated vector, 1 µg of pSQT1601-expressing Csy4 RNAse and RNA-guided FokI-dCas9 fusion nucleases, and 0.2 µg of pPABE Puro were transfected into luciferase-expressing HME2 and 4T1 cells. pSQT1313 and pSQT1601 were gifts from Keith Joung (Harvard Medical School, Boston, MA; Addgene plasmids #53370 and #53369; ref. 21). After 48 hours, cells were treated with 5 µg/mL puromycin for clonal selection. Genomic DNA and cell lysates from selected colonies were analyzed by the PAGE-genotyping method (22) and immunoblot analyses to screen for clones with SYK or ATG7 knockout. Disruption of both alleles was confirmed by DNA sequencing.

P-body formation and clearance

Cells were treated with TGFB1 (R&D Systems; 10 ng/mL) for the indicated times to induce P-bodies. For P-body clearance assays, cells were washed with PBS, and then allowed to recover in fresh media for the indicated times. In some experiments, the SYK inhibitor R406 (Selleckchem), or the autophagy inhibitors N2,4-bis(phenylmethyl)-2,4-quinoxalinediamine (DBeq; Sigma; 0.625–2.5 µmol/L), or chloroquine (Tocris; 10 µmol/L) were added during P-body clearance. For the detection of P-bodies and autophagosomes by immunofluorescence, cells were fixed with 10% ice cold methanol for 10 minutes, permeabilized with 1% Triton X-100 in PBS, and blocked with PBS containing 10% goat serum, 0.05% Tween 20, and 1 mg/mL BSA. Cells were immunostained using the indicated antibodies against DCP1A, p62, or phosphotyrosine. Bound primary antibodies were detected using AlexaFluor 488-conjugated goat anti-mouse IgG and/or AlexaFluor 594-conjugated goat anti-rabbit IgG secondary antibodies (Invitrogen). P-bodies were quantified using ImageJ to set a threshold mask (Otsu Thresholding Filter), which allowed only P-bodies (puncta) to be analyzed. The pixel range of P-bodies was set at a range between 30 and 270 units. This range was considered to have staining above background. The number of P-bodies and nuclei were counted in an image or field containing at least 25 cells to determine the number of P-bodies per cell. Data are expressed as the mean ± SEM from three independent biological replicates.

In vivo metastasis

Control and ATG7-deleted 4T1 cells engineered to express firefly luciferase were suspended in PBS (50 µL) and orthotopically engrafted onto the second mammary fat pad of 4-week-old Balb/c mice (2.5 × 104 cells/mouse; Jackson Labs). Primary tumor growth and metastasis development were

peptide was characterized by MALDI/MS. m/z: [M+H]+ at 1:100 concentration each in our sample preparation.
assessed via weekly bioluminescent imaging using the Advanced Molecular Imager (Spectral Instruments). In separate experiments, 4T1 primary tumors were engrafted onto Balb/c mice, and primary tumors were surgically excised when they reached 200 mm³. At this point, mice were split into cohorts and treated with fostamatinib (50 mg/kg; orally, once daily). Upon necropsy, lungs from all animals were removed and fixed in 10% formalin and dehydrated in 70% ethanol for visualization of pulmonary metastatic nodules and histologic analyses. All animal studies were performed in accordance with the animal protocol procedures approved by the Institutional Animal Care and Use Committee of Purdue University.

EMT score quantification

EMT metric described previously was applied to calculate EMT scores for various samples (23). A set of EMT-relevant predictors and cross-platform normalizer transcripts was extracted for each sample and then used to assign probabilities of belonging to E, hybrid E/M, or M phenotypes, as denoted by the ordered triple \( S_i = (P_E, P_{E/M}, P_M) \). Categorization was assigned on the basis of maximal value of this ordered triple, and then projected onto \([0, 2]\).

Statistical analyses

Two-way ANOVA or two-sided t tests were used where the data met the assumptions of these tests, and the variance was similar between the two groups being compared. \( P \) values of less than 0.05 were considered significant. No exclusion criteria were utilized in these studies.

Results

EMP drives metastasis

We recently established two mesenchymal cell conditions derived from a common, HER2-transformed, HME2 cell precursor (5). In one condition, EMT was induced upon prolonged continuous treatment (4 weeks) with the cytokine TGFβ1, and the other was induced upon a similar length of continuous treatment with the EGFR/HER2 kinase inhibitor, lapatinib. Importantly, while both mesenchymal states are robust, only TGFβ-induced EMT was capable of undergoing MET upon withdrawal of the exogenous stimuli (5). The resultant cell populations are illustrated in Fig. 1A via flow cytometric analysis of CD44 and CD24, where treatment and withdrawal of TGFβ1 resulted in the formation of a heterogeneous population of epithelial and mesenchymal cells (Fig. 1A). In contrast, LAPR cells derived from prolonged treatment with the kinase inhibitor remained in a mesenchymal, CD44闩 phenotype even after prolonged withdrawal of the compound. We sought to evaluate the impact of these reversible versus stable EMT events on tumor growth and metastasis. Therefore, we coengrafted the LAPR cells with the parental HME2 cells to create CD44 hi/CD44 low heterogeneous tumors, similar to the post-TGFβ1 population. The mixture of LAPR cells with parental HME2 cells did not affect primary tumor growth, and we did not observe any metastasis in mice bearing either of these tumors (Fig. 1B–D). In contrast, the post-TGFβ1-treated cells formed larger tumors, and we were able to image and isolate metastases within the long bones of one of these tumor-bearing mice (Fig. 1B–D). Ex vivo subculture of these bone metastases (HME2-BM) led to a culture that displayed a uniform epithelial phenotype.
morphology similar to that of the HME2 parental cells but with diminished cell surface expression of CD24 (Fig. 1D and E). Taken together, these data indicate that EMP is critical for the metastatic progression of this HER2 transformation model.

Reversible and irreversible EMTs have similar transition intensities

We next sought to characterize unique differences in gene expression between reversible and irreversible EMT events induced by TGFβ and kinase inhibition, respectively. Following 4 weeks of treatment with TGFβ1 or lapatinib, CD44hi cells were sorted by FACS and analyzed by RNA sequencing. Both of these cell populations were compared with nonstimulated, parental HME2 cells. Numerous established markers of EMT such as CDH1, Vimentin, Twist, and FGFR1 were similarly modulated by both TGFβ1 and lapatinib treatment (Fig. 2A). Only a limited set of genes were uniquely regulated between TGFβ1 and lapatinib-induced EMT events (Fig. 2B). Pathway analysis of these gene sets did not offer obvious mechanistic explanation for these reversible versus nonreversible EMT events (Fig. 2B; Supplementary Table S1). To investigate the notion that EMT becomes irreversible based on the intensity of an EMT, we utilized our recently developed inferential model of quantifying the extent of EMT in a given sample (23). This metric uses canonical epithelial and mesenchymal markers to compute the “EMT score” on a scale of 0 (fully epithelial) to 2 (fully mesenchymal). These analyses indicated that both TGFβ1 and lapatinib induced similar EMT intensities (Fig. 2C). These analyses suggest that the reversible nature of EMT is determined via the nature of the inducing stimuli as opposed to the intensity of the transition.

SYK activity is increased upon TGFβ-induced EMT

To further characterize posttranscriptional differences that may be regulating EMP, we conducted an analysis of kinase activity using a peptide-substrate microarray on the PamStation-12 platform. This platform is capable of quantifying the differential phosphorylation of 142 serine/threonine containing peptides and 140 different tyrosine-containing peptides upon incubation with cell lysates. This analysis detected a moderate decrease in phosphorylation of a peptide containing the autophosphorylation sites found within the activation loop of SYK, containing tyrosine 525 and 526 (Supplementary Table S2; ref. 24). However, consistent with previous studies, we observed SYK mRNA
expression to be drastically downregulated upon treatment with TGFβ (6). Moreover, use of the KM plotter analysis tool indicated that diminished SYK mRNA expression is strongly associated with decreased breast cancer patient survival (Fig. 3A; ref. 25). Therefore, we normalized the peptide phosphorylation values to SYK mRNA expression reads from our RNA sequence analyses. Use of this approach clearly demonstrated that, while SYK expression was downregulated by TGFβ-induced EMT, the activity of the remaining pool was dramatically increased (Fig. 3B). Consistent with our RNA sequence analyses, immunoblotting of whole-cell lysates demonstrated a robust decrease in the total levels of SYK in TGFβ-treated and LAPR cells (Fig. 3C). Differential phosphorylation of Y525/526 of endogenous SYK was not detectable in any of these samples, potentially due to the dramatic reduction in total SYK during EMT (Fig. 3C; Supplementary Fig. S1). Therefore, to verify enhanced SYK activity following TGFβ-induced EMT, we conducted a substrate phosphorylation assay using SYK PhageTIDE, a peptide sequence (EDPDYEQPSA) identified by phage display to be a highly specific and sensitive substrate for SYK as compared with the Y525/526 containing activation loop autophosphorylation peptide (19, 26). This peptide was conjugated to a DNA oligonucleotide, allowing for highly sensitive quantification of phosphorylated peptide via PCR following its capture using the anti-tyrosine antibody 4G10. Using this ultrasensitive approach, we were able to clearly demonstrate enhanced SYK activity specifically in HME2 cells induced to undergo EMT by TGFβ as compared with lapatinib-induced EMT (Fig. 3D). Taken together, these data clearly indicate that in contrast to diminished total expression levels of SYK, its kinase activity was upregulated in a reversible, but not in an irreversible EMT.

SYK is present in P-bodies and facilitates their clearance during MET

We have recently demonstrated that induction of EMT by TGFβ induces the formation of cytoplasmic RNA–processing complexes called P-bodies, and clearance of P-bodies through autophagy is required for reversal of EMT (15). On the basis of our previous identification of a role for SYK in promoting stress granule clearance through autophagy and our identification of multiple P-body proteins as SYK substrates, we next sought to assess a role for SYK in P-body formation and clearance (11, 27). Expression of mCherry-labeled SYK

Figure 3.
SYK activity is specifically increased following TGFβ-induced EMT. A, Kaplan–Meier analysis of recurrence-free survival based on the median expression value of SYK. Data were obtained from the indicated patient numbers using the KM plotter online analysis tool, resulting in the indicated P value. B, Substrate phosphorylation intensity determined for a peptide containing the SYK autophosphorylation (YS25/YS26) site in HME2 parental, LAPR, and TGFβ-treated cells. Data are normalized to SYK mRNA expression values determined by RNA sequence analysis for these same samples. Data are mean ± SD of three substrate phosphorylation analyses, resulting in the indicated P value. C, Immunoblot analysis of total SYK in HME2 parental, LAPR, and TGFβ-treated cells. β-Tubulin was assessed as a loading control. D, Quantification of SYK activity in the indicated cell populations using a DNA-conjugated SYK-specific substrate peptide (SYK-PhageTIDE). Data are mean ± SD of the signal for three unique 55-mer encoding constructs in a lysate sample, resulting in the indicated P values.
Control or EGFP-DCP1A to induce P-body formation. Localization of SYK into P-bodies was examined by treating the cells with TGF-β to visualize colocalization of SYK, DCP1A, and P-Tyr. After transfection, the cells were stained using antibodies against phosphotyrosine (P-Tyr), and cells were examined by fluorescence confocal microscopy to visualize colocalization of SYK, DCP1A, and P-Tyr. D, Control NMuMG cells and those stably expressing SYK-mC were left untreated (Control) or treated with TGFβ for 48 hours. Where indicated, cells were allowed to recover in the absence of TGFβ (Recovery) for an additional 24 hours. Cells were fixed and stained for DCP1A (green). E, Quantification of the average number of P-bodies per cell ± SEM (n > 150 cells/treatment) for triplicate experiments, resulting in the indicated P value. F, NMuMG cells stably expressing SYK-mC were treated with TGFβ and allowed to recover as in D. Where indicated, the autophagy inhibitor chloroquine was added during the recovery period, and cells were fixed and stained for DCP1A (green). P-bodies were quantified as above, resulting in the indicated P value.

SYK physically concentrates into P-bodies upon TGFβ-induced EMT and functions to promote their autophagic clearance during MET.

SYK activity is required for MET

To determine the necessity of SYK in the ability of cells to undergo EMT and MET, we deleted SYK from the HME2 cells using a genome editing approach (Fig. 5A; ref. 15). Complete absence of SYK led to an accumulation of P-bodies and prevented P-body clearance following TGFβ-induced EMT (Fig. 5B and C). To focus on the role of SYK kinase activity in P-body clearance and execution of the MET process, we treated cells with TGFβ and allowed them to recover in the absence or presence of the SYK inhibitor R406. Association of p62/Sequestosome-1 and LC3 targets these proteins to autophagosomes where they are degraded selectively through autophagy. Similar to DCP1A, when autophagy is inhibited p62-positive inclusion bodies accumulate (28, 29). Consistent with a role of SYK activity in autophagy-mediated clearance of P-bodies, we observed robust accumulation of p62 and DCP1A containing vesicles when R406 was included during recovery from TGFβ treatment (Fig. 5D). Following a 4-week treatment of the HME2 cells with TGFβ1, a 2-week recovery period allowed levels of Epithelial-cadherin (Ecad) to return (Fig. 5E). When R406 was added during the recovery phase, however, return of Ecad was
attenuated (Fig. 5E). Consistent with our previous report (14), addition of the autophagy inhibitor DBeQ similarly prevented cells from undergoing MET subsequent to TGFβ treatment and withdrawal (Supplementary Fig. S3). The onset of TGFβ-induced EMT can easily be visualized via bright-field microscopy with all cells displaying a mesenchymal morphology (Fig. 5F). This was also quantified by a CD44 flow cytometric phenotype (Fig. 5G). This approach clearly indicated that R406 prevents the ability of cells to undergo MET following TGFβ-induced EMT (Fig. 5E–G). These results suggest that SYK activity facilitates autophagy-mediated P-body clearance during MET.

Inhibition of autophagy stabilizes a mesenchymal phenotype and inhibits metastasis

Using an in vivo reporter system, we have previously established that highly metastatic 4T1 cells are very dynamic and undergo EMT and MET during initiation of growth in 3D culture and during in vivo tumor formation and metastasis (30, 31). To establish the necessity of autophagy-mediated MET for metastasis,
we again utilized a genomic editing approach to delete the autophagy mediator, ATG7 (Fig. 6A). Consistent with the dynamic EMP phenotype of the 4T1 cells and the requirement of autophagy for MET, deletion of ATG7 led to stabilization of a mesenchymal phenotype that included the accumulation of P-bodies (Fig. 6A and B; Supplementary Fig. S4). Correspondingly, deletion of ATG7 prevented efficient growth within a compliant 3D culture environment (Fig. 6C). Deletion of ATG7 had a minimal effect on primary tumor growth within the mammary fat pad (Fig. 6D). Histologically, ATG7-deleted tumors displayed a similar mesenchymal morphology, characteristic of 4T1 primary tumors, and cell proliferation was not affected (Fig. 6E). In contrast, deletion of ATG7 led to a complete inhibition of pulmonary metastasis as measured by longitudinal bioluminescence and endpoint enumeration of metastatic nodules (Fig. 6F–H). Taken together, these data strongly suggest that highly metastatic cells consistently transition between epithelial and mesenchymal phenotypes, and autophagy is required for the efficient execution of MET and metastatic progression.

Figure 6.
Autophagy is required for MET and metastasis. A, Genetic knockout of ATG7 (ATG7KO) in the 4T1 cells was verified by immunoblot. These same cell lysates were also analyzed for the EMT markers, Zo1, Zeb1, and Slug. Expression of actin served as a loading control. B, Control (WT) and ATG7KO 4T1 cells were stained with phalloidin to visualize differential organization of the actin cytoskeleton and DCP1A to visualize P-bodies. These cells were counterstained with DAPI to visualize the nucleus. C, Control (WT) and ATG7KO 4T1 cells were grown under single cell 3D culture conditions. Longitudinal cellular outgrowth was quantified by bioluminescence at the indicated time points. Data are normalized to the plated values and are the mean ± SD of three independent analyses, resulting in the indicated P value. D, Control (WT) and ATG7KO 4T1 cells were engrafted onto the mammary fat pad and primary tumor growth was quantified by bioluminescence at the indicated time points. Data are normalized to the injected values. E, IHC for Ki67 expression in control (WT) and ATG7KO primary tumors. F, Bioluminescent images and the corresponding gross anatomic views of lungs from control 4T1 (WT) tumor–bearing mice and those bearing ATG7KO tumors. Arrows, metastatic nodules. G, Quantification of bioluminescent radiance from the pulmonary region of WT and ATG7KO tumor–bearing mice at the indicated time points. H, Upon necropsy the numbers of pulmonary metastatic nodules were quantified for both control (WT) and ATG7KO 4T1 tumor–bearing mice. For D, G, and H, data are the mean ± SE of 5 mice, resulting in the indicated P values.
Pharmacologic inhibition of SYK stabilizes a mesenchymal morphology and inhibits metastatic outgrowth

Consistent with our ATG7 genetic data, previous studies indicate that inhibition of autophagy, through the use of chloroquine, effectively inhibits the pulmonary metastasis of 4T1 tumors (32). In attempts to improve the pharmacologic specificity of targeting autophagy as an antimetastatic therapy, we sought to evaluate the impact of SYK inhibition in the 4T1 model. In vitro, treatment of 4T1 cells with R406 stabilized a mesenchymal morphology, and potently inhibited tumor cell growth in 3D culture conditions (Fig. 7A and B). We next conducted in vivo studies using the clinically approved prodrug of R406, fostamatinib (33). To specifically focus on inhibition of metastasis, we established primary mammary fat pad tumors for a period of 2 weeks, and only after surgical resection of these primary tumors were mice treated with fostamatinib (Fig. 7C). This approach confirmed that systemic inhibition of SYK is capable of inhibiting the pulmonary metastatic outgrowth of 4T1 cells (Fig. 7D and F). Consistent with a role of SYK in MET, micrometastases that were located in the fostamatinib-treated group failed to regain Ecad expression as compared with similar-sized lesions in untreated animals (Fig. 7G).

Figure 7. Inhibition of SYK stabilizes a mesenchymal phenotype and inhibits pulmonary metastatic outgrowth. A, The 4T1 cells were treated with R406 (1 μmol/L) for 18 days, fixed, and stained with phalloidin (red) to visualize redistribution of the actin cytoskeleton and antibodies against DCP1A (green) to visualize P-body formation. Nuclei (blue) were counterstained with DAPI. B, The 4T1 cells were placed under single cell 3D culture conditions in the absence (DMSO) or presence of R406 (1 μmol/L) and cellular outgrowth within these cultures was quantitated by bioluminescence at the indicated time points. C, Schematic representation of the experimental approach. The 4T1 primary tumors were established and surgically resected. Following this procedure, mice were treated with fostamatinib (50 mg/kg; orally, once daily). D, Representative BLI images of mice treated as described in C. E, Pulmonary radiance values for control (DMSO) and fostamatinib-treated mice at the indicated time points. F, Upon necropsy, macroscopic pulmonary metastasis were enumerated in lungs of control (DMSO) and fostamatinib-treated mice. Data in E and F are the mean ± SE of 5 mice per group, resulting in the indicated P values. G, Histologic sections of similar-sized 4T1-metastases in control (DMSO) and fostamatinib-treated groups. Sections were stained with hematoxylin and eosin (H&E) or antibodies against Ecad.
Discussion

Transient suppression of a differentiated epithelial phenotype by breast cancer cells is strongly associated with an increased invasive phenotype and perpetuation of the early steps of metastasis (34). Mesenchymal cells also have an increased capacity to persist in the presence of chemotherapy (4, 35, 36). Our previous studies and our data herein are consistent with the notion that autophagy drives the MET process and efficient progression to macroscopic tumor formation (15). Our findings are strongly supported by recent studies indicating autophagy facilitates a dormant-to-proliferative switch by tumor cells within the lungs (16). Therefore, autophagy is intimately linked to the plasticity of tumor cells to transition between epithelial and mesenchymal states, processes that are critical for dissemination, drug resistance, and metastatic outgrowth.

In addition to chemotherapy, numerous studies indicate that when cells transition into a mesenchymal state, they also become highly resistant to kinase inhibitors, antibody therapies, and even immunotherapy (37–39). Therefore, the goal of complete eradication of these dormant, highly drug-resistant subpopulations may be unattainable. Herein, we explored approaches to pharmacologically maintain populations of disseminated cells in an asymptomatic state by preventing their reversion back to an epithelial state. By combined genetic and kinase activity analyses, we were able to identify SYK as being strongly activated in mesenchymal cells that are capable of reverting back to an epithelial phenotype and giving rise to lung and long bone metastases. The role of SYK in this process is to promote the removal of P-bodies through autophagy, an event that supports MET following initiation of metastatic outgrowth.

Our findings also emphasize the importance of properly interpreting data within publicly available databases that are largely based on mRNA and protein expression values (40–42). There are examples, such as HER2, where enhanced expression of kinases drives their auto activation and predicts for sensitivity to inhibitors. The corollary, however, is not well established. Indeed, mRNA expression analyses demonstrate a strong correlation between decreased SYK expression and improved patient survival, suggesting it functions as a tumor suppressor (8). However, our data clearly indicate that although SYK expression is dramatically inhibited with TGFβ, its activity is increased on a per-molecule level and it is spatially concentrated into P-bodies. The mechanisms of SYK activation and translocation to P-bodies, following TGFβ-induced EMT are yet to be established and are currently under investigation in our laboratory. However, the presence of high levels of phosphotyrosine in P-bodies and stress granules (27), suggests that SYK associates with ribonucleoprotein particles when it is in an activated state.

We also suggest the diagnostic utility of substrate kinase assays to overcome the pitfalls of gene expression analyses. Our oligonucleotide–peptide conjugate approach offers an extremely sensitive method to quantify kinase activity. The approach herein utilized a single peptide substrate optimized for SYK specificity (19). However, our qPCR-based readout presents the opportunity to utilize oligonucleotides with unique bar codes flanked by shared priming sequences linked to a variety of substrate-specific peptides, which is the subject of a forthcoming manuscript. This approach will allow for quantitative readouts of multiple kinase substrates all within a single reaction (11). Clearly, this type of enzymatic activity–based diagnostic that can be conducted in a single reaction on small amounts of tissue holds great promise for predicting personalized cancer therapeutics.

SYK is well-established as a key signaling molecule in B-cell activation. As such, fostamatinib was developed and has shown clinical efficacy for the treatment of B-cell–associated diseases such as rheumatoid arthritis and lymphoma (24, 43). Furthermore, fostamatinib was recently approved for the treatment of chronic immune thrombocytopenia. Our data suggest repurposing of fostamatinib as an effective treatment for the prevention of metastatic recurrence in breast cancer. The clinical trial parameters for this type of recurrence prevention strategy are difficult. However, fostamatinib might be particularly suited for this approach given it was developed as a long-term therapeutic for treatment of a chronic disease and thus has a very low toxicity profile (44, 45). In addition to the tumor cell autonomous effects presented herein, systemic treatment with fostamatinib also prevents B-cell–mediated promotion of a protumorigenic microenvironment (46, 47). Understanding this potential polypharmacology upon systemic treatment with fostamatinib clearly warrants further investigation.

Overall, these studies have utilized a variety of genetic, proteomic, and pharmacologic techniques to demonstrate that SYK activity and ATG7-mediated autophagy are required for MET, the vital final step in formation of macroscopic metastases. More broadly, our work also highlights an exit from the traditional pharmacologic goal of total tumor cell eradication and instead posits the concept of forced tumor dormancy for the management of stage IV breast cancer.

Disclosure of Potential Conflicts of Interest

C.D. Willey is a consultant/advisory board member for LifeNet Health. No potential conflicts of interest were disclosed by the other authors.

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References


Spleen Tyrosine Kinase–Mediated Autophagy Is Required for Epithelial–Mesenchymal Plasticity and Metastasis in Breast Cancer

Aparna Shinde, Shen D. Hardy, Dongwook Kim, et al.


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