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14. ABSTRACT

Our project aims to use a molecular signature strategy to develop new therapeutics against metastatic breast cancer by targeting the Epithelial-to-Mesenchymal Transition (EMT) pathway. The project has been pursued in a step-wise fashion: We first used RNA-seq to define the molecular signature linked to the gene expression program associated with EMT. We next conducted chemical screening by following the EMT program on a novel technology platform we developed. After successful accomplishment of the first two steps, we selected a panel of leading compounds identified from the initial screen to test on cell and animal models to evaluate their effects in inhibit breast cancer metastasis. At the conclusion of the project, we have confirmed the functional property of identified compounds in inhibiting specific EMT-associated gene expression program on our model cell line. However, as we pointed out in our previous annual reports, we may have sufficient time to compete the study on animal models, which takes time. Importantly, the support from DOD has played a vital role for us to initiate this important project and we intend to continue the project with whatever resources we can identify in the future.

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Introduction

This project was designed to use a molecular signature strategy to develop new therapeutics against metastatic breast cancer. The strategy is based on published pathway-centric technology developed in my lab on a prostate cancer model (Li et al., 2012b). The goal of this project was to define a panel of genes that are tightly associated with breast cancer transition from initial epithelial morphology to mesenchymal-like cells. This process, known as the EMT transition, has been shown to play a critical role during breast cancer metastasis.

While it has been generally accepted that EMT is critical for cancer cells to disseminate, the reversal of EMT known as MET is thought to also play a critical role for circulating cancer cells to settle down in distant organs. It both EMT and MET are important for cancer metastasis, inhibition of one may accelerate the other. However, it has been unclear whether EMT and MET are simple reversal of one another, which imposes a key scientific question in the field of cancer biology.

We propose to approach this problem by identifying small molecules that can block EMT and MET, which may allow us to understand differential requirements for the processes. This may lead to the discovery of new therapeutic strategies that may be uniquely applied in cancer treatment.

In our experimental strategy, we first selected a triple negative breast cancer cell line to define the EMT-associated gene expression program. We next prepared a set of probes that enabled us to detect the gene signature associated with the EMT program in a high throughput assay. Based on this functional readout, we conducted small molecular screens against such a collection of responsive genes to identify candidate hits capable of selectively blocking the expression of the cohort of the signature. We have now succeeded in this step and confirmed a set of leading compounds on the secondary screen. We are now at the stage of testing the leading compounds on multiple other cell and animal models to determine their biological effects in inhibiting breast cancer metastasis to distal organs.

Body

We initially proposed three aims for this project:

Aim 1: Define the gene signature associated with the EMT program.

Aim 2: Screen for small molecules that can specifically repress the EMT program.

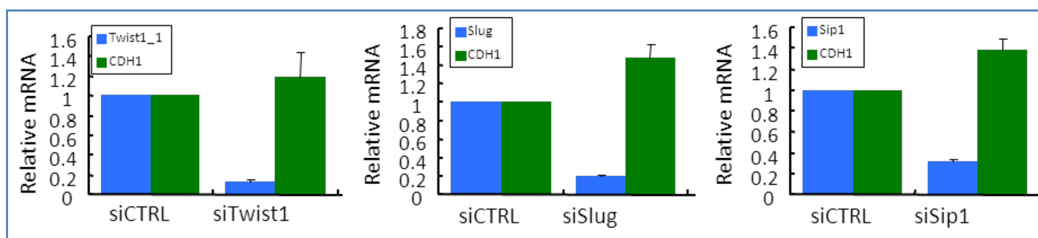
Aim 3: Test the effect of drug leads on cell and animal models.

Below, I described specific experiments we have performed toward the set goals in each specific aim:

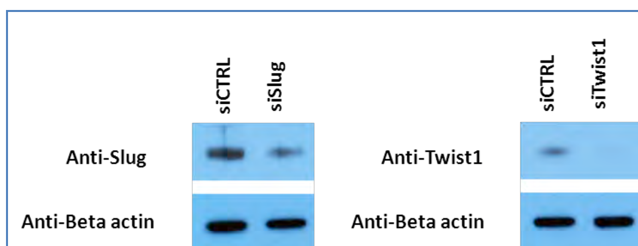
Aim 1: Define the gene signature associated with the EMT program

(1) Performed RNAi on SUM1315 cells against Twist, Snail, Slug, and SIP1
(2) Confirmed the RNAi efficiency by Western blotting
(3) Conducted RNA-seq analysis on both wt and RNAi knockdown cells
(4) Conducted bioinformatics analysis of the RNA-seq data to define regulated genes in each case
(5) Deduced specific and common gene signatures induced by individual EMT-associated transcription factors
(6) Validated a subset of regulated genes by real time PCR

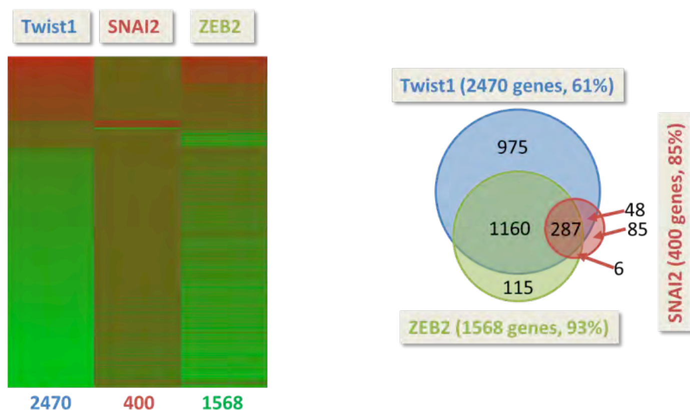
We have carried out RNAi against Twist1, Slug (SNAI2) and Sip1 (ZEB2) on SUM1315 cells (a cell line characteristic of triple negative breast cancer cells). As shown in the figure, the knocking-down efficiency determined by RT-qPCR is high in all three cases, while control RNAi showed little effect. The RNAi effects appear quite specific, as there were no measurable effects on a non-target gene (CDH1).



We confirmed efficient down-regulation of targeted Slug and Twist by Western blotting. As shown in the figure below, we achieved significant reduction of both proteins. The effect is especially striking on Twist. We have not yet done Western blotting with anti-Sip1 because of the poor quality of the antibody in our hands. However, the knockdown efficiency was confirmed by RT-qPCR.



The success of these initial experiments allowed us to initiate genome-wide analysis of genes regulated by these transcription factors. We developed a robust RNA-seq strategy by determining the digital information on the 3' end of each transcript expressed from the human genome (Fox-Walsh et al., 2011). We applied this technology to detect genes responsive to knockdown of Twist, Snail, and ZEB2. Heat map of altered gene expression is shown below on the left.



As expected, knockdown of each gene induced a unique spectrum of responses; however, there is a significant overlap among responsive genes to all three genes involved in the EMT transition (see the Venn diagram above on the right). By GO term analysis, these common 287 genes are enriched in translation and cellular metabolic processes.

We validated some of the responsive genes by RT-qPCR. In conclusion, we have accomplished all set goals in the first aim.

Aim 2: Screen for small molecules that can specifically repress the EMT program

(1) Designed and tested oligo pairs targeting the defined gene signature
(2) Tested cell culture conditions for chemical screening
(3) Treated the model cell line with the collection of 4,500 human experienced compounds library
(4) Identified a panel of lead compounds
(5) Confirmed and then determined EC50 for 40 lead compounds in a secondary screen

Based on the RNA-seq results, we selected 205 genes (202 common genes + Twist1, Sip1 and Slug) to design RASL-seq oligo pairs (Li et al., 2012a). We also included 26 house-keeping genes in this oligo pool as internal specificity controls. As described in the proposal, this is an evolving process. After initial design and

synthesis, we had to eliminate those that gave rise to too many counts (this will consume our sequence space) and compared the results with our genome-wide and RT-qPCR results to ensure that individual oligo pairs report the anticipated effects in regulated gene expression. On several cases, we had to re-design and re-synthesize those that are not efficient in targeting intended genes. Using the finally selected oligos, we prepared a working pool and conducted initial screen on our robot to determine if the cell culture system and the oligo pool could be adapted to high throughput operation.
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Having established the assay conditions, we shipped the cell line to Dr. Sheng Ding's lab at Gladstone/UCSF to test culture in the 384-well format and determine the growth/treatment conditions to ensure optimal cell density for drug treatment. They then carried out the screen with a collection of ~5000 human experienced compounds.

After the screen, they shipped the cell lysates back to my lab at UCSD, all in original 384 well plates. We performed RASL-seq based on our published technology (Li et al., 2012b) to identify lead compounds that can specifically intervene the EMT signature. We identified a collection of ~40 compounds, which we subjected them to the secondary screen to confirm their efficacy and specificity and determine their dosage-dependent effects (EC50).

In conclusion, we accomplished all intended experiments for the second aim, which took most of our time in the second year (2012-2013).

Aim 3: Test the effect of drug leads on cell and animal models

The third aim is to

(1) Conduct Western blotting of epithelial and mesenchymal markers on SUM1315, Bt594, and MDA157 cells before and after treatment with drug leads
(2) Perform cell migration and invasion assay on mock-treated and drug-treated cells
(3) Test the effect of leading candidates on breast cancer metastasis on nude mice

Unfortunately, at the end of the project, we run out of time to perform these assays. As described in the original proposal, we anticipated this problem because of potential delays. This turns out to be the case. In any case, the results we have obtained have set the stage for us to continue the project once additional resources become available. On the scientific front, we are still excited with the project..

Key Research Accomplishments

- Conducted RNAi on a breast cancer model
- Performed genome-wide analysis to define the EMT-linked molecular signature
- Designed, synthesized, and tested initial oligo pool for chemical screening
- Tested the screening procedure on our assay robot
- Adapted the cell model on screening robot
- Tested and optimized conditions for chemical screening
- Conducted both initial and secondary screens
- Confirmed the intended effects of a set of lead compounds on a model cell line.

Reportable Outcomes

We have published three papers on gene signature and chemical screening strategies during the duration of the project. However, those papers were largely supported by other resources prior to funding of this project. We presented our technology development efforts in a recent Society for Laboratory Automation and Screening (SLAS) meeting in San Diego.

Conclusion

We believe that we have accomplished most of what proposed to do except elaborated studies on additional cell lines and animal models. The delay was partly due to some problems in validating some of initially synthesized oligos, which might be related to pool quality of chemical synthesis and our custom built robotic

system. Re-test of lead compounds and determination of their dosage effects also took longer than anticipated. As a result of these delays, we have not yet obtained decisive information on the efficacy of our lead compounds on animal models. We thought to identify additional resources to continue the project. However, without showing some key in vivo effects as preliminary results, it is challenge to convince reviewers. We have thus decided to continue this project using other resources available to us until we obtain convincing preliminary results before applying for a NIH grant to push the project to the next level.

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