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Award Number: W81XWH-08-1-0306

TITLE: Characterizing an EMT Signature in Breast Cancer

PRINCIPAL INVESTIGATOR: Melanie C. Bocanegra

CONTRACTING ORGANIZATION:
Stanford University, Stanford, CA 94305

REPORT DATE:
April 2010

TYPE OF REPORT:

Annual Summary

PREPARED FOR:
U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-04-2010	Annual Summary	1 APR 2008-31 MAR 2010

4. TITLE AND SUBTITLE
Characterizing an EMT Signature in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0306

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Melanie C. Bocanegra

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
AND ADDRESS(ES)
Stanford University, Stanford, CA 94305

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. 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. 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 (ñtriple-negativeò) 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, suggesting that LYN is a likely target and invasion a relevant endpoint for dasatinib therapy. Our findings define a prognostically-relevant EMT signature in breast cancer, and identify LYN as a mediator of invasion and possible new therapeutic target of dasatinib.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	10	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction

Breast cancer is the second leading cause of cancer death in women in the United States, effecting women of all races and ethnicities. Breast cancer mortality results from the tumor acquiring the ability to invade and metastasize to different sites in the body. In order to invade and mestastasize, cancer cells must first dissociate from one another and become motile. This process mirrors the epithelial-mesenchymal transition (EMT), a highly conserved process in embryonic development that occurs during tissue patterning. During EMT, epithelial cells lose polarity and the ability to make cell-cell contacts by altering the expression of several genes, ultimately leading to a more migratory, fibroblast-like "mesenchymal" cell phenotype. There is strong evidence EMT occurs, at least transiently, during breast cancer progression [1] and we propose to exploit the phenotypic differences between "epithelial-like" and "fibroblast-like" breast cancer cell lines in culture to explore the mechanisms of EMT.

Body

Our first goal was to identify gene-expression signatures of EMT from breast cancer cells in culture. A discriminatory gene-expression signature was identified from the microarray data, using supervised analysis methods. The EMT signature comprised the top ranked 100 genes overexpressed in fibroblast-like breast cancer cells compared to epithelial-like breast cancer cells and normal breast fibroblasts, and in epithelial-like breast cancer cells compared to fibroblast-like breast cancer cells and normal breast fibroblasts (Figure 1A).

Our second goal was to determine the prognostic value of the EMT signature in breast cancer. The prognostic value of the EMT gene signature was evaluated by analyzing its coordinated expression and correlation with clinical outcomes in primary breast tumors, using publicly-available clinically-annotated breast cancer microarray expression datasets. Prognostic value was determined by clustering samples in the space of the set of 200 EMT signature genes, and comparing clinical outcomes (both recurrence-free survival and disease-specific survival) among the top sample clusters by Kaplan-Meier analysis. In published microarray cohorts there was significant association between the EMT signature and clinical outcome in patients with breast cancer, which was independent of established clinical and pathological variables $p<0.05$ (Figure 1B).

Our third goal was to assess the functional role of EMT signature genes in the EMT process. We attempted to identify candidate EMT "driver" genes by intersecting the EMT signature with genes found to be locally amplified by array-based comparative genomic hybridization (array CGH); however, no linear relationships were uncovered. Additionally, we evaluated the top 20 genes (Figure 1A) involved with key EMT processes and prioritized the LYN gene, a member of the Src-family kinases, for subsequent analysis. LYN expression, assayed by immunohistochemistry on tissue microarrays, was observed in 133 of 970 breast cancer cases (14.2%) (Figure 2A), and was significantly associated with shorter overall survival ($p=0.02$), and correlated with the basal-like/triple-negative phenotype (CK5/6 and/or EGFR positive, HER2 and ER negative) (Figure 2B). LYN overexpression in fibroblast-like breast cancer cell lines relative to epithelial-like cell lines was verified using western blot (Figure 3A).

In order to assess LYN's role as a regulator of EMT, we performed RNA interference (RNAi) on fibroblast like breast cancer cell lines to knockdown LYN levels. Western blot confirmed a 70% reduction of both active and total forms of LYN, as well as altered EMT molecular profiles (e.g. loss of vimentin) in response to LYN knockdown (Figure 3B). Although phase-contrast microscopy did not yield altered morphologes (i.e. loss of cell-cell contact), migration and invasion were significantly downregulated in response to LYN knockdown (Figure 3C). We also examined the expression profiles the Src-family of kinases in a cohort of 50 breast cancer cell lines and discovered that LYN expression is most is significantly correlated with the basal-like breast cancer cell lines (Figure 4).

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 (Finn *et al.*, 2007; Huang *et al.*, 2007). In our studies, dasatinib treatment of the mesenchymal (and also basal-like) breast cancer lines BT549 and Hs578T resulted in decreased cell growth/viability (Fig. 5A), with an IC_{50} (50% inhibitory concentration) of 1.6 μM and 0.30 μM respectively. Notably, these growth inhibitory concentrations were respectively 188- and 35-fold higher than the reported dasatinib IC_{50} of LYN tyrosine kinase activity *in vitro* (8.5 nM) (Nam *et al.*, 2005), suggesting 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_{50} for invasion was 0.028 μM and 0.026 μM (Fig. 5B), or respectively 57-fold and 12-fold lower than the IC_{50} for cell growth, and more comparable (only 3-fold higher) to the reported IC_{50} for LYN kinase activity. Western blot confirmed dasatinib inhibition of LYN activity (i.e. p-LYN) at nanomolar concentrations (Fig. 5C).

Key Research Accomplishments

- An EMT signature, derived by comparing morphologically distinct breast cancer cells, exhibits prognostic utility.

- LYN tyrosine kinase, a highly ranked EMT signature gene, is overexpressed in a subset of primary breast tumors, and is associated with basal-like phenotype and unfavorable outcome.

- RNA-interference (RNAi) knockdown studies demonstrate a role of LYN in promoting cell motility and invasion.

- 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 invasion a relevant endpoint for dasatinib therapy

Reportable Outcomes

"Prognostic signature of epithelial-mesenchymal transition in breast cancer highlights role of LYN in invasion and likely target of dasatinib." 2009 American Association for Cancer Research Conference, Denver, CO

Yoon-La Choi[1,2,*], **Melanie Bocanegra[2,*]**, Mi Jeong Kwon[3], Young Kee Shin[3], Seok Jin Nam[4], Jung-Hyun Yang[4], Jessica Kao[2], Andrew K. Godwin[5], Jonathan R. Pollack. LYN is a mediator of epithelial-mesenchymal transition and target of dasatinib in breast cancer**;** Manuscript Submitted 9/1/2009: *Cancer Research*

Conclusions

Our findings define a prognostically-relevant EMT signature in breast cancer, and implicate LYN as a mediator of invasion and possible new therapeutic target with particular relevance to clinically-aggressive basal-like/triple-negative breast cancer. Furthermore, LYN belongs to a class of tyrosine kinases (Src-family of protein kinases) which several small molecule inhibitors exist. We are currently focusing on understanding LYN's role as a theragnostic target for the drug dasatinib, presently in clinical trials for the treatment of basal-like breast tumors [2]. Additionally, future studies will center on supporting the RNAi studies with overexpression studies of LYN in "epithelial-like" breast cancer cell lines as outlined in the original statement of work.

Taken together, this body of work furthers our understanding of the molecular mechanisms tumors utilize to invade and metastasize. Due to LYN's significant role in cell motility and correlation to the invasive basal-like breast tumors, it's a promising molecular marker for predicting drug sensitivities which will ultimately improve clinical response.

References

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4. Nam et al. (2005). Action of the Src family kinase inhibitor, dasatinib (BMS-354825), on human prostate cancer cells. Cancer Res 2005;65:9185-9.

Appendices: None

Personnel Supported

Melanie C. Bocanegra

Supporting Data: *Figure Legends*

Figure 1. Cell line derived EMT signature shows prognostic relevance. (A) Expression profiling of morphologically distinct breast cancer cell lines defines signature of EMT. The EMT signature (heatmap shown) comprises the top ranked 100 genes overexpressed in fibroblast-like breast cancer cells compared to epithelial-like breast

cancer cells and normal breast fibroblasts, and in epithelial-like breast cancer cells compared to fibroblast-like breast cancer cells and normal breast fibroblasts. The top 20 genes, including the LYN tyrosine kinase (arrow), are shown. **(B)** EMT signature is prognostically relevant. Primary breast tumors from a publically-available microarray dataset (Sotiriou et al., JNCI, 2006) are clustered in the space of the EMT signature genes, then the two major sample clusters are compared by Kaplan-Meier analysis (*P*-values shown).

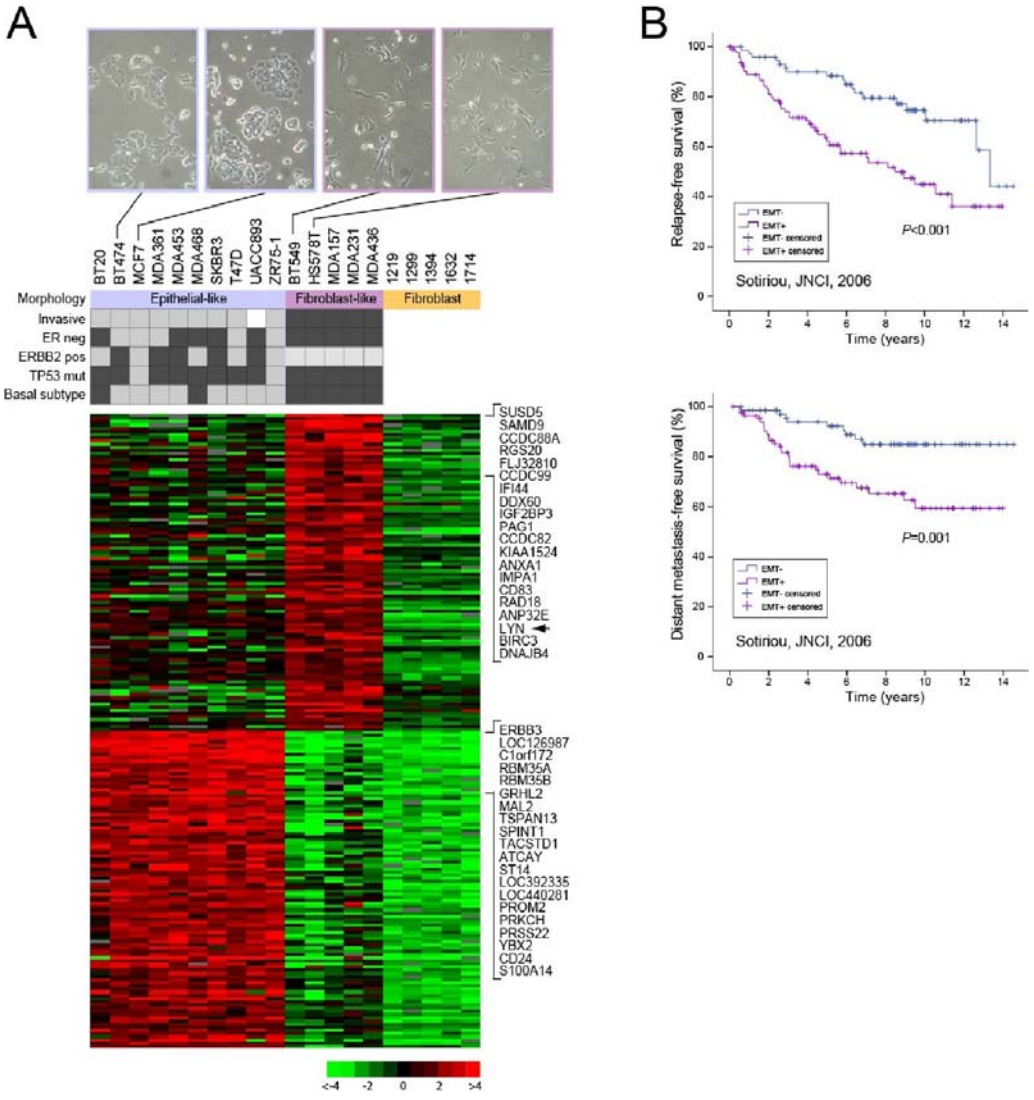
Figure 2. LYN immunostaining is associated with unfavorable outcome. (A) LYN immunostaining. Shown are representative breast cancer cases negative (arrow identifies region with tumor cells) or positive for expression of the LYN tyrosine kinase. **(B)** Kaplan-Meier analysis of LYN immunostaining in relation to relapse-free and overall survival.

Figure 3 LYN overexpression contributes to invasiveness. (A) LYN exhibits relative overexpression (by Western blot) in fibroblast-like breast cancer cell lines. **(B)** Validation of LYN knockdown by siRNA. **(C)** LYN knockdown leads to significantly decreased cell migration (*left*) and invasion (*right*) (*, $P<0.05$, **, P<.01).

Figure 4. LYN is overexpressed in basal-like breast cancer cell lines. Heatmap of microarray expression profiles of SRC family kinase genes in a collection of 50 breast cancer cell lines (clustering based on ~5000 variably expressed genes). LYN is significantly overexpressed in basal-like lines ($P<0.01$), previously reported to be selectively sensitive to dasatinib.

Figure 5. Dasatinib inhibits cell invasion at concentrations that inhibit LYN. Dasatinib effect (dose-response curve) on **(A)** cell viability and **(B)** cell invasion, for BT549 cells (*left*) and Hs578T cells (*right*). IC_{50} values (indicated) were determined from sigmoidal (four-parameter logistic) curves. **(C)** Verification in BT549 cells that dasatinib treatment leads to decreased phospho (activated)-LYN, assayed by Western blot.

Figure 1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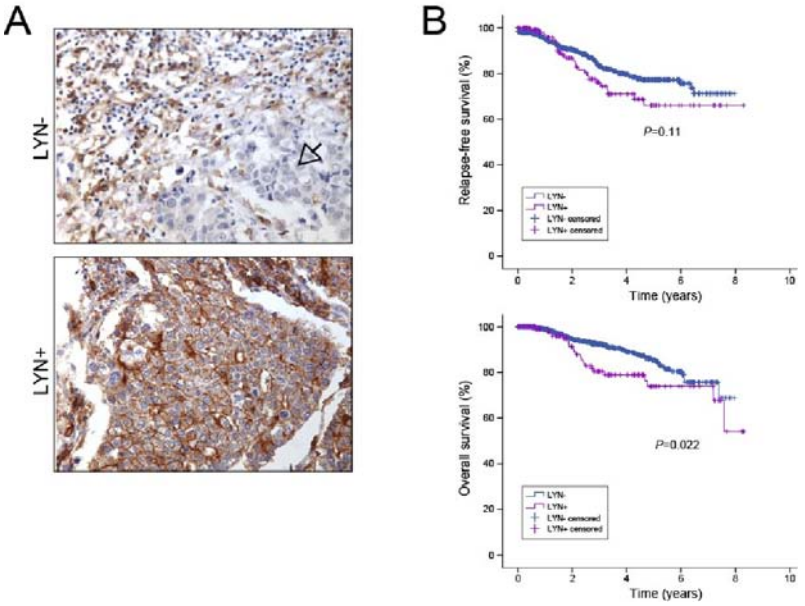
B
Relapse-free survival (%)
EMT-
EMT+
EMT- censored
EMT+ censored
P<0.001
Sotiriou, JNCI, 2006
Time (years)
Distant metastasis-free survival (%)
P=0.001
Sotiriou, JNCI, 2006
Time (years)

Figure 2



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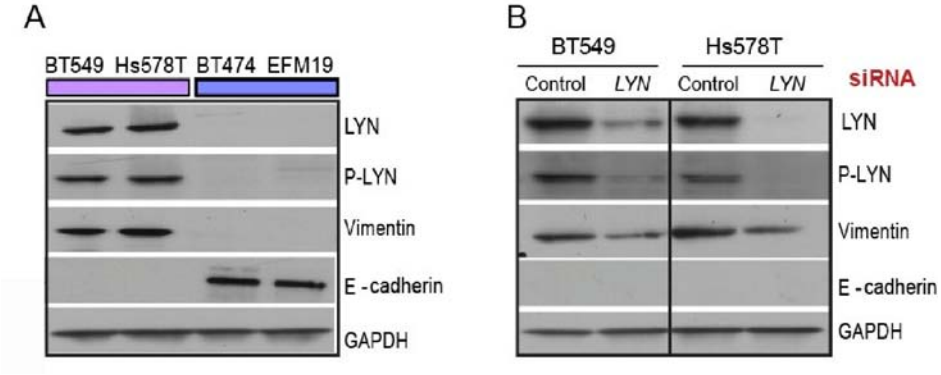

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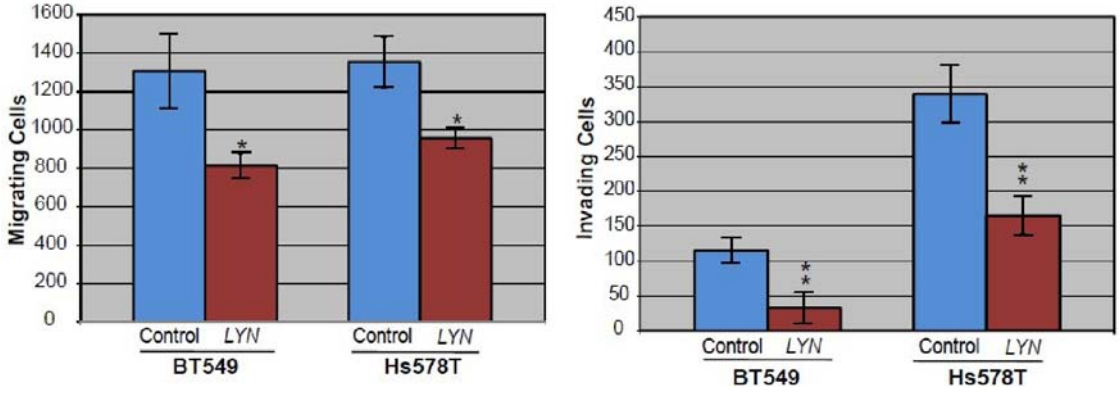

Figure 4

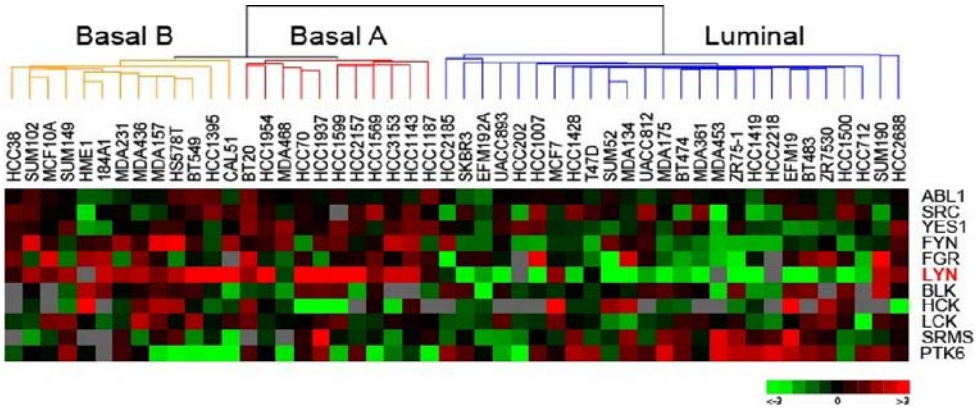

Figure 5