Elucidating mechanisms of tumor permeability in Triple Negative Breast Cancer

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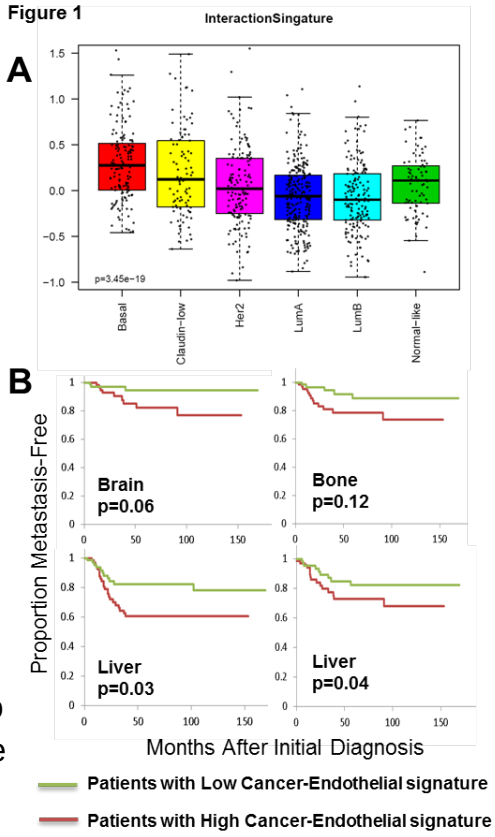
We are thankful for the support of the Triple Negative Breast Cancer Foundation to further our research efforts into identifying key targets that mediate breast cancer tumor cell endothelial-like morphologies. Since receiving this funding we have been focused on three major areas of research; 1) bioinformatic comparisons of multiple genomic datasets to further identify genes that mediate cancer cell-endothelial cell interactions and cancer cell tubule formation (i.e. vascular mimicry), 2) optimization of an *in vitro* screening assay to identify genes/pathways that mediate cancer cell tubule formation, 3) *in vivo* testing of two basal-like human-in-mouse models that exhibit differential sensitivity to chemotherapeutics.



The underlying hypothesis that we are testing is that Triple Negative Breast Cancers (TNBC) are highly aggressive in part because they have properties that facilitate their interaction and interdigitation within endothelial cells. Since breast cancer cells must interact with endothelial cells in order to intravasate into the vasculature and metastasize we have performed *in vitro* co-cultures with breast cancer cells lines and blood vascular endothelial cells. For these studies we utilized human TNBC cell lines (SUM149, SUM159, MDA231) and Luminal/Her2 cell lines (MCF7, T47D, SKBR3) and grew them in three-dimensional cocultures with human blood vascular endothelial cells. After 24 hours of coculture we harvested the cells and subjected their RNA to gene expression profiling to identify those genes that were significantly altered when in cocultures as compared to either cell type in monoculture. Overall, we found quite a great deal of cell-line specific expression changes when in coculture. Interestingly, there were 21 genes that were consistently upregulated in every coculture, suggesting these genes mediate the initial cancer cell-to-vasculature interaction that facilitates cancer cell entry into blood/lymphatic vessels and metastasis. When viewed across an 855 patient dataset,

collectively this 21-gene signature was the most abundantly expressed in the Basal-like and Claudin-low tumors (Figure 1A), and high expression of this signature can be used to predict relapse to several vital organs within TNBC patients (Figure 1B). Strikingly, one of the genes within this signature has been the focus of a collaboration with the Gregory Hannon lab at Cold Spring Harbor (see below), and we are hopeful that these efforts will be published by the end of the year.

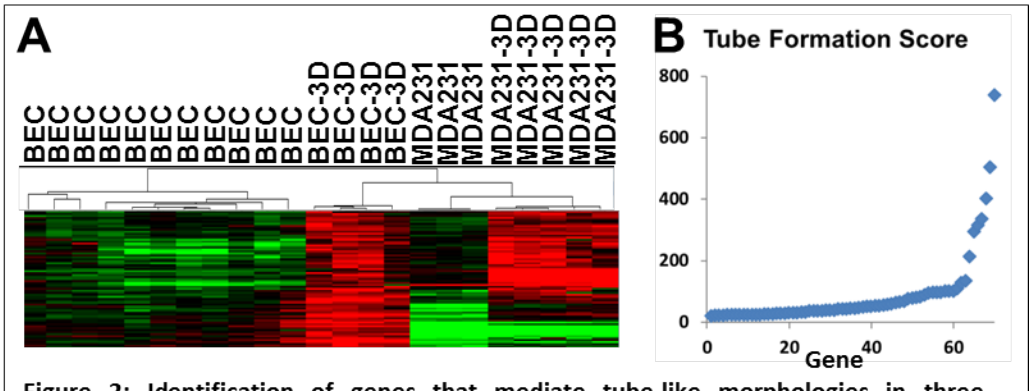


Figure 2: Identification of genes that mediate tube-like morphologies in three-dimensional culture *in vitro*. A) Primary blood vascular endothelial cells (BEC) and MDA231 claudin-low breast cancer cells were grown on plastic (2D) or matrigel (3D) and then after 24 hours gene expression microarrays were performed. Supervised hierarchical cluster of the top 70 genes identified as significantly upregulated in both types of 3D cultured cells. B) Tube Formation Score for each gene; defined by the product of two SAMs: [Claudin-low + Endothelial Cells versus Luminal+HER2 enriched cancer cells] x [One Class SAM Claudin-low + Endothelial Cells] = score.

Since we hypothesize that tube-forming abilities are also important for mediating metastasis we have also performed gene expression microarrays on TNBCs that were growing as monolayers in two-dimensions as compared to growing as tube-like structures in three-dimensional Matrigel based assays. With this approach we have identified genes that are commonly upregulated in blood vascular endothelial cells as well as in Claudin-low MDA231 breast cancer cells (Figure 2). These top genes identified are the main targets of our *in vitro* and *in vivo* screens that are the focus of the remainder of this proposal. We have optimized the *in vitro* screening procedure (Figure 3) with chemotherapeutics on Claudin-low cell lines and are now in position to determine if knocking out the genes we have identified inhibit tube formation.

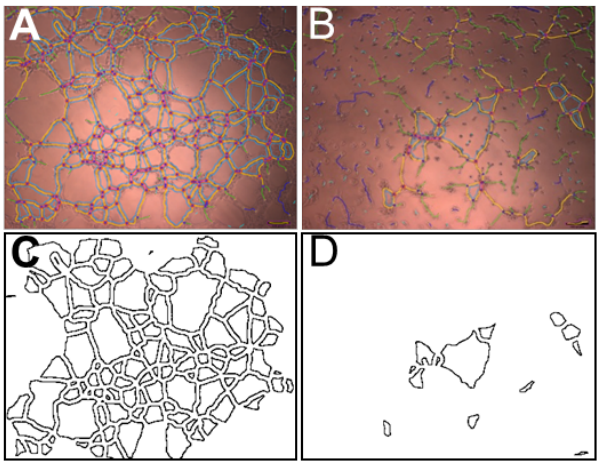
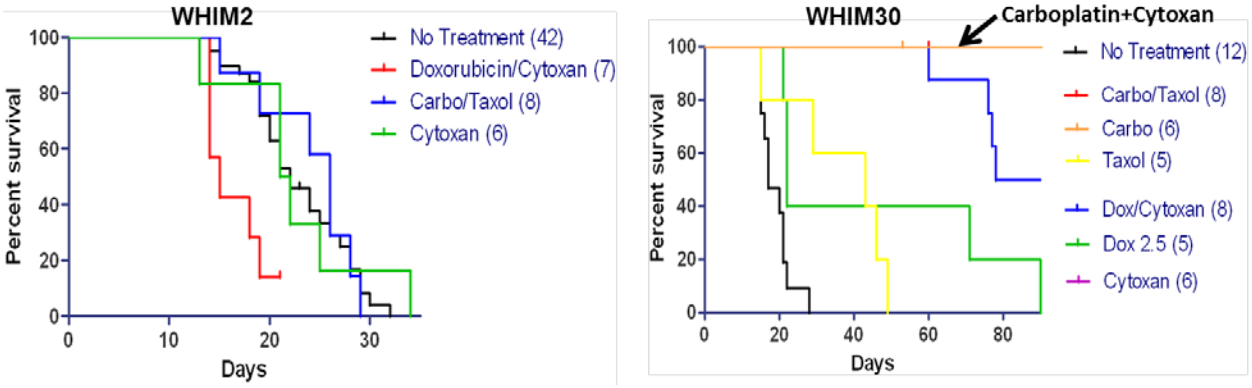


Figure 3. Tube formation assay with Claudin-low MDA231. Untreated cells (A,C) or carboplatin treated cells (B,D) after 8 hours. A&B) ImageJ automated loop, branch, segment counting, C&D) Loop network maps to quantitate drug/siRNA effectiveness.

Our experimental plan, which is underway, is to screen these genes of interest *in vitro* and if any successfully inhibit tube formation, to then grow these MDA231

cells *in vivo* +/- RNAi targeting and track their tumor growth and metastatic potential. We have previously identified that TNBC Claudin-low tumors, as compared to luminal tumors exhibit vascular leakiness, which is likely contributing towards for their strong metastatic potential. Therefore, the *in vivo* studies will explore vascular integrity through injecting systemic contrast agents and quantifying tumor vascular perfusion. To accomplish this goal we have initiated a collaboration with Dr. Paul Dayton at UNC, who is an expert on angiographic imaging with acoustic angiography. His expertise and technology will further allow us to identify how targeting these genes and chemotherapeutic drugs effect vascular flux and flow. These models will focus on using the established cell lines described above; however, if the initial studies prove informative, we also have great interest in targeting these genes within human-in-mouse patient-derived-xenograft (PDX) model systems. Therefore, in parallel to these *in vitro* studies we have been performing drug treatments on several PDX lines. Interestingly, we have identified two basal-like lines that have stark differences in their responses to chemotherapeutics (which is a great model for human disease as only some TNBC patients respond to chemotherapeutics). We have found that the WHIM2 PDX is not responsive to Carboplatin+Cytoxan regimen, whereas the WHIM30 PDX responds dramatically (Figure 4). Ideally these studies would identify a targetable link between tumor vascular permeability and chemotherapeutic (non)responsiveness observed in the WHIMs.

Figure 4.



In addition to these studies we have established TNBC focused collaborations with Gregory Hannon/Simon Knott at Cold Spring Harbor Laboratory, and Mark Pegram/Toby Ward at Stanford University. These collaborations have thus far revealed two distinct and novel mechanisms through which TNBCs metastasize. Thus far these efforts have resulted in a manuscript under revision for *Nature* and a second manuscript that is being prepared for a submission.

Thank you for your support, CHUCK