**Identification of biomolecules involved in CTC-endothelium interactions during early metastasis**

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Metastasis is responsible for the vast majority of cancer related deaths. However, due to long time scales associated with metastatic progression, the temporal cellular and molecular mechanisms of metastasis remain largely unknown. The voyage of tumor cells from primary to secondary sites consists of multiple steps governed by their unique spatial and temporal microenvironments, each of which can be rate limiting. Once in blood circulation, tumor cells are exposed to extremely stressful and hostile biochemical and biophysical conditions. These rare CTCs must survive and subsequently escape from the vasculature in order to successfully metastasize. Collectively, these unfavorable conditions make metastasis an extremely inefficient and slow process. Additionally, the short half-life of CTCs ranges from 1 to 2.4 hours [1, 2] with only 0.01% of cells succeeding in developing metastases [3]. Thus, the circulating phase is one of the most important rate-limiting steps in the overall metastatic cascade.

Several in vivo and in vitro models have been proposed to study various aspects of metastasis. While most in vivo models lack the ability to obtain spatial and temporal resolution of the metastasis process, majority of the in vitro approaches are significantly deviated from in vivo scenarios obscuring the validity of obtained results. For example, animal models are typically injected with $10^5$ to $10^6$ cancer cells (as compared to the critical value of 5-10 CTCs/7.5mL of blood in vivo) and provide quantitative information as an end result of the metastases development. On the other hand, most in vitro approaches utilize human umbilical vein endothelial cells (HUVECs) as the vasculature model obscuring the organotropic behavior of actual CTCs. In this study, we are investigating the dynamic involvement of some of the major biological molecules that participate in mediating the adhesion of circulating breast cancer cells to lung endothelium: CD44, CD47, and MET [4]. Utilizing microfluidic devices to closely mimic the physiological conditions such as flow and shear stresses, we have developed a more accurate in vitro model to study the initial course of metastasis (figure 1A & 1B).

Several measures were considered to ensure a more accurate representation of the in vivo microenvironment with respect to selection of more relevant cell types as well as flow conditions that exist within the vasculature. As stated above, most of the existing in vitro approaches investigating metastasis utilize HUVECs as their model endothelium. While HUVECs are robust and relatively easy to grow, they possess a generic endothelial phenotype as compared to the organotropic endothelium that has shown to be unique for most organs. Therefore, we utilized primary human lung microvascular endothelial cells (HLMVECs) to more closely emulate pulmonary vascular conditions where breast CTCs are known to metastasize. Furthermore, traditional in vitro studies utilize breast cancer line MDA-MB-231 for their experiments as model CTC cells due to their well-established aggressive metastatic characteristics. However, in vivo studies have demonstrated that this cell line comprises is comprised of different subsets of cell populations that selectively metastasize to specific target organs. The MDA-MB-4175 sub-line specifically targets lungs while a different subpopulation designated MDA-MB-231-BR targets brain tissue. Further studies found the expression of surface markers on MDA-MB-4175 cells similar to those on real lung-metastatic breast cancer CTCs validating the relevance of this cell line as a lung-specific breast CTC model cell line that we have utilized for this study [5]. Additionally, shear stress plays a critical role in regulating EC characteristics as well as in CTC-EC interactions. Such interactions (rolling, weak adhesion etc.) are obscured when transwell assays are used or when cells are infused within microchannels and allowed to bind to the substrate under stagnant conditions. Hence, we to add further accuracy to our system, we investigated CTC-EC interactions under shear stress induced physiological flow conditions as seen in vivo.

We first determined the basal binding efficiencies for E-selectin, SIRP-α, and HGF. The ligands were immobilized within microfluidic channels and MDA-MB-4175 cells were introduced to the channels and cell adhesion was recorded as a function of time. Additionally, cytokine and chemokine stimulation of the major biological markers was performed to ensure controlled expression on their respective cell types (Figures 2 and 3). From these

experiments, we determined the basal expression of CD44, CD47, and MET and their ligands that allow for cell adhesion.

Following the above experiments, we then performed similar experiments with CTCs flowed over an HLMVEC layer cultured within microfluidic channels (figure 1C). Using chemokine and cytokine concentrations determined from the above experiments, we were able to control the expression of the proteins of interest to determine the role they played in CTC binding to their target endothelium. ICC was performed on these channels examining the expression of relevant proteins (figures 2 and 3). Preliminary data suggests that CD44 and its ligand E-selectin depict a time-dependent increase in their expression following TNF-α stimulation. Both of these effects appear to happen on a relatively short time scale but long enough to allow CTCs to eventually find its way to its primary target organ. Further experiments are underway to fully explore the role of CD44, CD47, MET, and possibly other markers on cancer metastasis and how CTCs interact with their target tissue.

With the utilization of an assay that closely resembles pulmonary vascular conditions as well as simulating physiological flow conditions, this approach accurately reveals the temporal and spatial expression of biomolecules involved in metastasis. Additionally, this in vitro model offers precise control over various conditions allowing further experimentation for identifying other ligands providing greater understanding of molecular interactions during the circulation phase of metastasis.

**References**


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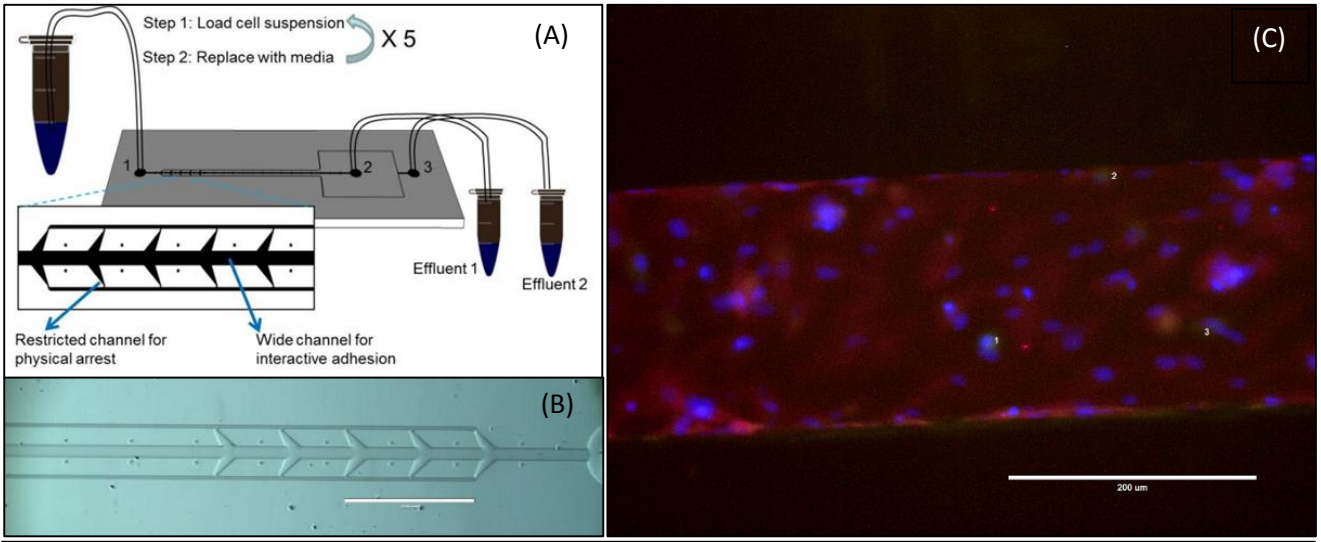



**Figure 1:** Images of the microfluidic devices that will be utilized for these experiments

(A) Image shows the schematic of the microfluidic device with its inlets and outlets.

(B) An image of the central part of the actual device.

(C) Images of HLMVECs grown inside a microfluidic channel with MDA-MB-4175 cells that have been flowed over top and bound. Stained with E-cadherin and counterstained with DAPI. Numbers 1, 2, and 3 point to MDA-MB-4175 cells that have bound to the HLMVEC layer. The MDA-MB-4175 cells were transfected with eGFP-Actin.

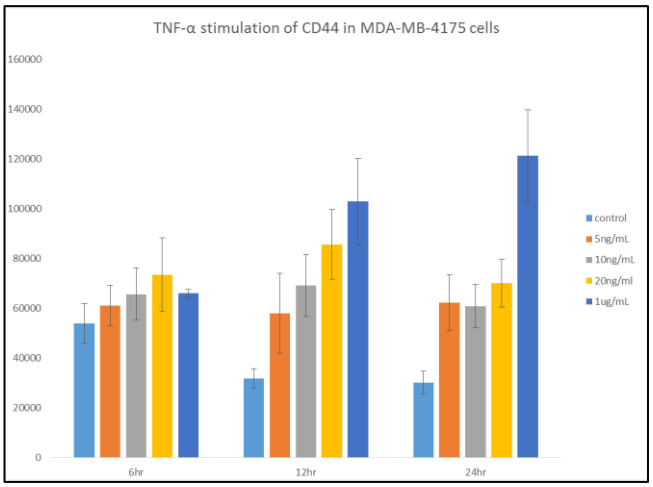



**Figure 2:** TNF-α stimulation of CD44 in MDA-MB-4175 cells. Cells show a significant increase of CD44, a protein found to be upregulated on lung-metastatic breast cancer, after 12 hrs of incubation with varying concentrations of TNF-α. When compared to the control group. The elevated levels of CD44 remain even after 24 hrs of exposure.

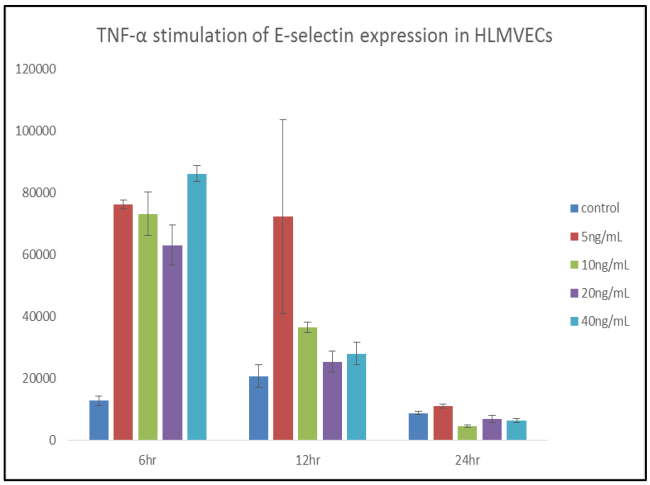



**Figure 3:** TNF-α stimulation of E-selectin, the protein target of CD44, on HLMVECs. Significant elevation of E-selectin is seen in all groups that received TNF-α when compared to the control group at 6 hrs. At 12hrs E-selectin expression has decreased, and, at 24hr, the levels in the TNF-α groups are comparable to the control group.