Study of the Role of Circulating Tumor Cells (CTC) and Circulating Cellular Microenvironment Components in Metastasis Formation and their Clinical Value

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STUDY OF THE ROLE OF CIRCULATING TUMOR CELLS (CTC) AND CIRCULATING CELLULAR MICROENVIRONMENT COMPONENTS IN METASTASIS FORMATION AND THEIR CLINICAL VALUE

By
Zheng Ao

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STUDY OF THE ROLE OF CIRCULATING TUMOR CELLS (CTC) AND CIRCULATING CELLULAR MICROENVIRONMENT COMPONENTS IN METASTASIS FORMATION AND THEIR CLINICAL VALUE

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Circulating Tumor Cells (CTC) are tumor cells found in cancer patients’ peripheral blood. They hold significant clinical value for cancer prognosis and management. While enumeration of CTC has been proven to provide reliable prognosis value for cancer management, further molecular and functional characterization of CTC may provide insights into both the biology of metastasis and critical clinical information such as the dynamic changes in molecular expression of tumor cells during treatment. Here, we will discuss how molecular and functional characterization of CTC can be utilized for such clinical applications, and the methodology development for such applications including a thermo-responsive release method of viable CTCs for downstream characterization. Another aspect of CTC is their interactions with circulating cellular microenvironment components. Tumor microenvironment components, such as Cancer Associated Fibroblasts (CAF) and Myeloid Derived Suppressor Cells (MDSC), play important roles in tumor growth and metastasis formation. Here, we will discuss the identification of their interactions with CTC in circulation and how they are associated with metastasis in both murine models and clinical settings.
DEDICATION

This thesis is dedicated to my dear parents, Ao Quanhong and Zheng Xuehui, for their selfless support over the past 26 years, the education they provided me with, their encouragement to be curious about the world, and the ethical standards they taught me to apply in both life and science.

This thesis is also dedicated to my mentor, Dr. Ram Datar. He has always guided me in research and in life. He mentored me when I was new to research; he encouraged me when I had adventurous thoughts; and he guided when I was lost. It is truly a blessing to have him as my mentor, and he will always be the beacon that guides me through my future career.

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## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>viii</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>xii</td>
</tr>
</tbody>
</table>

Chapter

1 **INTRODUCTION** ................................................................. 1
   1.1 Clinical Significance of CTC ........................................... 2
   1.2 Methodologies for CTC isolation ........................................ 7

2 **MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF CTC** .. 13
   2.1 Molecular characterization of CTC to interrogate metastasis initiation gene signature ................................................................. 14
      2.1.1 Development of molecular assays to analyze metastasis initiating gene signatures: uveal melanoma as a model .......................... 19
      2.1.2 Study of tumor tropism gene signature in circulating tumor cells isolated from breast cancer patients ................................. 23
   2.2 Molecular characterization of CTC to interrogate dynamic change during treatment ................................................................. 46
      2.2.1 HOG GU12-160 Trial: Phase 2 Study of Docetaxel +/- OGX-427 in Patients With Relapsed or Refractory Metastatic Bladder Cancer ................................................................. 46
      2.2.2 LCI-GU-BLA-SPEC-001 Trial: Aurora Kinase Expression in Muscle-Invasive Bladder Cancer ....................................................... 49
   2.3 Establishing CTC culture to study in vitro drug sensitivity and develop personalized treatment for cancer patients ................................. 53
      2.3.1 Temperature sensitive release of viable Circulating Tumor Cells (CTCs) following size-based capture ................................. 57
3 CHARACTERIZATION OF CIRCULATING TUMOR MICROENVIRONMENT COMPONENTS .................................................. 72

3.1 Identification of circulating cancer associated fibroblasts as a potential biomarker for metastasis ................................................................. 73

3.1.1 Validation of cCAF enumeration using microfilter technology .................................................................................. 76

3.1.2 cCAF identification and enumeration from human patients........................................................................... 78

3.2 Characterization of Circulating Tumor Cell (CTC) -Myeloid Derived Suppressor Cell (MDSC) clusters in mouse models............................... 85

4 CONCLUSION ...................................................................................................................................................... 92

References .......................................................................................................................................................... 96
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Brief illustration of existing microfabricated filter membrane structures for capture and characterization of CTC.</td>
<td>9</td>
</tr>
<tr>
<td>1.2</td>
<td>Membrane Microfilter Technology for CTC Capture.</td>
<td>10</td>
</tr>
<tr>
<td>2.1</td>
<td>MCF-7 cells probed for NCOA-3 transcript by RNA-FISH.</td>
<td>21</td>
</tr>
<tr>
<td>2.2</td>
<td>Uveal Melanoma cells probed with GAPDH house-keeping gene.</td>
<td>22</td>
</tr>
<tr>
<td>2.3</td>
<td>Tumor tropism related genes in breast cancer.</td>
<td>24</td>
</tr>
<tr>
<td>2.4</td>
<td>Gel Electrophoresis images of tumor tropism primers tested.</td>
<td>26</td>
</tr>
<tr>
<td>2.5</td>
<td>Gradient PCR to optimize Tm for qRT-PCR reaction.</td>
<td>26</td>
</tr>
<tr>
<td>2.6</td>
<td>Positive controls slides to test shortened IF protocol.</td>
<td>29</td>
</tr>
<tr>
<td>2.7</td>
<td>Cultured cancer cells seeded in blood are captured on slot filter and subjected to short double immunofluorescence staining protocol.</td>
<td>30</td>
</tr>
<tr>
<td>2.8</td>
<td>Folate-FITC labeling of tumor cells.</td>
<td>30</td>
</tr>
<tr>
<td>2.9</td>
<td>Test of Folate-PEG-FITC on cancer cells.</td>
<td>32</td>
</tr>
<tr>
<td>2.10</td>
<td>Test of specificity of folate-FITC staining for tumor cells spiked into blood.</td>
<td>33</td>
</tr>
</tbody>
</table>
2.11 PC3 prostate cancer cells were laser captured onto the cap from the slot filter ................................................................. 34

2.12 qRT-PCR analysis of GAPDH expression from LCM samples of PC3 cells captured on filter .................................................. 34

2.13 LCM on HeLa cells captured on slot filter and stained using folate-PEG-FITC protocol ................................................................ 37

2.14 PC3 cells post Folate-PEG-FITC staining was picked up from filter using CellCelector ................................................................. 38

2.15 PC3 cells picked from microfilter and analyzed for gene expression ........ 39

2.16 Gel electrophoresis analysis of qPCR products. hMAM, CEA, KRT19, MUC1 gene expression in SKBr-3, MCF-7, MDA-MB-468 cell lines ..... 43

2.17 Triple marker staining on Cytospins of MCF-7 and PMBC cells .......... 48

2.18 Optimization of LCI staining protocol ........................................... 51

2.19 Representative image of Patient CTC ........................................... 52

2.20 PIPAAm coated slot filters to capture and release circulating tumor cells from blood ........................................................................ 59

2.21 Measurement of pore parameters pre- and post- PIPAAm coating ......... 60

2.22 Illustration of the experimental workflow to calculate tumor cell capture and release efficiency using PIPAAm coated slot filter .............. 61
2.23 SK-Br-3 breast cancer cells captured and released from blood remained viable and expanded in culture rapidly ................................................. 64

2.24 Capture and release of LMTS-GFP CRC cells using PIPAAm coated slot filter .................................................. 65

2.25 Proliferation rates of SKBr-3 cells were compared before and after PIPAAm filter capture and release, as measured using EdU assay .......... 67

2.26 Measurement of proliferation rates and metabolism rates of tumor cells before and after PIPAAm coated slot filter capture and release ........... 68

2.27 Test of PIPAAm coated filter in patient samples ............................................. 70

3.1 Validation of microfilter capture of CAF cells ............................................. 77

3.2 Identification of CTC and cCAF on microfilter ........................................ 79

3.3 Swarm plots indicating the enumeration of cCAFs and CTCs from blood samples ............................................................................... 80

3.4 Identification of CTC and cCAF clusters on microfilter ......................... 81

3.5 Illustration of hypothesized CTC-MDSC interactions. CTC express RAGE receptor and MDSC expression S100A8/A9 ligand ....................... 86

3.6 CTC-MDSC in 4T1 mouse model. 4T1 tumor cells were xenografted into NOD/SCID mice .................................................................. 86

3.7 Illustration of experimental plan to interrogate CTC-MDSC interactions in MDA-MB-231 xenograft model .................................. 87
3.8  CTC-MDSC interactions observed in MDA-MB-231 xenograft models

3.9  CTC in culture from MDA-MB-231 xenograft model at Day 4-Day 5
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>29</td>
</tr>
<tr>
<td>2.2</td>
<td>35</td>
</tr>
<tr>
<td>2.3</td>
<td>36</td>
</tr>
<tr>
<td>2.4</td>
<td>41</td>
</tr>
<tr>
<td>2.5</td>
<td>41</td>
</tr>
<tr>
<td>2.6</td>
<td>42</td>
</tr>
<tr>
<td>2.7</td>
<td>62</td>
</tr>
<tr>
<td>2.8</td>
<td>62</td>
</tr>
<tr>
<td>2.9</td>
<td>66</td>
</tr>
<tr>
<td>3.1</td>
<td>77</td>
</tr>
<tr>
<td>3.2</td>
<td>80</td>
</tr>
</tbody>
</table>
3.3 CTC and CTC-leukocyte interaction events enumeration in AT-3 and E-0771 syngeneic model........................................................................................................ 91
Chapter 1. Introduction

Metastasis disease accounts for 90% of cancer-related mortality, and a great majority of life-threatening cancers occur in epithelial tissues, yielding carcinomas. The metastasis of epithelial cancer is a highly complicated, multi-step event and is depicted as the invasion-metastasis cascade. The cascade includes localized invasion, intravasation, transport through circulation, arrest in microvessels of various organs, extravasation, formation of micrometastasis and colonization (Hanahan & Weinberg, 2011). While tumor cells successfully extravasate into the peripheral blood and circulate, they are called Circulating Tumor Cells (CTCs) before they intravasate and form micrometastasis. Circulating Tumor Cells (CTC) are considered the pivotal component of the metastatic cascade and are being extensively studied only in the last decade or so. Understanding the biological and clinical impact of CTC is likely to reveal important information of the metastatic process and contribute to better management of cancer (Alix-Panabieres & Pantel, 2013).

1. This chapter is based on previous publication: Datar, R., Ao, Z., Cote, R., (2015). Significance of Studying Circulating Tumor Cells.: Springer.
1.1 Clinical Significance of CTC

Most of the clinical data pertaining to clinical utility of CTC was collected utilizing CellSearch (see below). One well-validated clinical application of CTC is assessment of their prognostic value by CellSearch assay at pre-treatment baseline. Patients with higher than 5 CTC per 7.5 mL blood were shown to have shorter progression-free survival and shorter overall survival in a study analyzing baseline CTC level in a cohort of 177 metastatic breast cancer patients in 2004 (Cristofanilli et al., 2004). Subsequently, similar results were seen for metastatic prostate cancer in 2008 (de Bono et al., 2008) and metastatic colorectal cancer in 2009 (Cohen et al., 2009). Several follow-up studies have confirmed the prognostic value of CTC, and as a result, CTC has been proposed in the new 2010 edition of the tumor-node-metastasis (TNM) cancer staging system manual as cM0(i+) (Edge & Compton, 2010) which is yet to be included in the clinical guidelines.

In addition to the prognosis value of CTC, other studies are being carried out to explore the further clinical applications of CTC such as utilizing CTC as surrogate endpoint for clinical trials, as predictive marker to guide treatment, as marker for early detection of solid tumors and as companion diagnostic.

1) CTC as surrogate endpoint for clinical trials:

One important potential clinical utility of CTC is their use as a surrogate endpoint for clinical trials. If approved to be informative, interrogating CTC with minimally invasive blood draws at follow-up time points to monitor treatment will greatly benefit the cancer
management, and several clinical trials are designed to test the feasibility of this notion. For example, CTC enumeration analysis at follow-up visits for the same cohort of patients examined by Cristofanilli et al above showed CTC to be prognostic of progression-free survival and overall survival (Hayes et al., 2006). Similarly, another clinical trial in breast cancer also indicated that CTC as a surrogate endpoint is more reproducible and robust than radiographic response (Budd et al., 2006). In prostate cancer, a clinical trial conducted in a cohort of 263 metastatic castration resistance prostate (mCRPC) cancer patients – the SWOG S0421 trial also indicated that, patients with rising CTCs at week 3 have significantly worse overall survival as compared with those with less or equal number of CTC at the week 3 follow-up visit (Goldkorn et al., 2014). In another study in mCRPC setting, CTC in combination with serum lactate dehydrogenase (LDH) level was shown to be a better surrogate for survival than PSA level (Scher et al., 2013). Thus, so far, clinical trials attempting to interrogate CTC as a surrogate endpoint for clinical trials have shown some promising results, prompting further extensive follow-up studies. These attempts are discussed point-by-point as following:

2) CTC as Predictive Marker to Guide Treatment:

As similarly encouraging evidence supports the value of CTC as prognostic markers in various cancers, an obvious question is: Can we use CTC to guide treatment selection? More specifically, can we use CTC measurement at baseline, or at follow-up time-points, to predict patient’s response to treatment and thus guide therapy? An example is the SWOG S0500 clinical trial (J. B. Smerage et al., 2014).
Results from the SWOG S0500 Phase III clinical trial (See Figure 2, Chapter 12, J. Smerage, for Trial Schema) were presented at the 2013 ASCO San Antonio Breast Cancer Conference. The trial was designed primarily to determine in a first line chemotherapy setting whether women with metastatic breast cancer and elevated CTCs by CellSearch assay ($\geq 5$ per 7.5 mL of whole blood) after 3 weeks of first-line chemotherapy derive increased benefit (overall survival and progression-free survival) from changing to an alternative chemotherapy regimen at the next cycle, instead of waiting for clinical evidence of progressive disease before changing to an alternative chemotherapy regimen. The trial was not designed to compare chemotherapies. The underlying hypothesis was that treatment decisions can be made based on CTC levels, with the belief that a significant number of patients resistant to their first line of therapy would respond to a second-line therapy. Patients may benefit by switching early to a new line of therapy through avoiding the cumulative toxicities of ineffective therapy while spending more time on active therapy, thus improving quality of life and potentially tolerating future therapies better. Contrary to expectation, the patient data from randomized arms did not differ with respect to progression-free or overall survival. Given the very poor survival outcomes for this population, it was concluded that this population likely has a disease that is generally resistant to cytotoxic mechanisms. However, trial data did demonstrate a large, clinically significant, and statistically significant difference in prognosis for patients in whom the CTC remained elevated after one cycle of first-line chemotherapy. This is a population that should be considered for clinical trials of novel agents or novel treatment strategies early in the course of their disease.
In summary, the SWOG-S0500 trial validated the hypothesis that the group of patients with elevated CTC at baseline and 21 days after starting the first chemotherapy has a worse prognosis with regard to progression-free and overall survival, while low baseline CTC levels indicate a very good prognosis. The trial also showed that switching to a different chemotherapy sooner does not improve outcomes. For these patients, a clinical trial to investigate new targeted therapies should be considered, since chemotherapy is not effective in this population of patients.

To address the clinical utility of CTC in another direction, the ongoing METABREAST trial aims at identifying patients without the need for aggressive treatment if they have low CTC at baseline level. In this study, CTC were measured at baseline, and patients receive chemotherapy if they are detected with >5 CTC, otherwise they will receive endocrine therapy (Alix-Panabières & Pantel, 2014).

3) CTC as a Marker for Early Detection of Solid Tumors:

In addition to investigating CTC as a surrogate endpoint and predictive marker, other studies focus on the possibility of using CTC for early detection for solid tumors. As reported in mouse model breast cancer research, tumor cells can “leave home early” (R. A. Weinberg, 2008) and establish metastasis without the necessity of experiencing the steps of transformation at primary sites (Podsypanina et al., 2008). Another study in pancreatic cancer transgenic mouse model revealed that CTC can enter blood stream even before tumor formation (Rhim et al., 2011). These observations encourage the notion that CTC could be used for early detection of cancer, as harbingers of impending malignancy. However, preliminary data from pilot clinical trials has stimulated some disputes. For
example, a study probing for CTC in patients with benign colon diseases has detected CTC in 11.3% of the 53 patients analyzed, which could be false-positive results (Pantel et al., 2012). Another potential problem of using CTC for early detection of cancer is the extremely low CTC count in early stage patients. The cut-off of CTC count in a non-metastatic breast cancer setting by CellSearch is determined to be 1 per 7.5 mL blood draw, which, although prognostic (Rack et al., 2014), can be easily missed depending on the sampling of the blood and the analysis process. One solution to interrogate such a low level of CTC is to examine larger volume of blood. This can either be achieved by an in vivo CTC capture probe –CellCollector® (Saucedo-Zeni et al., 2012) or by taking advantage of a standard clinical procedure –leukapheresis (J. C. Fischer et al., 2013) to harvest CTC from a much larger volume of blood. In general, emerging technologies with ability to interrogate larger volume of blood, or those with higher sensitivity to detect CTC in smaller blood volumes, might shed light on this clinical application of using CTC for early detection of cancer.

In conclusion, although prognostic utility of CTC has been well-validated for various cancers, their clinical application as a surrogate endpoint, as predictive marker to guide therapy or as an early diagnostic marker is still largely unexplored and will require large scale clinical trials for validation. Although still in development awaiting further validation, the future vision of a CTC test is to serve as a “liquid biopsy” that can provide clinicians comprehensive clinical information of the patient in a minimally invasive blood draw.
4) CTC as Companion Diagnostic:

As some of the technical hurdles around CTC enumeration and suitability of various CTC capture and analytic platforms for evaluation of biomarkers get resolved, there are ongoing efforts in parallel that address development of CTC assays as companion diagnostic to assess the efficacy, toxicity and successful targeting of anti-cancer therapeutics in real time as they are being developed, both in pre-clinical studies as well as Phase I and II clinical trials. Needless to say, such use of CTC assays must stand the rigor of regulatory hurdles. In a 2012 publication, Punnoose and Lackner review these developments and suggest a path for co-development of anti-cancer therapeutics with CTC-based diagnostics that could enable clinical validation and qualification of CTC-based assays as companion diagnostics (Punnoose & Lackner, 2012). Chapter 15 of this volume also addresses this concept in detail.

1.2 Methodologies for CTC Isolation

Ashworth first reported tumor cells in a patient’s peripheral blood over one and a half century ago (Ashworth, 1869). However, the study of these tumor cells has always been hampered by the rare existence of this cell population amid the excess of hematopoietic cells in blood. Various CTC isolation technologies have been developed only relatively recently, based on various principles such as affinity-based capture technologies including CellSearch™ (Cristofanilli et al., 2004), or non-affinity based technologies such as size based microfiltration (Zheng et al., 2007), density-based gradient centrifugation (Gertler et al., 2003) or electrical property-based dielectrophoresis (DEP).
(Becker et al., 1995), etc. Most of the clinical data pertaining to clinical utility of CTC was collected utilizing CellSearch™, the only FDA-cleared technology for CTC enumeration for breast, colon and prostate cancers.

Affinity based CTC capture platform, such as CellSearch system, although effective, can lead to neglect of certain subpopulations of CTC (Mikolajczyk et al., 2011). And this concern is shared among other affinity based CTC capture platforms, as affinity based CTC capture relies on expression of certain antigen on tumor cells, which can vary due to the heterogeneity observed among CTC (Powell et al., 2012). In contrast, non-affinity based techniques isolate CTC based on their physical properties. These antigen agnostic capture platforms may address the concerns of neglect of certain CTC sub-populations and give us a more comprehensive picture of CTC. Size based CTC capture is one of the strategies employed to realize antigen-agnostic capture of CTC.

The size-based isolation of circulating tumor cells was initially being explored by using track-etched polycarbonate filters. These polycarbonate filters are fabricated in a fashion that 235U fission fragments were used to bombard the polycarbonate membranes, which is then subjected to warm sodium hydroxide etching to generate pores with a uniform size (Fleischer, Alter, Furman, Price, & Walker, 1972). Utilizing this type of technology, isolation by Size of Epithelial Tumor Cells (ISET) was developed to isolate CTC from cancer patients’ peripheral blood (Vona et al., 2000). After collection of peripheral blood from patient, sample was drawn through ISET filter by negative pressure. Using a cut-off of 8 µm, larger Circulating Tumor Cells were retained on the filter, while majority of the
leukocytes and erythrocytes will pass through the filter. Post-filtration, the filter is subject to immune-histochemistry (IHC) so that CTC can be distinguished from the rest of normal blood components retained on the filter. Although effective and has been tested in several disease settings (including breast cancer, lung cancer, pancreatic cancer cutaneous melanoma and uveal melanoma (De Giorgi et al., 2010; Hofman, Bonnetaud, et al., 2011; Khoja et al., 2012; Mazzini et al., 2014; Pinzani et al., 2006). Track-etched filters can be limited due to the randomized deposition of pores, which can lead to less effective filtration area and fused pores [Fig 1.1 A]. This can potentially yield lower capture efficiency and higher clogging events.

![Figure 1.1 Brief illustration of existing microfabricated filter membrane structures for capture and characterization of CTC. (A) Track etched polycarbonate filter (B) Parylene C filter with evenly distributed pore arrays (C) 3D Bi-layer membrane filter (D) Slot shaped pore filter membrane(Ao et al., 2015)]

To alleviate this concern, our group has developed a parylene based filter with uniformly deposited pores generated with oxygen plasma etching in reactive ion etching (RIE) technique. Briefly, parylene C was deposited to 10 µm thickness, which is then masked with photoresist material (Cr/Au or AZ 9260). Parylene C membrane is then etched with RIE, and pores were generated before the final step to strip off the photoresist with acetone. By employing this novel technique, our group has manufactured filters with 40,000 pores with 8 µm diameter evenly distributed in a 6x6 mm filtration area (Zheng et
al., 2007) [Fig 1.1 B]. This pore deposition in a controllable fashion will both increase the effective filtration area and ensure uniform filtration pore sizes on the whole filter.

To use this parylene based filter for CTC enumeration, a blood sample needs to be first diluted 1:1 with PBS and fixed with an end concentration of 1% formalin for 10 minutes. Post fixation, blood sample is positively pressured through the filter using a bench-top syringe pump at a rate of 200 mL per hour [Fig 1.2 E]. Following sample filtration, an equal volume of PBS is filtered to further clean the filter and reduce unwanted leukocytes retaining on filter. The filter can be then subjected to Immunofluorescence (IF) or IHC and other

Figure 1.2 Membrane Microfilter Technology for CTC Capture. Eight µm diameter round pore microfilters (A) are used to capture fixed cells for CTC enumeration and characterization, while slot pore microfilters (6 µm X 40 µm, B) are used for viable CTC capture in the Next-Gen microfilter device. Scanning electron micrographs of fixed tumor cells captured by the round pore microfilter (C) and of viable tumor cells captured by the Next-Gen microfilter device in a model system. (E) Filtration system set-up, microfilter is assembled between 2 acrylic pieces and clamped. Syringe with blood sample is then mounted on top of the cassette. Blood is processed through the microfilter using a standard syringe pump at the flow rate of 200 mL/hour.
downstream analysis. We have tested this filter in several disease settings including breast cancer, prostate cancer, colorectal cancer and bladder cancer. In a pilot cohort of 57 samples collected from these 4 cancer types, our filter demonstrated better performance in sensitivity as compared with CellSearch™ platform for the blood draws collected from the same time points in all 4 disease settings (Lin et al., 2010). And currently our efforts have been using this microfiltration platform to characterize CTC from a Phase 2 clinical trial in patients with relapsed or refractory metastatic bladder cancer clinical trial to monitor therapeutic responses (Noah Hahn, OncoGenex, & Hoosier Cancer Research, 2016). Additionally, employing this microfabricated filter, urine samples can also be examined to detect urothelial carcinoma of the bladder as an alternative to the standard urine cytology with lower cost and potentially better performance (Birkhahn et al., 2013).

Although this novel design of microfilter with well controlled pore deposition is effective in enumeration of CTC, the pre-fixation step of sample with 1% formalin, although mild, prohibits downstream functional characterization of CTC such as establishing CTC culture and drug sensitivity test. Also, the fixation can potentially lead to sub-optimal conditions for RNA profiling of CTC due the chemical modification of RNA by fixatives and also loss or degradation of RNA during the fixation process. This problem can be more evident in single CTC RNA profiling since single cell analysis usually requires high quality RNA. Thus, retrieval of live CTC from blood can be vital to enable these types of analysis. In order to accommodate this need of live CTC capture, novel designs of microfilter focus on decreasing shear pressure during filtration process to maintain CTC
viability. One design that meets this need is a double layered 3D membrane structure that our group has developed. As shown in [Fig 1.1 C], on the top layer, pores of 9 µm diameter are deposited, while on the bottom layer, pores of 8 µm diameter are deposited. The distance between the double layers is well engineered to be 6.5 µm. Thus during filtration process, larger CTCs will be trapped at the edge of the gap. This design allows us to capture viable CTC, and as model system experiments demonstrated, cultured tumor cells spiked into healthy donor’s blood can be retrieved and maintain in culture for at least 2 weeks (Zhou et al., 2014).

Another design of the microfilter that can be employed for viable CTC capture is achieved by modification of pore geometry. Using a design of “slot” pores instead of round pores, CTC can be captured without pre-fixation due to the larger fill factor of the new design that reduces the shear pressure during filtration. By employing this capture platform, cultured tumor cells in 1mL of healthy donor’s blood can be recovered with 90% capture efficiency and 90% cell viability as validated by measuring telomerase activity by qRT-PCR [Fig 1.1 D] (Lin et al., 2010).

Given the background and the clinical relevance of CTC above, my thesis will address the need and new data about the molecular and functional characterizations of CTC as well as the circulating tumor microenvironment components.
Chapter 2. Molecular and Functional Characterization of CTC$^2$

As CTC research evolves, it is noteworthy that as exhaustive molecular and functional characterization of CTC studies have been carried out, CTC population has been shown to be heterogeneous and the gene expression levels among CTC vary even within the same patient sample (Powell et al., 2012). As we already know from primary tumor, tumor heterogeneity greatly impact the course of cancer development and treatment effectiveness, it is natural to assume that such heterogeneity within CTC population might impact cancer treatment as well. As demonstrated by Lohr et al, whole-exome sequencing of CTC in metastatic prostate cancer revealed that CTCs possess unique mutations, which were not found in corresponding primary tissues (Lohr et al., 2014). Analyzing CTC at single cell level may reveal novel drug targets and provides a window for prostate cancer management. To address this heterogeneity, an increasing number of studies have begun to look beyond CTC enumeration to CTC molecular characterization in order to elucidate the subpopulations within CTC.

---

2. This chapter is based on previous publication:
2.1 Molecular Characterization of CTC to Interrogate Metastasis Initiating Gene Signature

Molecular characterization of CTC will help us to understand better the metastasis process, notably, answering questions regarding epithelial-mesenchymal transition (EMT), cancer stem cells, and the fate of CTC.

One important process involved in tumor metastasis that calls EpCAM-based CTC capture in question is the Epithelial-Mesenchymal Transition (EMT). EMT phenomenon has been described as the process whereby tumor cells gradually transition from epithelial phenotype into mesenchymal phenotype during metastatic progression, ostensibly via down-regulation of expression of epithelial markers (EpCAM, E-cadherin, cytokeratin, etc.) and up-regulation of mesenchymal gene expression (e.g. Vimentin), to achieve a more invasive phenotype (Thiery, 2002). EMT process has been extensively investigated in primary tumors but to a much lesser extent in CTCs. EpCAM-based technologies may tend to capture and enrich “epithelial” CTC, thus potentially missing the CTCs with mesenchymal phenotype that may be metastasis-initiating. As mentioned earlier, a study by Lin et al demonstrated that by employing a size-based isolation strategy without relying on EpCAM expression, CTC can be detected at higher sensitivity (Lin et al., 2010). In addition, a study by Harouaka et al has demonstrated that a mesenchymal phenotype CTC can be detected using size-based isolation technologies (Harouaka et al., 2014). A 2013 study by Zhang et al isolated viable breast cancer CTC using 4 target markers –HER2+/EGFR+/HPSE+/Notch1+, cultured the CTC and derived a population
that metastasized to brain in a mouse model (Zhang et al., 2013); this specific population was EpCAM-negative, and would have been missed if EpCAM was employed as the sole target molecule for CTC capture. In contrast, it is likely that antigen-agnostic CTC capture methods (such as those based on cell size) or capture methods that exploit other target antigens (including epithelial and/or mesenchymal antigens) will likely provide more insights into this phenomenon. For example, various studies employing cell size-based CTC capture have reported mesenchymal-like CTCs expressing the mesenchymal marker Vimentin (Harouaka et al., 2014; Hofman, Ilie, et al., 2011). Another study investigated EMT status of CTC captured from breast cancer patient samples using EpCAM, HER2 and EGFR as capture target antigens, and discovered that mesenchymal cells were highly enriched in the CTC population. The proportion of mesenchymal CTC increased during chemotherapy treatment (Yu et al., 2013). A converse interesting notion barely examined in the context of cancer but worth studying in CTC is the concept of Mesenchymal-Epithelial Transition (MET), wherein mesenchymal CTC may revert back to an epithelial phenotype once at the secondary site, expressing cell attachment protein such as such as E-cadherin, thereby regaining ability to form proliferative epithelial growths in distant organ sites. In contrast, cells without this capability to revert back to epithelial status seem to be unable to initiate metastasis effectively (Kang & Pantel, 2013). This hypothesis could be the explanation for the observation that many EpCAM-based CTC capture technologies seem to be capturing CTC in an intermediate status that is neither epithelial nor mesenchymal but rather a transitional status, also referred to as epithelial-mesenchymal plasticity (EMP). (Brabletz, 2012) Cells that have the EMP capability seem to be able to switch between epithelial and mesenchymal status and
might be population of the utmost importance in circulation (Alix-Panabières & Pantel, 2014). One of the many studies that supports this hypothesis is a clinical study looking at EMT status on CTC captured from metastatic breast cancer and mCRPC patients, where 75% CTCs were found to co-express Cytokeratin (epithelial), Vimentin (mesenchymal) and N-cadherin (mesenchymal), along with a stem cell marker CD133 expressed at a high frequency (Armstrong et al., 2011). Although the association between CTC EMT status and clinical outcome is still unclear, such studies will be critical not only to choose an appropriate CTC capture technology (EpCAM versus non-EpCAM-based capture) but also to elucidate the biological nature of CTC and the clinical relevance of mesenchymal CTC subpopulations.

Another potential phenomenon that is worth studying in CTC besides EMT is the existence of cancer stem cell subpopulation. It has been well demonstrated that the CD44+/CD24-/low population can form tumor with much higher efficiency as compared with the other subpopulations in breast cancer (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003). It has been previously shown that bone marrow Disseminating Tumor Cells (DTC) possess such putative stem-like phenotype (CD44+/CD24-/low) (Balic et al., 2006). It will be of interest to look for this subpopulation in breast cancer CTCs. A study in a pilot cohort of 30 breast cancer patients analyzed for CTC sub-populations found 35.2% of the CTCs to be CD44+/CD24-/low, while another cohort was shown to contain 17.7% CTCs that were ALDH1+/CD24-/low (Theodoropoulos et al., 2010). A different group of researchers attempting to detect metastasis-initiating cells (MICs) using a xenograft assay
demonstrated a subpopulation of CTC from luminal breast cancer patients that could initiate metastasis in mice, where they manifest a EpCAM+, CD44+, CD47+, MET+ phenotype (Baccelli et al., 2013). Thus, preliminary data has shown that there is a subpopulation of CTC, which possesses “stem-like” phenotype and can be responsible for metastasis initiation. Further studies interrogating these features in larger cohort of clinical trials and their correlation with patient clinical outcome can be informative and reveal more information about the “real culprit” CTC subpopulation that is responsible for metastasis, and the one that potentially could prove to be the valuable therapeutic target.

Another important question to be answered is the fate of CTC in circulation. It is reasonable to assume that there can be 3 potential fates for tumor cells in circulation. The first fate is that a given CTC will be “permanently non-productive” such that it will undergo either anoikis, apoptosis, or necrosis, be eliminated by immune surveillance or simply remain unable to home to metastatic niche or unable to initiate the intravasation process. The second fate is that a CTC can be “temporarily non-productive”, either successfully invading into secondary site and staying “dormant” (either stay in G0 phase of cell cycle or maintain an equilibrium of proliferative and apoptotic rates) or staying locked in mesenchymal status (Kang & Pantel, 2013) and failing to colonize and form metastasis. The third fate is that the CTC is “productive”, not only capable of invading into a secondary site but also forming metastasis by rapid proliferation. It is possible that CTC subpopulations are not committed to one certain fate. Thus, cells from “temporarily
non-productive” fate can transform into “productive” fate and form metastasis after long-term dormancy under certain environmental cues or additional genetic mutations.

To interrogate the fate of CTCs, one study looked at expression of apoptotic marker (M-30) and proliferative marker (Ki-67) in breast cancer CTC, and the data supported the hypothesis that there were proliferative as well as apoptotic subpopulations of CTC in circulation. Apoptotic CTC were seen more in early stage breast cancer patients (Kallergi et al., 2013). Another study looking at M-30 and Bel-2 expression in CTC indicated that, surprisingly, apoptotic CTC with M-30 expression is associated with worse prognosis in patients with elevated CTC level, whereas patients with Bel-2 CTC had better clinical outcomes in contrast to the notion that Bel-2 will lead to anti-apoptotic effect on CTC and worse outcomes (Jeffrey B. Smerage et al., 2013). While the data on apoptotic CTC looks confounding, other groups have also examined proliferative subpopulations in CTC. Thus, a study investigating Ki-67+ CTC concluded that proliferative CTC, independent of disease stage or treatment, is a rare population in circulation, and a fraction of non-proliferative CTC seem to be more chemo-resistant (Muller et al., 2005).

Since data on apoptotic CTC remains elusive, and proliferative CTC seems to be a rare population in circulating, the key distinctive characteristics between the first “permanently non-productive” fate and the other two fates could lie in the homing to secondary site and initiation of extravasation process. Mechanisms of establishing micrometastasis at secondary sites can be a combination effect of physical trapping and chemical homing. Whereas physical trapping at secondary organ can be correlated to
organ vasculature and tumor cell clustering (Circulating Tumor Microemboli lodging),
chemical homing can be correlated with chemokines, micro-RNAs and other tumor
microenvironment signaling (Miles, Pruitt, van Golen, & Cooper, 2008). In 1889,
Stephen Paget first brought up the notion that tumor cells form metastasis at secondary
organs as “seeds” on congenial “soils” (Paget, 1889). Later on, recent research has
indicated that, certain “tumor tropism” signatures can be established to predict the
potential secondary sites (Kang et al., 2003; Minn et al., 2007). Studying these signatures
on CTC might reveal important traits that could shed light on the homing mechanisms of
CTC to secondary organs.

It has been indicated that the half-life for CTC was 1-2 hours. However, CTC can be
detected in dormancy patients 8-22 years post mastectomy (Meng et al., 2004). It is
highly likely that CTC can be shed from micro-metastasis and circulates in the blood,
even seed back to the primary/metastatic lesions (M. Y. Kim et al., 2009). This indicates
that, secondary organs, especially bone (Shiozawa et al., 2011), can possibly serve as
reservoirs for CTC, and thus will be critical to monitor for patients with metastatic
dormancy.

2.1.1 Development of Molecular Assays to Analyze Metastasis Initiating Gene
Signatures: Uveal Melanoma as a Model

Dr. William Harbour’s group at University of Miami has discovered a gene expression
panel that categorize uveal melanoma into 2 unique subgroups (Class I and Class II) that
have distinct phenotypes and behavior resulting in distinct prognosis by interrogating the
biopsy from the tumor (Harbour & Chao, 2014). However, although benefiting patients with this information, biopsy from the eye is an extremely invasive method. In collaboration with Dr. Harbour’s group, we are developing the “liquid biopsy” method to interrogate CTC from a minimal invasive blood draw. Our hypothesis is that the phenotype of CTC will mimic or better represent the class of the uveal melanoma, since CTC is likely the “real culprit” causing metastasis. By molecular characterization of CTC from uveal melanoma patients, we hope we can gain knowledge on both prognosis and treatment target of the disease.

CTC gene expression can be interrogated by RNA-FISH. Although being a low throughput method, RNA FISH benefit the CTC analysis as a fast, easy and cost-effective method. Interrogating up to 4 transcripts at a time allowed by spectrum unmixing, we can both identify CTC captured on microfilter and interrogate 1-2 markers of interest.

To achieve this goal, in collaboration with Stellaris-FISH –Biosearch, we attempted a RNA-FISH protocol on tumor cells captured on filter to interrogate specific transcripts on CTC. As a model system, we first tested RNA-FISH protocol with NCOA-3 probe in MCF-7 cell line.

MCF-7 cells were seeded onto glass coverslips and cultured in DMEM for 48 hours before it was fixed with ice-cold methanol. The cells were then incubated with NCOA-3 probe –Q570, a well-validated probe for this cell line provided by our collaborator at Biosearch. Samples were then imaged using Nikon Ti-E microscope under 60X oil lens
[Fig 2.1 A]. The red dots in the cell are labeled single molecule transcripts of NCOA-3 expressed by MCF-7 cells.

Figure 2.1 MCF-7 cells probed for NCOA-3 transcript by RNA-FISH. (A) MCF-7 on coverslips probed for NCOA-3 Q570, imaged under 60X oil lens. (B) StellarVision image of microfilter at 20X dry lens. (C)(D) StellarVision image in B zoomed in for single CTC resolution. Image quality at 20X equals traditional microscope 100X oil lens enabled by StellarVision platform.

Since the protocol of MCF-7 RNA-FISH is validated. Our next goal was to test if this works with cells captured on microfilter. To test this, we harvested MCF-7 cells from culture. Following trypsinization, ~1,000 MCF-7 cells were spiked into sterile 1XPBS and fixed with 1% formalin. The sample is then processed through the microfilter and
tumor cells were captured on filter by size. The sample is immediately subject to RNA-FISH probing for NCOA-3. Post the sample processing, the sample was also shipped to Stellaris for Stellar Vision imaging platform analysis. Stellar Vision is an innovative platform, which applies Synthetic Aperture Optics (SAO) technology to generate images of the filter at 20X magnification and achieves 100X resolution and the data output can be automatically analyzed by imaging software [Fig 2.1 B]. This allows for efficient analysis of CTC eliminating the complication of manual enumeration of transcript copies in CTC using traditional microscope and laborious process of re-focusing during imaging due the waviness nature of the filter as demonstrated by other imaging technologies (Williams et al., 2014). Using this assay, we were able to quantify gene expression at single molecule level [Fig 2.1 C, D].

Figure 2.2 Uveal Melanoma cells probed with GAPDH house-keeping gene. Uveal Melanoma cell line MEL-270 and MEL-290 were probed with GAPDH probe labeled with Q570 dye. Cell nucleus were counter-stained with DAPI dye.

To validate that this assay is also applicable to our proposed disease type –uveal melanoma, we also tested two uveal melanoma cell line –MEL-270 and MEL-290.
Briefly, both cell lines were cultured on glass coverslip. The samples were then probed for GAPDH using the same protocol as described before for MCF-7 cells. Post-RNA-FISH, the samples were imaged under 60X oil lens. As seen in Fig. 2.2, the RNA-FISH protocol worked well on both the MEL-270 [Fig 2.2 A] and MEL-290 [Fig 2.2 B] cells lines.

Concluding Remarks:
In conclusion, we have developed a methodology potentially applicable as a “liquid biopsy” of cancer to analyze gene expression signature of CTC \textit{in situ}. Following up with this study, we are in the process of optimizing probes for genes of interest in Uveal Melanoma including MITF and DCT transcripts to move this study forward, and eventually test these probes on CTC from uveal melanoma patient blood samples, where the RNA-FISH assay will be employed to analyze the metastasis initiating molecular signatures in these CTCs.

2.1.2 Study of Tumor Tropism Gene Signature in Circulating Tumor Cells Isolated from Breast Cancer Patients

Understanding the distribution patterns of metastasis of cancer has been a challenge for centuries. There are two prevailing hypotheses attempting to explain this phenomenon: the mechanical hypothesis and the “seed-and-soil” hypothesis. The mechanical model tries to explain the pattern of metastasis by attributing this to the layout of the vessels connecting the site of a primary tumor and the site of metastasis, e.g. colon cancer
metastasize to liver and breast cancer metastasize to lung. However, this hypothesis fails to explain cases such as duodenal carcinoid tumor have a high rate of lymph node metastasis but rarely metastasize to the liver, which is the common site of metastasis of the neighboring colon cancer (Robert A. Weinberg, 2014). In 1889, Stephen Paget proposed the “seed-and-soil” hypothesis that the metastasizing cancer cells (the seed) found a compatible home only in certain especially hospitable tissues (the soil) (Paget, 1889). Recently, a few studies have partially validated this hypothesis by discovering that certain gene expression signature will predict the sites of metastasis known as “tumor tropism” signatures (Kang et al., 2003; Lorusso & Ruegg, 2012; Minn et al., 2005; Smid et al., 2006) [Fig. 2.3]. In these studies, the gene expression signatures of primary tumors are proven to be of predictive values of secondary sites. Confirming the concordance or discrepancies of these signatures in CTCs with primary tumors will be of high interest since CTC analysis holds the advantages against primary tumor of minimal invasive test,

<table>
<thead>
<tr>
<th>Bone tropism:</th>
<th>Lung Metastasis:</th>
<th>Brain Metastasis:</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-11</td>
<td>IL13Ro2</td>
<td>ST6GALNAC5</td>
</tr>
<tr>
<td>CTGF</td>
<td>SPARC</td>
<td>COX2</td>
</tr>
<tr>
<td>CXCR-4</td>
<td>VCAM1</td>
<td>HBEFG</td>
</tr>
<tr>
<td>MMP-1</td>
<td>MMP-2</td>
<td>ANGPTL4</td>
</tr>
<tr>
<td>Osteopontin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Tumor self-seeding:**
- FSCN1
- MMP-1
- CXCL1

**General Breast Tumorigenicity**
- ID1
- CXCL1
- COX2
- MMP-1
- EREG

Figure 2.3 Tumor tropism related genes in breast cancer. We summarized 21 total of tumor tropism gene panels of interest from past publications. References used to generate

possibility of real-time monitoring of relapse after the primary tumors have been resected and the potential of more refined information of metastasis initiating tumor cell population instead of the more heterogeneous primary tumor. Studying of tropism signatures in CTCs will be of prognostic value and potential application in treatment selection – e.g. biophosphonates treatment for breast cancer patients with high incidence of bone metastasis.

Optimization of Primers for Tumor Tropism Panel Analysis: We first designed tropism signatures panels of interest to be tested based on recent publications [Fig 2.3]. We have designed 21 pairs of primers to target these 21 relevant transcripts in 5 relevant panels: bone tropism, lung metastasis, brain metastasis, tumor self-seeding and general breast tumorigenicity.

To test the best Tm for these 21 primers to work together, we first extracted RNA from MDA-MB-231 cell line. We then performed qRT-PCR to determine which were compatible with the PCR protocol. After first round of testing, we have determined that the following primers pairs word fine without further optimization: from MMP-1, IL13Ra2, SPARC, HBEGF, Osteopontin, ANGPTL4, ID1 [Fig 2.4]. For the primers that didn’t work for the first round, we then tested using gradient PCR to determine the optimal Tm that might work for them. The optimal Tm was determined to be at 64.4 Celsius degree for MMP-2 and CXCR-4 primers. [Fig. 2.5] The rest of the primers were then re-ordered with different designs.
Figure 2.4 Gel Electrophoresis images of tumor tropism primers tested. We were able to amplify transcripts from MMP-1, IL13Ra2, SPARC, HBEGF, Osteopontin, ANGPTL4 and ID1 genes from the first design. Amplicons with desired length of amplification were marked with red rectangles.

Figure 2.5 Gradient PCR to optimize Tm for qRT-PCR reaction. Replicate of PCR reactions were tested with gradient PCR Tm from 57.6 Celsius degree to 64.4 Celsius degree.

Following this, we then seek an optimal protocol that allows us to interrogate CTC expression at single cell level. However, due to the rare presence of CTC in high background of lymphocytes, even post- enumeration by microfiltration platform, CTC
gene expression analysis can be hampered by RNA quality and quantity harvested from the limited material and the extensive process of staining and cell isolation which could potentially lead to RNA degradation. Several optimization procedures of the protocol were attempted to minimize loss of material and RNA degradation during CTC identification on filter as well as isolation of CTC from filter steps and are discussed below:

**Identification of CTC from the Filter:** Although microfiltration platform is able to enumerate CTC from $10^7$ scale of lymphocytes per 7.5 ml of blood sample, there are still approximately $10^3$ scale of lymphocytes which will be captured on the filter. In order to identify CTC among the background of these non-CTC cells captured on filter, distinctive staining for CTC is required prior to isolation of pure CTC population for gene expression analysis. However, a traditional protocol of immunofluorescence staining we apply to identify CTC often take place at room temperature for 4-12 hours and utilize permeabilization and fixation reagents (e.g. 1% formalin, Triton X-100) which will lead to high degradation and RNA loss. In order to achieve the goal to preserve RNA for downstream analysis, two major staining processes were attempted: a shortened immunofluorescence protocol to minimize RNA degradation and a live cell labelling protocol utilizing folate-FITC to label folate uptaking tumor cells. For RNA analysis, the slot pore filter to capture live CTC is applied instead of the traditional round-pore filter to avoid potential RNA loss during the pre-fixation required in round-pore filtration process.
In order to label the CTC without hampering the RNA quality for single cell analysis, we explored the possibility of staining using the following two protocols:

1) Shortened IF Protocol: In order to utilize the same immunofluorescence panel to identify CTC by CK/CD45 staining while greatly reduce the time required for staining and replace the permeabilization process, the shortened IF protocol is designed as following: the filter is first subject to 70% ice-cold ethanol to fix and permeabilize CTC for 3 minutes on 4°C cold block, then it is subject to 3 washes with PBS, 2 minutes primary antibody incubation with 15X more concentrated antibody (as compared with normal IF staining protocol) diluted in RNase inhibitor containing buffer on 4°C cold block, 3 washes with PBS, 2 minutes secondary antibody incubation with 10X more concentrated antibody (as compared with normal IF staining protocol) diluted in RNase inhibitor containing buffer on 4°C cold block, 3 washes with PBS. At last, the filter is dehydrated with acetone for 30 seconds if the following protocol for isolation is Laser Capture Microdissection (LCM). This new protocol successfully reduced the staining time from at least 4 hours in traditional staining protocol to 8-9 minutes, utilizing a concentrated antibody solution and a fixative (70% ethanol) more compatible with downstream RNA analysis. Also, the whole staining process is completed on a 4°C cold block in RNase free environment to better avoid RNA degradation.

To test that this protocol also works with cells captured on microfilter, we also spiked these tumor cells into 7.5 mL of healthy donor’s peripheral blood and processed them
through the slot filter. After the cells were captured on the filter, we then subject the samples to the shortened IF protocol. Results were shown in Fig 2.7.

<table>
<thead>
<tr>
<th>Immunofluorescence Staining Procedure</th>
<th>Duration (in minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 70% ethanol fixation</td>
<td>3</td>
</tr>
<tr>
<td>2 Phosphate buffered saline (PBS) wash</td>
<td>0.5</td>
</tr>
<tr>
<td>3 Concentrated primary antibody incubation (7.5 mg/L Monoclonal Mouse anti CD45 246 mg/L and Polyclonal Goat anti-CK)</td>
<td>3</td>
</tr>
<tr>
<td>4 PBS wash</td>
<td>0.5</td>
</tr>
<tr>
<td>5 Concentrated secondary antibody and DAPI incubation (40 mg/L Alexa Fluor 488 Goat Anti-Mouse and 40 mg/L Alexa Fluor 594 Goat Anti-Rabbit)</td>
<td>3</td>
</tr>
<tr>
<td>6 PBS wash</td>
<td>0.5</td>
</tr>
<tr>
<td>7 Dehydration</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 2.1 Shortened IF staining protocol. Samples were fixed with ice-cold 70% ethanol for 3 minutes and then subject to a concentrated primary and secondary antibody incubation for 3 minutes each. The sample was then dehydrated with ascending concentrations of ethanol and xylene.

Figure 2.6 Positive controls slides to test shortened IF protocol. Cultured cancer cells were harvested and mixed with lymphocytes isolated from healthy donor’s blood using density gradient centrifugation and spun onto glass slides. The slides were subject to the short double immunofluorescence staining. Cancer cells were stained as CK positive (red fluorescence), CD45 negative (green fluorescence). Lymphocytes are detected as CD45 positive (green fluorescence) and CK negative (red fluorescence).
Figure 2.7 Cultured cancer cells seeded in blood are captured on slot filter and subjected to short double immunofluorescence staining protocol. Cancer cells are detected as CK positive (red fluorescence), CD45 negative (green fluorescence) (indicated by red arrows). Lymphocytes are detected as CD45 positive (green fluorescence) and CK negative (red fluorescence) (indicated by green arrows).

Figure 2.8 Folate-FITC labeling of tumor cells. (A) Folate is conjugated with FITC dye using a PEG linker. (B) Folate-FITC is internalized into a folate receptor positive cells and the tumor cell is thus labeled with FITC dye.
2) Folate-FITC Staining Protocol: Although shortened IF protocol described above can be utilized as a more efficient way to label CTC and minimize the degradation of RNA during the labeling process, the ultimate choice will be labeling the cell alive which will not introduce RNA damage during labeling process. To achieve this goal, we focused on a recently developed technique, which has been tested in intraoperative tumor labeling and CTC labeling in blood stream, the fluorophore tagged folic acid (folate-FITC) (van Dam et al., 2011). [Fig 2.8 A] Folate-FITC can be internalized via folate receptor and viably label tumor cells that express folate receptors such as ovarian cancer cells [Fig 2.8 B]. In addition, prostate cancer cells can also intake folate-FITC via a PSMA mediated process and get labeled by this compound. (He et al., 2008) To test this, we purchased commercially available Folate-PEG-FITC which was a conjugate of folate and the FITC dye by PEG linker. Different concentrations and incubation period was tested with folate receptor positive HeLa cells and folate receptor negative MCF-7 cells. The optimized staining was achieved using Folate-FITC at 200 U/ml concentration added into folate deprived culture medium for overnight incubation. With this protocol, we were able to label HeLa ovarian cancer cells as well as PC3 prostate cancer cells but not the MCF-7 cells. [Fig 2.9]

To test is this protocol will allow us to label folate receptor positive CTC from ovarian or cervical cancer alive without labeling the background lymphocytes post live-cell capture process. We harvested HeLa cells from culture and pre-labeled the cells with Calcein AM Red-Orange dye. The pre-labeled cells were then spiked into healthy donor’s blood and processed through the slot filter for viable cell capture. Post-capture, the filter is subject
to the Folate-PEG-FITC staining overnight. The sample was fixed and counter-stained for DAPI at the end of the staining on the next day. [Fig 2.10 A] As expected, all pre-labeled, Calcein AM Red-Orange positive HeLa cells were labeled also with Folate-PEG-FITC, whereas other nucleated cells, i.e. PBMC caught on filter, were not labeled with Folate-PEG-FITC.

Figure 2.9 Test of Folate-PEG-FITC on cancer cells. Folate-PEG-FITC specifically labels folate receptor positive HeLa ovarian cancer cells and PSMA positive PC3 prostate cancer cells but not MCF-7 breast cancer cells which express neither folate receptor nor PSMA.

Isolation of CTC from the Filter: In order to analyze the gene expression profile of the pure CTC population, labeled CTC needs to be isolated from the filter. Due to the static charge and surface chemistry of the filter, release of the cells from the filter is not efficient without special coating. Thus, we propose to utilize two different methods to accomplish this task, Laser Capture Microdissection (LCM) and Aspiration (CellCelectorTM).
Figure 2.10 Test of specificity of folate-FITC staining for tumor cells spiked into blood. (A) Workflow of the experiment. HeLa cells were harvested and labeled with Calcein AM Red-Orange before spiked into healthy donor’s blood and captured on filter. The HeLa cells were then labeled with folate-PEG-FITC. (B) Chart of distinctive staining on HeLa cells and PBMC. HeLa cells should be labeled with both Calcein AM Red-Orange and Folate-PEG-FITC and DAPI, while PBMC should only be labeled with DAPI. (C) Results of Folate-PEG-FITC staining indicating only HeLa cells take up Folate-FITC dye whereas PBMC were not labeled.

1) Laser Capture Microdissection:

Laser capture microdissection (LCM) is a precision microdissection system used extensively with tissue dissection and genetic analysis. VERITAS™ system is a LCM system that uses laser to micro-dissect cells/tissues of interest. Briefly, a cap with ethyl-vinyl acetate membrane is placed on top of the sample of interest. After the sample is
thoroughly analyzed under the imaging platform within the system and the area of interest is defined, a laser pulse will be applied to the spot of interest. Heated by the laser pulse, the membrane coating on the cap will anchor the cell/area of interest. Then, when the LCM cap is lifted from the sample, the cells/tissue sections of interest with then be picked up from the sample and the cap can now be subject to DNA/RNA analysis.

Figure 2.11 PC3 prostate cancer cells were laser captured onto the cap from the slot filter. PC3 cells were spiked into PBS and captured onto slot filter, stained with H&E and dehydrated. Selected cells were picked by laser and transferred onto LCM cap for analysis.

Figure 2.12 qRT-PCR analysis of GAPDH expression from LCM samples of PC3 cells captured on filter. Positive amplification was observed in positive controls, unstained samples and H&E stained samples. No amplifications were detected in negative controls.
We managed to analyze house-keeping gene (GAPDH) expression in LCM material from as little as RNA isolated from 200 cells seeded in blood and captured on filter [Fig. 2.12]. We then combined the shortened IF protocol with the LCM protocol. We used SKBr-3 breast cancer cells representing CTC and Raji cells representing contaminating PBMC as controls. A mixture of both cell line was spun onto the glass slide and subject to shortened IF protocol. We then first analyzed the LCM sample with Agilent Bioanalyzer for RNA quality. The results were shown in Table 2.2. RNA quality decreased significantly after dehydration and went below acceptable RIN number after LCM. We still analyzed this RNA for 6 tumor tropism genes transcripts in our proposed panel: GAPDH (House Keeping Gene), KRT7 (Cytokeratin 7), and tumor tropism markers MMP-1, HBEGF, CTGF and CXCR4 by qRT-PC. The results were shown in Table 2.3.

<table>
<thead>
<tr>
<th>RNA extraction source</th>
<th>RIN number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Fresh cell suspension of SKBr-3 and Raji cells</td>
<td>5.9</td>
</tr>
<tr>
<td>2 SKBr-3 and Raji cells spun onto glass slides, dehydrated</td>
<td>3.3</td>
</tr>
<tr>
<td>3 SKBr-3 and Raji cells spun onto glass slides, stained, dehydrated</td>
<td>2.8</td>
</tr>
<tr>
<td>4 SKBr-3 and Raji cells spun onto glass slides, stained, dehydrated, LCM</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Table 2.2 RNA quality analysis of LCM samples. Cultured SKBr-3 and Raji cells were harvested, spun onto glass slides, subjected to the short staining process, dehydrated and microdissected using Arcturus Laser Capture Microdissection System (Veritas Model). Parallel samples were acquired at different steps for RNA quality monitoring purpose. RNA sample quality was analyzed by Agilent Bioanalyzer 2100 and evaluated by RNA integrity number (RIN) number.
Table 2.3 qRT-PCR analysis of RNA from LCM samples. RNA extracted as described above was subjected to cDNA synthesis and SYBR Green based q-PCR analysis for 6 genes: GAPDH, KRT7, MMP-1, HBEGF, CTGF and CXCR4. All six gene transcripts could be successfully amplified except for the sample after Laser Capture Microdissection.

As shown in row 4 of table 2.2, SKBr-3 and Raji cells yielded limited RNA integrity (RIN 2.4) following IF staining and LCM, which reflected in failure of downstream q-PCR as seen in row 4 of table 2.3.

In addition to attempt LCM on shortened IF protocol stained samples, we also attempted samples stained with Folate-PEG-FITC protocol. In consistence with the shortened IF protocol, RNA quality of samples went down drastically post-dehydration and LCM process (RIN 2.4). [Fig. 2.13]

<table>
<thead>
<tr>
<th>RNA extraction source</th>
<th>Gene transcripts detected/Target genes attempted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  Fresh cell suspension of SKBr-3 and Raji cells</td>
<td>6/6</td>
</tr>
<tr>
<td>2  SKBr-3 and Raji cells spun onto glass slides, dehydrated</td>
<td>6/6</td>
</tr>
<tr>
<td>3  SKBr-3 and Raji cells spun onto glass slides, stained, dehydrated</td>
<td>6/6</td>
</tr>
<tr>
<td>4  SKBr-3 and Raji cells spun onto glass slides, stained, dehydrated, LCM</td>
<td>3/6</td>
</tr>
</tbody>
</table>
In summary, the limitation of LCM is due to several factors, 1. LCM efficiency is comparatively low (usually downstream assay require 5X to 10X more material when the samples are from LCM). 2. LCM requires fixation and dehydration of samples. Although acetone is a milder dehydration method as compared with the traditional ethanol gradient and xylene dehydration method, it still results in cell deformation, which increases the difficulty for post-dehydration cell identification as well as reduced cell retrieval rate. 3. LCM material needs to be purified by a specially designed RNA purification kit, although this kit performs better the other commercially available kits to isolate RNA from LCM samples, further RNA loss and degradation could still occur in this column based purification process. Thus, LCM is not ideal for this specific requirement to isolate small amount of RNA from CTC. Thus we continue to pursue methods to better this result.
2) Automated Single Cell Harvesting System:

CellCelector\textsuperscript{TM} is an automated single cell harvesting system, which utilizes aspiration and/or scraping to transfer single cell alive from one platform to downstream analysis tool. This will eliminate the problems of requirements of fixation, dehydration for LCM and relatively low efficiency of LCM.

To test this system, we first spiked PC3 cells in 1XPBS and captured cells on slot filter. The sample is then subject to either the shortened IF staining protocol or the Folate-FITC staining protocol. 100 cells from each filter was picked using CellCelector\textsuperscript{TM}. [Fig 2.14] Picked cells were then lysed and subject to RNA extraction, and RNA amplification. RNA samples isolated from 100 PC3 cells demonstrated positive qRT-PCR results for KRT7, GAPDH and weak/negative results for KRT20, PTPRC, showing consistency

![Figure 2.14](image)

Figure 2.14 PC3 cells post Folate-PEG-FITC staining was picked up from filter using CellCelector. (Left) the target cell was selected, marked by red circle. (Right) Post-picking, the target cell was picked up, sparing the surrounding cells.
Figure 2.15 PC3 cells picked from microfilter and analyzed for gene expression. 100 PC3 prostate cancer cells were captured on filter and stained either with shortened-IF protocol, or Folate-FITC protocol. After RNA purification and amplification, q-PCR was performed to analyze for GAPDH, KRT-7, KRT-20 and PTPRC expression analysis. Similar results were seen in positive control using PC3 cell pellet and 100 cells retrieved from filter by CellCeletter. No amplifications were seen in negative controls which were PCR reactions with no template and PCR reactions using blank amplification product while no template was loaded for amplification.

with results from RNA sample extracted from PC3 cell pellet harvested using standard protocol. [Fig 2.15] This provides another solution for us to pursue CTC gene expression with the limited amount of RNA can be isolated. Since the cells are alive after isolation process, this is highly likely to be compatible with single cell analysis platforms such as Fluidigm™.

In summary, the automated single cell harvesting system, CellCeletter™ seems to be an ideal solution to isolate viable CTC from patient samples post-capture and identification.
of CTC. This workflow we established here using CellCelector™ can be easily applied for single CTC genomics/transcriptomics study.

3) Nanodroplet PCR

Our collaborator, Dr. Greg Faris’s lab at Stanford Research Institute (SRI) international has developed a nanoliter droplet laser-based heating PCR method (Kim, Vishniakou, & Faris, 2009) based upon a microfluidic optical microfluidic platform. With this platform, a single near infrared laser is used for both droplet manipulation and droplet heating for PCR.

By combining this technology with our microfilter platform, we aim to develop a platform that enables us to analyze single CTC gene expression profiles in situ by multiplex q-PCR in droplet.

In order to perform multiplex qRT-PCR in the droplet, TaqMan probe PCR method was chosen. Fluorescent probes were chosen according to the fluorescent filter combinations at SRI for spectral unmixing for quadruple signal analysis. The fluorescent filters available on SRI laser assisted nanoliter droplet PCR system are as follow:

Excitation Filter: 89101x (EX) (Chroma)

Beam Splitter: 89100bs (BS) (Chroma)
Emission Filter: ET455/50m (EM), ET525/36m (EM), ET605/52m (EM), ET705/72m (EM) (Chroma)

Thus we chose fluorescent dyes to label the TaqMan probes that are compatible with these filter sets. The detailed information is listed as below:

<table>
<thead>
<tr>
<th>Target</th>
<th>Probe Label</th>
<th>Ex</th>
<th>Em</th>
<th>Compatible emission filter at SRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMAM</td>
<td>Pacific Blue</td>
<td>416</td>
<td>451</td>
<td>ET 455/50m</td>
</tr>
<tr>
<td>CEA</td>
<td>FAM</td>
<td>494</td>
<td>525</td>
<td>ET 525/36m</td>
</tr>
<tr>
<td>KRT19</td>
<td>Rhodamine Red-X</td>
<td>560</td>
<td>580</td>
<td>ET 605/52m</td>
</tr>
<tr>
<td>MUC1</td>
<td>Cy5</td>
<td>646</td>
<td>667</td>
<td>ET 705/72m</td>
</tr>
</tbody>
</table>

Table 2.4 Choice of fluorescent dyes to label TaqMan probes that are compatible with SRI platform.

Following the choice of fluorescent dyes, we further designed the primer and probe sequences to perform quadruple PCR to analyze for hMAM, CEA, KRT19 and MUC1 gene expression. The primer and probe sequences are listed as below:

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Primer</th>
<th>Length</th>
<th>Reverse Primer</th>
<th>Length</th>
<th>Probe</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMAM</td>
<td>CAAGACCTTGTGTCACAGAAC</td>
<td>22</td>
<td>GAAGGTGTTGGTTGCAAGCAATC</td>
<td>22</td>
<td>TGCAAGGAATGATGTTGAAGACCAACTA</td>
<td>26</td>
</tr>
<tr>
<td>CEA</td>
<td>GCCATCTGGCGCAATGATGAGATG</td>
<td>21</td>
<td>CCACACCCAGACCCCAATCAG</td>
<td>21</td>
<td>AGTCAAGAAGCAGATCACGTCTCTGCATC</td>
<td>27</td>
</tr>
<tr>
<td>KRT19</td>
<td>CGCACAGAGTGTGATTGCAATG</td>
<td>21</td>
<td>CGACCTGCGGTCAATCCTC</td>
<td>22</td>
<td>CCAATATCGAGCTCATGGCCGAGCA</td>
<td>24</td>
</tr>
<tr>
<td>MUC1</td>
<td>CGCGTGCCATTTCTATCTCATTTG</td>
<td>24</td>
<td>ATCCCGGCCCGTGGAAAGATG</td>
<td>19</td>
<td>CTGTCTGACATGCGGCCGAA</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 2.5 Sequence of probe and primer pairs to detect hMAM, CEA, KRT19 and MUC1 mRNA.
In order to validate the primer sequences, we tested the performance of qRT-PCR analysis using the primer pairs designed above using cDNA synthesized from RNA extracted from the three model system breast cancer cell lines as proposed in the Statement of Work: SKBr-3, MCF-7 and MDA-MB-468.

Briefly, breast cancer cells were cultured till they reached 70-80% confluence. Cells were harvested via trypsinization, and RNA was extracted from cell pellet using QIAGEN RNAeasy Mini Kit. RNA quality and quantity was analyzed using biophotometer and 0.5 µg RNA from each cell line was used to synthesize cDNA using Quanta qScript cDNA Synthesis Kit, qRT-PCR result was detected using Quanta PerfeCTa SYBR Green FastMix, the PCR products were further confirmed using gel electrophoresis. The results are as below:

<table>
<thead>
<tr>
<th>Target/Cell Line</th>
<th>SKBr-3</th>
<th>MCF-7</th>
<th>MDA-MB-468</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMAM</td>
<td>24.84</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CEA</td>
<td>27.80</td>
<td>22.46</td>
<td>-</td>
</tr>
<tr>
<td>KRT19</td>
<td>16.29</td>
<td>16.80</td>
<td>17.94</td>
</tr>
<tr>
<td>MUC1</td>
<td>22.65</td>
<td>21.67</td>
<td>22.56</td>
</tr>
<tr>
<td>Quadruple</td>
<td>16.41</td>
<td>17.07</td>
<td>17.95</td>
</tr>
<tr>
<td>Negative Control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.6 qRT-PCR analysis of hMAM, CEA, KRT19, MUC1 gene expression in SKBr-3, MCF-7, MDA-MB-468 cell lines. 20 µL cDNA was synthesized from 0.5 µg RNA extracted from each cell line and 1 µL of the cDNA was used for each qPCR reaction. In the quadruple PCR reaction, all primers for the four genes are loaded into single reaction. All signals are detected using SYBR Green since not all the probe dyes can be detected using the real-time PCR platform at University of Miami.
In order to determine the specificity of the qPCR reactions, products of the qPCR were analyzed using gel electrophoresis and results are as below:

Figure 2.16 Gel electrophoresis analysis of qPCR products. hMAM, CEA, KRT19, MUC1 gene expression in SKBr-3, MCF-7, MDA-MB-468 cell lines. Data demonstrated that specific band at target length was amplified for each of the target genes. In quadruple PCR reaction, multiple bands were present, the different bands are close in length, thus unable to be unmixed at current resolution.

For SRI to perform nanoliter droplet PCR on site, we extracted RNA from 500,000 MCF-7 and SKBr-3 cells. We used NanoDrop system to measure RNA concentration after extraction. The measurement is as follow:
MCF-7 634.1 ug/mL (A260/A280: 1.93)
SKBr-3 901.2 ug/mL (A260/A280: 2.03)

cDNA synthesis was then performed using Quanta™ cDNA synthesis kit.

The cDNA concentration is then measured using NanoDrop system. The measurement is as follow:

MCF-7 13.9 ug/mL (A260/A280: 1.76)
SKBr-3 13.8 ug/mL (A260/A280: 1.79)

In order to mimic single cell cDNA content in the nanodroplet, we calculated how much cDNA material will be encapsulated in a typical 1 nL reaction at SRI. Based on the fact that we extracted 14 uL of RNA from 500,000 cells, and from this stock RNA, we used 1 uL of RNA to synthesize of 20 uL of cDNA, then we used 5 uL of this cDNA to constitute 20 uL of PCR mix at SRI, then we generated 1 nL of droplet for droplet PCR reaction, we would be working with 500,000/14/20/(20/5)/2,000 cells, which is 0.22 cell per droplet. Thus, this indicated the current cDNA concentration would only yield subcellular level of material for droplet PCR.

Thus, the cDNA was concentrated using SpeedVac and the volume of the cDNA was reduced from a combined 100 uL to roughly 20 uL. Then the cDNA concentration was measured using NanoDrop. The measurement is as follow:
MCF-7 63.6 ug/mL (A260/A280: 1.77)

SKBr-3 57.5 ug/mL (A260/A280: 1.82)

Based on this concentration, we would have roughly $0.22 \times (63.6/13.9) = 1$ cell for MCF-7, and $0.22 \times (57.5/13.8) = 0.92$ cell for SKBr-3 in the 1 nL reaction. This concentrated cDNA is shipped to SRI for Nanodroplet PCR analysis.

In summary, we have achieved optimizing the protocol for analysis of 4 breast cancer related transcripts simultaneously as multiplex q-PCR. As for future plan on this study, we are planning to optimize this multiplex PCR reactions in droplet and eventually employ CellCelector™ to isolate single CTC captured on microfilter from breast cancer patient blood and sending this patient single CTC cDNA to SRI for droplet analysis.

Concluding Remarks:

To sum up, in this chapter, we aim at analysis of CTC at single cell level, which will allow us to interrogate multiple gene expression in single CTC, including the tumor tropism panels. We addressed this by establishing a complete workflow that enabled such applications from 3 different aspects: 1) establishing primers that will detect tumor tropism transcripts; 2) establishing methods to label CTC without hampering RNA for single cell gene expression analysis; 3) establishing methods to isolate identified CTC for single cell analysis.
2.2 Molecular Characterization of CTC to Interrogate Dynamic Change During Treatment

There has been a recent surge in the CTC capturing technology industry. Molecular characterization of CTCs are becoming “hot-beds” for researchers seeking better utilization of CTCs in the clinical setting. Although the identification and quantification of CTCs alone can be effective in prediction of disease outcome (Balic, Williams, Lin, Datar, & Cote, 2013), molecular and functional characterizations of CTCs are likely to increase the specificity of the CTC assay, leading to personalized targeted therapies.

Results from a recent clinical trial, SWOG 0500, indicates that chemotherapy treatment decisions based on elevated CTC numbers after a first cycle of chemotherapy, does not benefit metastatic breast cancer patients (J. B. Smerage et al., 2014). Although, the clinical utility of CTC numbers alone in determining treatment options needs further validation, these results exemplify the importance of, and demand for, molecular characterization of CTC for personalized therapies. Here, we will discuss several ongoing clinical trials in the lab that discuss such applications.

2.2.1 HOG GU12-160 Trial: Phase 2 Study of Docetaxel +/- OGX-427 in Patients With Relapsed or Refractory Metastatic Bladder Cancer

Although the utility of CTC analysis in other common malignancies has been widely demonstrated, few studies have investigated the utility of CTCs to predict therapeutic
response in bladder cancer, and there are currently no studies that directly evaluate the expression of a therapeutic target on CTCs to predict and monitor treatment efficacy.

In collaboration with Memorial Sloan Kettering Cancer Center, Dana Farber Cancer Institute and Johns Hopkins Medical Institute, our lab is investigating CTC in a multicenter Phase 2 clinical trial in bladder cancer conducted under the auspices of the Hoosier Cancer Research Network (HCRN), formerly the Hoosier Oncology Group, and Oncogenex (Noah Hahn et al., 2016). Oncogenex has developed OGX-427, an antisense oligonucleotide inhibitor of production of heat shock protein 27 (Hsp27). Hsp27 protein is elevated in bladder cancer and many other cancers and is associated with chemoresistance. This randomized study is comparing efficacy of docetaxel alone to that of docetaxel combined with OGX-427 in patients with relapsed or refractory metastatic bladder cancer after a platinum-containing regimen. Our ongoing companion CTC studies analyze changes in CTC numbers, as well as CTC characterization by immunofluorescence-based quantification of changes in protein expression of Hsp27. Our hypothesis is that efficacy of targeted therapy will be evident from both decreased CTC numbers and down-regulated expression of targets of therapy (Hsp27 for OGX-427), while non-responsive patients will fail to show such decrease and/or down-regulation.

To conduct this study, we first optimized the immunofluorescence staining protocol for 3 marker, Cytokeratin, CD45 and Hsp27. Since anti-human pan-cytokeratin antibody and anti-human Hsp27 antibody were both raised in rabbit, we took to strategy of pre-conjugating the pan-cytokeratin antibody with Alexa 488 dye. Sample is then subject to first the primary antibody anti-human Hsp27 antibody (rabbit) and anti-human CD45
antibody (mouse) incubation, then anti-rabbit Alexa 594 (goat) and anti-mouse Alexa 680 (goat) secondary antibody labeling and then pre-labeled anti-human pan-cytokeratin antibody with Alexa 488 dye. The sample is lastly counterstained with DAPI for nuclear staining. Thus, the sample will be labeled as DAPI, Cytokeratin (Alexa 488), Hsp27 (Alexa 596) and CD45 (Alexa 680). [Fig 2.17]

Figure 2.17 Triple marker staining on Cytospins of MCF-7 and PMBC cells. MCF-7 cells were labeled with CK (Red, Alexa 488), Hsp27 (Yellow, Alexa 594), PBMC were labeled with CD45 (Green, Alexa 680). Image kindly provided by Anthony J. Williams.

Applying this protocol, to date, 357 samples have been collected from 4 time points (screening, Cycle 2 Day 1, Cycle 3 Day 1 and Cycle 5 Day 1) from 105 patients and processed for CTC enumeration and Hsp27 expression analysis. 30 samples from screening time point have been analyzed so far and CTCs were detected from 30/30 (100%) patients. Also, Hsp27 positive CTCs were detected from 30/30 (100%) patients. Further analysis will be conducted to correlate the results with clinical outcome (response, progression, survival) in this cohort.
Concluding Remarks:

This study is one of the first of its kind to monitor not only CTC counts, but also dynamic changes of drug target expression on CTC during treatment. Analysis of the results will reveal critical information on how this kind of molecular test of CTC will benefit treatment decision and inform clinicians about the effectiveness of cancer treatment.

2.2.2 LCI-GU-BLA-SPEC-001 Trial: Aurora Kinase Expression in Muscle-Invasive Bladder Cancer

Similar to the goal we are seeking in the HOG trial, we are also collaborating with Dr. Earle Burgess at Levine Cancer Center (LCI) on another clinical trial in bladder cancer: the LCI-GU-BLA-SPEC-001 Trial (Burgess, 2016).

Bladder cancer is the fourth most common malignancy in men in the United States, and it leads to significant morbidity and mortality. For these patients, neoadjuvant cisplatin-based chemotherapy (NAC) increases median survival by up to 3 years and the likelihood of cure by approximately 5% (Advanced Bladder Cancer Meta-analysis, 2005).

However, due to concerns over treatment related toxicity, the use of NAC remains low (Raj et al., 2011). For this reason, identifying patients that potentially will benefit from NAC treatment will be of great interest.
The three members of the aurora kinase family of serine/threonine kinases (aurora kinase A, B and C) regulate cell division and maintenance of genomic stability. Overexpression of AK-A and AK-B can be transforming and induce chromosomal instability in vitro and is frequently observed in many human tumors (Lens, Voest, & Medema, 2010). Overexpression of AK-A can also promote tumorigenesis through inhibition of p53 pro-apoptotic functions (Katayama et al., 2004).

Overexpression of aurora kinases A and B have been reported in urothelial carcinoma of the bladder (Sen et al., 2002). In one series of 205 previously untreated patients with bladder cancer, 74% of invasive tumors demonstrated AK-A protein overexpression by immunohistochemistry (IHC) in contrast to only 7.5% of superficial tumors (Sen et al., 2002). Tumors with strong AK-A expression were associated with an increased risk of distant recurrence and decreased overall survival (OS). Thus, it is of our interest to interrogate both CTC level and aurora kinase expression on CTC in this bladder cancer clinical trial.

To optimize staining that allows us to interrogate 3 markers simultaneously on a sample, we employed a similar staining strategy as reported in the previous section regarding the HOG GU12-160 trial. Briefly, tumor cells were labeled with pre-conjugated CK-Alexa 488 and Aurorakinase A/B-Alexa 594, whereas PBMC were labeled with CD45-Alexa 680. [Fig. 2.18]
Figure 2.18 Optimization of LCI staining protocol. Cytospins of MCF-7 cells and PBMC were stained with Pan-cytokeratin (Green) and Aurorakinase A/B (Red) and CD45 (White), cell nucleus were counterstained with DAPI (Blue).
Figure 2.19 Representative image of Patient CTC. CTC on the top left corner is Aurora Kinase A/B positive whereas CTC on the bottom right corner is Aurora Kinase A/B negative.
Concluding Remarks:

To summarize, we have used triple immunofluorescence protocol to analyze 11 samples from 5 patients from 3 different time-points (Baseline, after neo-adjuvant therapy and after surgery) so far. [Fig. 2.19] Further samples are being analyzed for CTC enumeration and aurora kinase expression. This will be correlated with primary tumor pathology analysis as well as the clinical information of the patients. Similar to the HOG trial, this study will be one of the first of its kind trials to reveal critical information on how molecular characterization of CTC will impact cancer management, in the aspects of treatment decision-making, surrogate end-point for clinical trial and treatment response monitoring.

2.3 Establishing CTC Culture to Study in vitro Drug Sensitivity and Develop Personalized Treatment for Cancer Patients.

Current techniques for CTC capture and identification suffer from significant barriers including multiple procedural steps and high cost, limiting both the biological study and widespread clinical utility of CTCs. Functional characterization of CTCs is limited due to the inability to isolate viable CTCs and lack of efficient and robust methods for the reliable and faithful expansion of tumor cells.

A fundamental understanding of the biology of CTC, and their role in metastasis and treatment response prediction is limited. Virtually all CTC studies have been performed on fixed cells, and thus are static. These limitations are due in part to the approaches used
to capture CTCs, such as the by the FDA-approved CellSearch assay, as well as by the underlying problem of the inability to maintain continuous cultures of human epithelial cells in vitro, regardless of the tissue of origin. But recent developments have yielded more encouraging results. For example, a recently reported microfluidics device combines excellent CTC capture and “on chip” treatment with the therapeutic taxol drugs as seen in the microtubule organization alterations (Kirby et al., 2012).

Meanwhile, clinically relevant cell lines for cancer research and drug development were lacking. There is a reliance on a small number of transformed cancer cell lines and inaccurate animal models, many of which fail to effectively reflect the in situ human tumor. We believe these “unmet needs” translate into a deficiency in our understanding of the events leading to cancer initiation, of predictive biomarkers disease aggressiveness and of cellular behavior during and after treatment. Armed with the increasing knowledge of the heterogeneity of cancer, where each tumor is contains a unique series of molecular derangements, there is a growing realization that major advancements in cancer therapy may require a wholly new approach to using primary tissue for biomarker discovery, validation and drug development and screening.

Thus an important direction to pursue in CTC study is the functional characterization of CTC. Technologies that allow viable CTC capture enable such assays (Gallant et al., 2013; Karabacak et al., 2014; Kirby et al., 2012; Zhou et al., 2014). One study demonstrated development of oligoclonal CTC culture from 6 metastatic luminal breast cancer patients, indicating that CTC culture can be potentially used for Next-Gen
sequencing and, more importantly, for drug sensitivity screening (Yu et al., 2014). Another study tested drug sensitivity on chip without establishing CTC culture in prostate cancer, where CTCs from docetaxel resistant CRPC patients do not respond to on-chip docetaxel and paclitaxel treatment (Kirby et al., 2012). A third study showed that xenograft assays of patient-derived CTC into immuno-compromised mice can provide valuable prognosis information (Baccelli et al., 2013). However, attempts to culture CTC are still at very early stage and have low efficiency. The CTC culture method reported by Yu et al, managed to establish 6 CTC culture from 36 patient samples attempted, while the xenograft assay only managed to establish CTC xenograft mice from patients pre-screened with more than 1,000 CTCs per 7.5 mL blood. If CTC culture can be developed into a high-efficiency method and validated in larger cohorts to faithfully reflect patient treatment response without introducing culture-induced artefacts, it could be a powerful tool to guide therapy and greatly benefit cancer management.

In collaboration with Dr. Chris Albanese and Dr. Richard Schlegel at Georgetown University Medical Center, studies are under way to explore the idea of applying their Conditional Reprogrammed Cell (CRC) culture method to effectively establish CTC culture from cancer patients. GUMC group has developed the CRC culture conditions that allow for the long-term culturing of primary mammalian epithelial cells (human, mouse, rat, dog, and horse tested to date) from various organs, including the prostate, breast and lung (Chapman, Liu, Meyers, Schlegel, & McBride, 2010; Liu et al., 2012; Palechor-Ceron et al., 2013). These conditions permit cells to bypass replicative senescence and become “conditionally” immortal without detectable cell crisis. Our
studies have established that the combined culturing of mammalian epithelial cells with irradiated murine fibroblast feeder cells and treatment with the Rho kinase (ROCK) inhibitor Y-27632 \(((+)-(R)-\text{trans-4-(1-aminoethyl)-N-(4-pyridyl)}\text{-cyclohexanecarboxamide dihydrochloride})\) increased the proliferative capacity of primary human epithelial cells, resulting in a conditional immortalization. The efficiency of tumor immortalization depends upon the tissue of origin, but in general it approaches 100%. For example, 100% of samples from breast, prostate, lung, head and neck, and bladder cancers establish into stable cell cultures. The efficiency for pancreas and colon was originally less (50%), but now approximates 100% with the use of low oxygen conditions for culture. These conditions also protected against genomic instability, resulting in cell lines with morphological and genetic profiles similar to the initial primary cells. Further, we have demonstrated that CRC can be generated from as few as 2-4 tumor cells (Chapman et al., 2010). Because the CRCs re-differentiate along their original committed lineage upon removal of feeders and Y-27632 (Chapman et al., 2010; Liu et al., 2012), we have termed these cells “conditionally reprogrammed cells” (CRC).

The CRC culture system enables the first inexhaustible, patient-specific, biobank of primary epithelial cells, which have been propagated in excess of 100 population doublings with no gross genetic changes (Liu et al., 2012). Further, cells derived from prostate lesions formed tumors in SCID mice while those from the normal epithelium did not, recapitulating the original cellular phenotype (Liu et al., 2012). A study led by Yuan et al, showed that the in vivo response to drugs in a patient with recurrent respiratory
papillomatosis could be predicted by in vitro chemosensitivity analysis of CRC grown from the tumor (Yuan et al., 2012).

2.3.1 Temperature Sensitive Release of Viable Circulating Tumor Cells (CTCs) following Size-Based Capture

As we previously stated, slot filter is a very successful size-based strategy for viable CTC capture and analysis (Xu, Lu, Tai, & Goldkorn, 2010). These Parylene-C based microfilters not only capture and retain CTCs with high efficiency but also allow in situ analyses, which require extensive manipulation of CTCs. However, the strong retention of CTCs hinders those experiments that require that the captured cells be transferred onto other platforms. For example, microfluidic platforms, such as the FluidigmTM single cell analysis system (Powell et al., 2012) or the DEPArrayTM system (Peeters et al., 2013) require suspended cells as input, and novel ex vivo culture methods require CTCs to be cultured on specialized surfaces, such as ultra-low attachment plates. All of these platforms, which have great promise as a companion chemo-sensitivity test, are incompatible with many of the capture platforms, including our microfilters. Thus, a facile route to viably recover CTCs captured from the blood of cancer patients is highly desirable.

3. This chapter is based on a previous publication:
Several platforms that allow for viable CTC capture and release have been reported based on immobilized antibodies conjugated to a cleavable linker (Deng et al., 2014), Poly (N-iso-propylacrylamide) (PIPAAm) (S. Hou et al., 2013), or electroactive films (Xiao et al., 2014). However, these systems are affinity-based and can be potentially biased by the target antigen. For example, the most commonly targeted antigen for CTC capture, EpCAM, can be absent within certain CTC populations possessing mesenchymal phenotypes (Yu et al., 2013). In contrast, label free, size-based isolation and release of CTCs can provide a method to study the CTC population in a potentially more comprehensive manner, such as the centrifuge-on-a-chip method reported by Mach et al (Mach, Kim, Arshi, Hur, & Di Carlo, 2011). Here, we demonstrated that by combining our filtration-based platform with the PIPAAm coating method, we could achieve an antigen-agnostic, efficient method to effectively capture and isolate viable CTCs from blood.

PIPAAm is a polymer that undergoes a reversible lower critical solution temperature (LCST) phase transition at a solution temperature of 32°C (Okano, Bae, Jacobs, & Kim, 1990). Traditionally, this property of PIPAAm has been widely explored for tissue engineering applications. Typically, cells are cultured on PIPAAm coated surfaces at 37°C when PIPAAm is hydrophobic. The cells can then be detached as a sheet when the culture temperature is shifted to below 32°C, where the PIPAAm coated surface becomes hydrated (Kumashiro, Yamato, & Okano, 2010; Yamada et al., 1990).
In our formulation, epithelial cancer cells are bound to the parylene C membrane by non-specific electrostatic interactions instead of extracellular matrix (ECM) mediated adhesion. This necessitated a modification of the conventional PIPAAm based release strategy. The epithelial cell capture is performed at room temperature (below 32°C) and cell release is enabled by placing the filter in our culture media maintained at 37°C. At this temperature, the PIPAAm polymer layer becomes hydrophobic, thereby releasing the electrostatically bound cells.

Figure 2.20 PIPAAm coated slot filters to capture and release circulating tumor cells from blood. (A) Microscopic view (400X magnification) of the slot filter post PIPAAm coating; (B) Filtration set-up with syringe pump to capture CTCs from blood using PIPAAm coated slot filter (C) The set-up for the filtration cassette: the PIPAAm coated slot filter is sandwiched between the top cassette and the bottom cassette with two PBMS pieces to seal the cassette. (D) Scheme of using temperature change to release captured CTCs from PIPAAm coated slot filter (E) Characterization of pore dimensions before and after PIPAAm coating.
To coat PIPAAm onto the slot filter, we first dissolved PIPAAm in butanol at 10% (w/v) concentration. The PIPAAm solution was then spin coated onto the slot filter at a top speed of 6000 rpm for one minute\textsuperscript{19}. The coated filter was air-dried and stored at room temperature before use [Fig. 2.20 A,B]. We compared the pore sizes before and after coating by phase-contrast microscopy [Fig. 2.21] and as anticipated, found a 7% decrease in pore length and 15% decrease in pore width after PIPAAm coating [Fig. 2.21]. The Table 2.1 shows the measured pore parameters before and after PIPAAm coating.

<table>
<thead>
<tr>
<th>Filter</th>
<th>Average Length</th>
<th>Average Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-coated</td>
<td>38.08±0.82</td>
<td>6.35±0.34</td>
</tr>
<tr>
<td>PIPAAm-coated</td>
<td>35.31±0.79</td>
<td>5.37±0.35</td>
</tr>
<tr>
<td>Decrease Percentage</td>
<td>7.3%±2.1%</td>
<td>15.3%±5.6%</td>
</tr>
</tbody>
</table>

Figure 2.21 Measurement of pore parameters pre- and post- PIPAAm coating. A) Slot filter pre- and B) post- PIPAAm coating was imaged using Nikon Eclipse Ti-E microscope C) Pore length was found to be decreased by 7.3%±2.1% after PIPAAm coating and pore width was found to be decreased by 15.3%±5.6% after PIPAAm coating. The effect of this pore size decrease was enhancement in capture efficiency [Fig 2.21], along with a decrease in enrichment factor, with more erythrocytes and leukocytes seen post
capture (data not shown). This is consistent with our previous report on slot filters with a 5µm pore width\(^8\). However, this decrease in enrichment factor does not hamper the functionality of the PIPAAm coated slot filter for viable CTC capture and release, since the additional erythrocytes and leukocytes captured can be easily removed by a gentle wash of fresh media at day 1. This did not affect cell viability, proliferation or metabolic activity.

Figure 2.22 Illustration of the experimental workflow to calculate tumor cell capture and release efficiency using PIPAAm coated slot filter.

Methodologically, CTCs are captured onto PIPAAm coated slot filters at room temperature with PIPAAm coating in its hydrophilic state. Post capture, a mild reverse flow was applied to release cells trapped in the pores and then the filter was incubated in...
the release medium at 37°C to induce the phase transition. The cells bound to filter are then released [Fig. 2.20].

To test the PIPAAm coated slot filters, we first labeled SK-Br-3 breast cancer cells (ATCC, Manassas, VA) with green fluorescence using CellTrace CFSE Cell Proliferation Kit (Gibco, Life Technologies, Grand Island, NY). The experimental procedure is illustrated in details in Fig. 2.22. Briefly, ~1,000 of the pre-labelled fluorescent SK-Br-3 cells, based on cell count using Scepter Automated Cell Counter (Millipore, Gibbstown, NJ), were spiked into 3 mL of healthy donor’s blood. One third of the cell suspension

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<th>D</th>
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Table 2.7 Calculation of CTC capture and release rates using pre-labelled SK-Br-3 cells spiked in healthy donor’s blood as a model system. Three replicates were done on different dates, and calculations were done within each set of replicates.

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<td>78</td>
<td>89%±3%</td>
<td>7%±1%</td>
<td>6%±1%</td>
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</tbody>
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Table 2.8 Calculation of SKBr-3 capture and release rates using non-coated filters with SKBr-3 cells spiked in healthy donor’s blood as a model system.
was spun onto a glass slide for cell counting under fluorescence microscopy (Zeiss Axiovert 200M, Jena, Germany) to quantify and verify the number of cells in the blood (Count A). The remaining spiked blood was then diluted 1:1 with 3 mL of Hank’s Balanced Salt Solution (HBSS) (Gibco, Life Technologies, Grand Island, NY) and two equal aliquots of the cell suspension were processed in parallel through PIPAAm coated slot filters at a constant flow rate of 75 mL/hour. Post capture, 1 mL of sterile HBSS was filtered through at the same flow rate to remove and remaining red blood cells and debris. Next, one filter was directly mounted on a glass coverslip and examined under the fluorescence microscope to enumerate the number of cells captured on filter (Count B). The flow-through of the sample was also examined for cell loss during filtration (Count C). For the second sample, we reversed the filtration cassette and used 1 mL of McCoy’s 5A culture medium (Gibco, Life Technologies, Grand Island, NY) to flush out cells trapped in the pores at a constant flow rate of 100 mL/hour. Post reverse-flow, the filter was then incubated in culture medium at 37°C (VWR symphony incubator, Radnor, PA) for 20 minutes. Post incubation, the release medium was spun onto the glass slide using a Statspin Cytofuge (Beckman Coulter, Miami, FL) for enumeration of the cells released from the filter (Count D). The filter was examined, post cell release, to insure effective cell collection (Count E) Results from 3 replicates are shown in Table 2.7. The coating method did not hamper the capture efficiency of the filter itself. Overall, we achieved capture, release and retrieval efficiency averages of 94%±9%, 82%±5% and 77%±5% respectively. The release and retrieval efficiency was increased as compared with those of non-coated filter (7%±1% release efficiency and 6%±1% retrieval efficiency) [Table 2.8].
To test the viability of the cells released from the filter, we spiked ~1,000 SK-Br-3 cells into 7.5 mL of healthy donor’s blood, captured and released them from PIPAAm coated slot filter using the method described above. A Live-Dead® assay (Life Technologies, Grand Island, NY) was performed to evaluate the cell viability before spike into blood and after release. The pre-spike viability was 98% (592 out of 602 cells counted) and the viability of cells captured and released from blood was 95% (540 out 567 cells counted) [Fig. 2.23 A]. We also cultured, in parallel experiment, the cells released from blood

![A: Before Release](Image)

**A**

**Before Release**

**After Release**

![B: Day 3 and Day 10](Image)

**B**

**Day 3**

**Day 10**

Figure 2.23 SK-Br-3 breast cancer cells captured and released from blood remained viable and expanded in culture rapidly. (Top Panel) SKBr-3 cells released from the filter was tested with Live-Dead Assay, 95% (540 out of 567 cells counted) of cells remained viable (Green) after PIPAAm release, whereas dead cells were labelled as red. (Bottom Panel) Cells released from PIPAAm coated slot filter remained viable and expanded in a petri dish. Scale Bar: 100 µm
McCoy’s 5A culture medium (Gibco, Life Technologies, Grand Island, NY). Images were taken at day 3 and day 10. As shown in Fig. 2.23 B, cells released from the filter remained viable and expanded rapidly in culture establishing their viability post cell capture from blood and release from the filter.

Figure 2.24 Capture and release of LMTS-GFP CRC cells using PIPAAm coated slot filter. (Top Panel) Comparison of LMTS-GFP cells cultured on Parylene C surfaces for 5 days (A) and on culture plate for 5 days (B). Parylene C surfaces do not favour the growth of LMTS-GFP CRC cells. (Bottom Panel) LMTS-GFP cells were spiked into healthy donor’s blood and retrieved by PIPAAm coated slot filter. Images were taken under phase contrast microscopy and FITC channel and merged for display. Images were taken at day 1 (C) and day 5 (D). Scale Bar 100µm.

The ultimate application of this technology is to capture patient-derived CTCs from blood and release them with minimal impact on their viability. The released CTCs can then be subject to optimal culture surfaces/conditions, which would have been impossible
on cells retained on filter. To demonstrate the feasibility of this application, we used
conditionally reprogrammed cells (CRCs) (Liu et al., 2012) as a model system. The CRC
approach enables the establishment of continuous primary cell cultures from the majority
of the epithelial tissues and could potentially be applied for CTCs culture establishment.

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<td>87%±10%</td>
<td>79%±14%</td>
<td>69%±12%</td>
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Table 2.9 Calculation of capture and release rates with LMTS-GFP cells spiked in healthy donor’s blood as a model system. Three replicates were done on the same date with the same aliquots, so calculations were done on averaged numbers with each replicate.

Using the CRC method, a tumor cell line was established from a non-small cell Lung carcinoma that had Metastasized To the Spine and termed LMTS. This line was stably labeled with green fluorescent protein (GFP) and termed LMTS-GFP. Although Parylene C can be favorable for culturing certain immortalized cell line (Chang et al., 2007; Kaminska et al.), it was not the optimal surface to culture LMTS-GFP cells [Fig. 2.24 A]. Thus, in order to retrieve LMTS-GFP cells from blood and expand them in culture, we tested the PIPAAm coated slot filter for viable cell release, post capture. First, we tested the capture, release and retrieval efficiency of LMTS-GFP cells on the PIPAAm coated filters. ~1,000 LMTS-GFP cells were spiked into 7.5 mL of healthy donor’s blood and the capture and release experiment was performed in triplicate. As indicated in Table 2.9, efficiencies of capture, release and retrieval produced averages values of 87%±10%, 79%±14% and 69%±12%, respectively. With the confirmative results showing high
efficiency retrieval of LMTS-GFP cells from blood, we then tested the culture of these cells retrieved from blood. We spiked ~1,000 LMTS-GFP cells into 3 mL of healthy donor’s blood. The blood was then diluted to 6 mL volume using PBS. Post capture, the released cells [Fig. 2.24 C] were then cultured in a 48 well plate (Greiner Bio-One, Monroe, NC) in irradiated J2 conditioned F+Y media prepared as described20, 23. As shown in Fig. 2.24 D, released cells remained viable at Day 5 and expanded in culture.

Figure 2.25 Proliferation rates of SKBr-3 cells were compared before and after PIPAAm filter capture and release, as measured using EdU assay. (A) SKBr-3 cells plated as controls (B) SKBr-3 cells retrieved from blood by PIPAAm coated slot filters. Scale Bar: 100 µm

In order to confirm that the process of retrieving tumor cells from blood does not alter the cells’ proliferation rate, metabolism and biochemical properties, we analyzed the tumor cells retrieved from blood by MTT and EdU assays as shown Fig 2.25. For the EdU assay, the modified thymidine analog EdU (Life Technologies, Grand Island, NY) was integrated into newly synthesized DNA and then labeled with Alexa Fluor 594® dye. Cells were incubated with EdU for 2 hours and then fixed for labeling. We then
enumerated the total number of cells (Hoechst+ cells) and newly proliferated cells (Hoescht+, EdU+ cells) and calculated the percentage of EdU+ cells. For SKBr-3 cells, EdU+ cells constituted 29%±6% of the control cell population and 32%±8% of the PIPAAm released cells.

Figure 2.26 Measurement of proliferation rates and metabolism rates of tumor cells before and after PIPAAm coated slot filter capture and release. Proliferation rate of the LMTS-GFP cells was measured using an EdU assay. All cells were labeled with Hoechst (Blue) and express GFP (Green), and newly proliferated cells were labeled with EdU (Red). Comparable proliferation rates were observed between LMTS-GFP cells plated as controls (A) and spiked in LMTS-GFP cells reretived from blood by PIPAAm coated slot filters (B). Metabolism rate and growth curves of SKBr-3 cells and LMTS-GFP cells were measured using an MTT Assay. No significant differences in metabolism rate and proliferation rate were seen between released cells from PIPAAm coated slot filter and control cells for either (C) SKBr-3 cells or (D) the LMTS-GFP CRC line. We also probed the captured and released LMTS-GFP cells for mutated p53 protein as seen in control cells. (E) Extracts from parental control (lane 1) and LMTS-GFP cells cultured following release from the PIPAAm filter (lane 2) were probed by immunoblot for the p53 tumor suppressor protein. The LMTS line harbors an E298* truncation mutation with a predicted molecular weight of approximately 43 kDa (asterisk). LNCaP cells (Lane 3) were run as a control for wild type p53. β-actin was probed as loading control as seen on bottom panel of E.
population, with no significant difference between groups (p value=0.44) [Fig. 2.25]. For
LMTS-GFP cells, EdU+ cells constituted 48%±2% of the control cell population [Fig. 2.26 A] and 46%±4% of the PIPAAm released cells population [Fig. 2.26 B], with no significant difference between groups (p value=0.38). To test the growth curve and metabolism rate of the PIPAAm released cells, we have also performed MTT assay (Life Technologies, Grand Island, NY) on released SKBr-3 cells and LMTS-GFP cells. As seen in Fig. 2.26 C and D, the growth curves and metabolism rates remained the same for both SKBr-3 and LMTS-GFP cells pre- and post- filter capture and release. To verify that the biochemical markers of LMTS-GFP cells were not altered, we probed for p53 protein that has an E298* truncation mutation harboured by LMTS-GFP cell line in cells we spiked into blood and retrieved using PIPAAm coated slot filters. As seen in Fig. 2.26 E, LMTS-GFP cells retrieved from blood by PIPAAm coated slot filters harbor the same truncated p53 as seen in control LMTS-GFP cells.

To test the applicability of our strategy for human patient samples, we tested this technology with 4 metastatic breast cancer patient samples. Briefly, we collected 15 mL of peripheral blood by venipuncture from each patient. Blood samples were split evenly into 2 tubes of 7.5 mL blood for each patient. One tube of blood was processed using our round-pore microfilter, which has been demonstrated previously to capture greater number of CTCs as compared with CellSearch® in the same cohort of patients (Lin et al., 2010). The other tube was processed using the PIPAAm coated slot filter. Post- release, the release medium was spun onto a slide for CTC enumeration. Both the microfilter and the slide
Figure 2.27 Test of PIPAAm coated filter in patient samples. (Top Panel) CTC were captured using round-pore filter or PIPAAm coated slot filters from metastatic breast cancer patients. Samples were subject to immunofluorescence staining for markers of Pan-cytokeratin (Alexa 488 -Green) and CD45 (Alexa 680 –White). The sample was then counter-stained with DAPI (Blue). CTCs were identified as DAPI+ CK+ CD45- cells. Scale Bar 20 µm (Bottom Panel) CTC enumeration and release using round-pore filters and PIPAAm coated slot pore filters. With released cells were then subjected to immunofluorescence staining for pan-cytokeratin and CD45, markers for CTCs identification. As data shown in Fig. 2.27, the CTC enumeration using PIPAAm coated slot filter or round-pore filter in parallel samples were comparable. Thus, with the PIPAAm coating, we showed the ability to recover CTCs captured from human patient samples with a high retrieval rate.
Concluding Remarks:

Circulating Tumor Cells (CTCs) have been established as an important biomarker in cancer and they may play promising roles in cancer management. Molecular and cellular analyses of CTCs can reveal valuable information for cancer prognosis and may help drive precision medicine. Several platforms commonly used in CTC capture and analyses have limitations in allowing for molecular and cellular analyses of CTCs since the cells captured are either fixed or immobilized. Further, affinity-based systems, which allow for viable CTCs capture and release, can potentially be biased by the choice of target antigen. In contrast, by combining our antigen-agnostic filtration based platform with the versatile PIPAAm coating method, we can achieve capture and release of viable CTCs from blood at a high efficiency. This will lay the foundation for the in depth characterization of viable CTCs, including single cell phenotypic and genomic analysis as well as *ex vivo* CTCs culture. Based on our proof-of-principle demonstration with human patient samples, future work is under way to employ this technology for clinical applications.
Chapter 3 Characterization of Circulating Tumor Microenvironment Components

While studies in sub-populations in CTC can be riveting, another interesting observation is the CTC clusters, also known as Circulating Tumor Microemboli (CTM). Their existence was first reported in Small Cell Lung Cancer (SCLC) patients using a size-based CTC isolation strategy. In this study, presence of CTM was shown to correlate with worse clinical outcome as an independent prognostic marker (J. M. Hou et al., 2012). In addition, recent studies have revealed that CTC travel with other blood components as heterogeneous clusters including immune cells (Balic et al., 2013), macrophages (Adams et al., 2014), and platelets (Aceto et al., 2014). In addition, mouse model studies have shown tumor cells traveling with stromal cells, potentially cancer associated fibroblasts as its own “soil” to establish distant metastasis (Dan G. Duda et al., 2010), although these are to date not shown to exist in peripheral blood in human cancer patients. Study of CTC companion cells in circulation could reveal important information on metastasis initiation and expand the definition of “liquid biopsy” to include other cell types beyond CTC.
3.1 Identification of Circulating Cancer Associated Fibroblasts as a Potential Biomarker for Metastasis

Tumor metastasis is the leading cause of cancer related deaths. Metastasis is a multi-step process that not only depends on tumor cell invasion but also depends on assistance from other cellular components of the tumor microenvironment. Cancer associated fibroblasts (CAFs) have been implicated to play an important role in metastasis formation. Thus, it is of great interest to study their participation in the metastatic process including their significance in the circulation.

A human tumor is a complex tissue composed of malignant cells and tumor associated stromal cells. Circulating Tumor Cells (CTCs) are tumor cells found in cancer patients’ peripheral blood. Studies of CTC have revealed their promising prognostic value in several cancers including breast cancer, colorectal cancer and prostate cancer (Alix-Panabières & Pantel, 2014). Recently, several groups have reported existence of CTC clusters and their clinical relevance (Alix-Panabières & Pantel, 2014). Although the prognostic value of CTC has been well validated, there are still limitations of enumeration of CTC being used as a routine clinical biomarker (Alix-Panabières & Pantel, 2014).

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4. This chapter is based on previous publication:
These limitations exist in using CTC as a clinical marker for early detection of cancer or using CTC as a surrogate endpoint in interventional studies (Alix-Panabières & Pantel, 2014). These findings indicate either the limitations in specificity of CTC detection assays employed, or that detecting CTC alone can be misleading or inadequate, especially when applied for early detection of metastasis. Additional biomarker assays may result in enhanced specificity and broaden the application of “liquid biopsy”.

Tumor associated stromal cells or cells of the tumor microenvironment are made up of various types of cells, including fibroblasts, endothelial cells, immune cells, adipocytes, pericytes and extracellular matrix (Mueller & Fusenig, 2004). Contrary to the previous belief that cells of the tumor microenvironment are innocent bystanders, there is substantial evidence highlighting the role of stromal cells in tumor progression and metastasis (Mueller & Fusenig, 2004; Pietras & Ostman, 2010).

The wounded epithelial cells and immune cells including monocytes and macrophages induce an activated fibroblasts phenotype by various growth factors and chemokines such as TGF-β, EGF, PDGF, FGF2, MCP-1, reactive oxygen species and ECM proteases (Kalluri & Zeisberg, 2006). The activated fibroblasts are identified using various markers, such as alpha-Smooth Muscle Actin (alpha-SMA), fibroblasts specific protein (FSP), vimentin, prolyl 4-hydroxylase and fibroblast activation protein (FAP), with FAP and alpha-SMA double positivity being the most definitive markers to identify CAFs (Kalluri & Zeisberg, 2006). These activated fibroblasts are called CAFs (cancer associated fibroblasts) (Kalluri & Zeisberg, 2006). FAP is currently the most specific
marker used to identify “activated fibroblasts” or CAFs, and is a type II transmembrane serine protease (E. Fischer et al., 2012). Its specific function is unknown, but the enzyme activity has been implicated in tumor progression, extracellular matrix (ECM) remodeling and metastasis (E. Fischer et al., 2012).

CAF-secreted cytokines and growth factors can act in a paracrine fashion and regulate a multitude of critical steps in tumor metastasis. CAFs are involved in breast cancer initiation by over-expression of TGF-β and HGF (Hepatocyte Growth Factor). CAFs promote tumor progression and invasion by secreting ECM degrading proteases, the MMPs (Matrix Metalloproteinases). CAFs have been implicated in promoting angiogenesis through secretion of VEGF, FGF2 and SDF-1. CAFs are known to induce EMT through secretion of TGF-β and HGF that also facilitates metastasis (Kalluri & Zeisberg, 2006). CAF-secreted cytokines also support immune evasion and confer a survival advantage to tumor cells (Liao, Luo, Markowitz, Xiang, & Reisfeld, 2009). CAFs collectively serve as an “incubator” for cancer cells by providing a favorable “soil” that supports growth of cancer cells at distant sites (D. G. Duda et al., 2010; Kalluri & Zeisberg, 2006). Depletion of CAFs has shown to decrease metastasis by altering cytokine profiling, and repressing angiogenesis and recruitment of immunosuppressive cells (Liao et al., 2009).

The above-mentioned data underline the importance of CAFs in the process of metastasis. It is natural to assume that “liquid biopsies” of tumor components from blood would not only contain tumor cells but also contain other cellular components from
tumor microenvironment. Supporting this notion, Duda et al, have demonstrated that CTCs can carry stromal cells as “soil” to facilitate metastasis formation (Dan G. Duda et al., 2010). In addition, Hjerpe et al, have demonstrated the existence of CAFs in ascites of breast cancer patients by protein marker expression (Hjerpe, Lundqvist, & Shoshan, 2012). Yet, in spite of all the supporting literature, there has been no direct evidence showing the presence of CAFs in circulation in clinical settings. Thus, in this chapter, we will discuss how we detect the presence of CAFs in circulation using our microfilter platform and how their presence is highly associated with metastasis.

3.1.1 Validation of cCAF Enumeration using Microfilter Technology

Hypothesizing that cCAFs would be a rare cellular population in patients’ peripheral blood and hence realizing that we might face the same technical challenge as studying CTCs, we explored the possibility of employing our current cell-size-based CTC isolation microfilter (Zheng et al., 2007) to isolate the cCAFs that are similarly large relative to hematopoietic cells. To test the possibility of this notion in a model system, we used CAF23 cells, which we established from a triple negative breast cancer patient primary tumor which like all CAFs is FAP+ and alpha-SMA+, as well as vimentin+ and pan-cytokeratin-negative (Drews-Elger et al., 2014). We spiked 3,000 CAF23 cells together with 3,000 MCF-7 breast cancer cells resuspended into 1XPBS. The sample was then processed through our microfilter using the same protocol as previously reported for CTC capture (Lin et al., 2010). Following cell capture, the microfilter was subjected to a double immunofluorescence staining for pan-cytokeratin and FAP to identify epithelial
MCF-7 and fibroblastic CAF23 cells, respectively. The results showed that CAF23 cells could be captured on filter based on their larger size (~20 µm – 40 µm) [Figure 3.1]. Further evaluation of CAF capture efficiency was also done by spiking 100 CAF-23 cells into 7.5 mL of healthy donor’s blood. An average capture efficiency of 95.0%±2.8% was demonstrated [Table 3.1].

![Figure 3.1 Validation of microfilter capture of CAF cells. 3,000 CAF-23 and 3,000 MCF-7 cells were spiked into 5ml of PBS, fixed with formalin, processed through microfilter device and immunofluorescently stained with anti-FAP-Alexa 488 and anti-CK-Alexa 594 antibodies (Top Panel) Merged Picture (Bottom Panel) Picture split into individual channels.](image)

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<td>CAF-23 cells captured on filter</td>
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Table 3.1 Validation of filter’s capture efficiency on CAF cells. 100 CAF-23 cells were spiked into 7.5 mL of blood, processed through microfilter and immuno-fluorescently stained for FAP. The samples were enumerated under fluorescent microscope.
3.1.2 cCAF Identification and Enumeration From Human Patients

Having demonstrated that CAFs could be captured from human blood with a very high efficiency, we employed the microfilter capture process to attempt identification of cCAFs in cancer patients’ peripheral blood. Seven and a half milliliters of peripheral blood samples were collected from a total of 30 breast cancer patients, 20 patients with metastatic breast cancer (Stage IV, MET group) and 10 patients with localized breast cancer with > 5 years of long term disease free survival (Stage I patients treated with curative therapy, LOC group). This latter group of patients is predominantly composed of individuals who will not recur although a small number of these patients will develop recurrences late. Utilizing a triple staining for pan-CK, FAP and the lymphocyte marker CD45, cCAFs are identified as CK-, FAP+, CD45- cells whereas CTCs are identified as CK+, CD45- cells [Figure 3.2 A, B]. FAP/alpha-SMA double staining in parallel clinical samples demonstrated that these FAP+ cCAFs are also alpha-SMA positive confirming their identification as CAFs [Figure 3.2 C]. Also, we maintained some of the viably captured cCAFs in parallel samples in culture, and these cells showed the typical spindle morphology of cells of mesenchymal origin [Fig. 3.2 D]. As shown in Table 3.2, cCAFs can be detected in 30/34 (88.2%) patients from the MET group but in only 3/13 (23.1%) of patients from the LOC group and at very low level (<2 cCAFs). It should be noted that CTCs are detected at a much higher incidence (84.6%) in the LOC group. These data once again question the use of CTCs as a standalone biomarker for metastasis and also highlights the potential importance of cCAFs as a biomarker for metastasis.
Figure 3.2 Identification of CTC and cCAF on microfilter. (A) cCAF identified by FAP staining in breast cancer samples. (Scale Bar: 20 µm) (B) CTC identified by pan-CK staining in breast cancer samples. (Scale Bar: 20 µm) (C) Representative Picture of cCAF double stained by FAP and alpha-SMA. (Scale Bar: 20 µm) (D) cCAFs possess a spindle morphology in culture. Picture was taken at 20X magnification at Day 8 in culture. (Scale Bar: 20 µm)

We also analyzed cCAFs in colorectal cancer patients with metastasis to liver as well as patients with localized prostate cancer. We demonstrated that cCAFs are detectable in both colorectal and prostate cancer setting, and not detectable in healthy donor’s blood [Table 3.2]. And again, although further validation with comparable controls is needed,
Table 3.2 CTC and cCAF enumeration from cancer patients. (A) Table showing cCAF and CTC enumeration from metastatic breast cancer patients (MET group), localized breast cancer patient with >5 years disease free survival (LOC group), metastatic colorectal cancer patients with metastasis to liver, localized prostate cancer patients and healthy donors.

Figure 3.3 Swarm plots indicating the enumeration of cCAFs and CTCs from blood samples. Samples from healthy donors (H; n=7), patients from the LOC group (C; n=13), and patients from the MET group (M; n=34) were analyzed for CTC and cCAF enumeration. p values for Student’s t-test are indicated for comparison of counts of cCAFs and CTCs between patients from the LOC and MET groups.
Metastasis is the underlying cause for most cancer-related deaths. In order for metastases to successfully occur, the tumor cells have to overcome various hurdles (Joyce & Pollard,
2009), including invading the basement membrane to reach the circulation, surviving in circulation, extravasating, and finally seeding and growth at the distant site (Joyce & Pollard, 2009). The microenvironment plays a critical role in determining if the cancer cells will undergo senescence, apoptosis, dormancy or if they will form metastasis at distant site (Joyce & Pollard, 2009). There is extensive data demonstrating the involvement of numerous cellular and molecular players within the TME and their role in metastasis (Mueller & Fusenig, 2004; Pietras & Ostman, 2010). Recent studies have also showed how stromal cells like macrophages cluster with the circulating tumor cells and promote tumor metastasis (Adams et al., 2014).

Our results show for the first time direct evidence of circulating Cancer Associated Fibroblasts present along with the circulating tumor cells in metastatic patients’ blood. These cells were both FAP and alpha-SMA positive, showed spindle morphology in culture and were of the appropriate size range for mesenchymal/epithelial cells. Our findings support previous observations indicating the role of CAFs in tumor metastasis where circulating tumor cells might carry their own “soil” during circulation, thus evading cell death in circulation and creating a metastatic niche at the distant site (D. G. Duda et al., 2010). Our results establish a strong association of the presence of cCAFs in patients’ blood samples with tumor metastasis.

To date, it has been widely documented that CTCs are of important prognostic value in several cancer types (Cohen et al., 2009; Cristofanilli et al., 2004; de Bono et al., 2008). However, using CTCs for early detection of solid tumors is not established. This is
primarily due to the “false positive” detection of circulating epithelial cells in circulation caused by other diseases, such as benign colon disease (Pantel et al., 2012). In addition, CTCs are detected in both early stage and metastatic breast cancer (Nakagawa et al., 2007) making it difficult to use their presence or number as a standalone biomarker for metastasis. A companion biomarker such as cCAFs could benefit the early detection of solid tumor and prove to be an efficient biomarker for metastasis. In our case, cCAFs were only detected in 3 of the 13 samples analyzed from the LOC group, and in both of these the cCAF number was 2 or fewer, while in the MET group, cCAFs were detected in 30 of 34 samples with only 12 of the 30 having 2 or fewer. Similarly, cCAF number was significantly higher in metastatic colorectal cancer samples compared with localized prostate cancer samples. Also, in 1 metastatic breast cancer patient, 2 metastatic colorectal cancer patients and 1 localized prostate cancer patient, cCAFs are detected but CTCs were not, thus further highlighting the importance of this newly discovered CAF population in circulation and whether the presence of them is clinically relevant. In general, cCAF enumeration could be a companion/superior biomarker for early detection of cancer metastasis. We are testing this hypothesis in a larger cohort of patients to support this notion. With the ability to establish the cCAFs in culture, further characterization of cCAFs both in *in vitro* assays and *in vivo* co-xenografts of cCAFs together with tumor cells would define and validate their role in facilitating metastasis. We hope these findings stimulate further studies of this novel population in circulation.

The findings from our study support the potential of employing the presence and/or enumeration of cCAFs as a biomarker for tumor metastasis. Expanded cohorts are
necessary to determine both the sensitivity of cCAF detection as well as a potential threshold of cCAF number for a minimally invasive liquid biopsy biomarker. Our findings also support the rationale for targeting CAFs as a single target population for treatment of multiple different cancers. A FAP-targeting DNA vaccine has been shown to specifically eliminate CAF populations and mitigate tumor metastasis in a mouse model of breast cancer (Liao et al., 2009). Sibrotuzumab, anti-human FAP antibody, has shown remarkable tumor stromal targeting properties in humans (E. Fischer et al., 2012). Though Phase I/II clinical trials results of sibrotuzumab failed to show therapeutic activity, FAP radioimmunoconjugates and antibody drug conjugates may have a promising potential for diagnostics and therapeutics (E. Fischer et al., 2012).

Concluding Remarks:

In summary, we have demonstrated that using our novel cell-size based microfilter technology, circulating CAFs (cCAFs) can be detected along with CTCs in breast, colorectal and prostate cancer patients’ peripheral blood. In addition, our results show that the presence of cCAFs is highly associated with metastasis in breast cancer by comparing the enumeration data from metastatic breast cancer patients with that from patients with ductal carcinoma in situ (DCIS) or early stage 1 invasive breast cancer with no relapse for 5 years. These findings establish a significant association of cCAFs with metastasis and support cCAFs as a promising metastasis biomarker that can be easily accessed minimally invasively in peripheral blood and quantified by enumeration.
3.2 Characterization of Circulating Tumor Cell (CTC) -Myeloid Derived Suppressor Cell (MDSC) Clusters in Mouse Models

Myeloid Derived Suppressor Cells (MDSCs) are cells that are known to suppress T- cells, and linked with poor prognosis in cancer (Talmadge & Gabrilovich, 2013). Study of CTC interactions with MDSC will be of interest to interrogate their roles in survival in circulation and distant metastasis formation.

It is known that via interactions between S100A8 ligand expressed on MDSC and RAGE receptor on tumor cells, metastasis and tumor formation were promoted (Riehl, Nemeth, Angel, & Hess, 2009). Thus, we hypothesize that MDSC will interact with CTC in circulation via this ligand-receptor interaction and promote metastasis. [Fig. 3.5]

To study this, in collaboration with Dr. Barry Hudson and Dr. Marc Lippman at University of Miami, we investigated this interaction between CTC-MDSC in mouse models. First, to validate the staining protocol for S100A8 and RAGE, we analyzed blood samples from 4T1 mouse model. 4T1 tumor cells were xenografted into NOD/SCID mice. Mice were sacrificed at week 6. Blood sample was obtained via intracardiac puncture, and analyzed for CTC using microfilter technology. Filter was stained for DAPI, S100A8 and RAGE analysis. As seen in Fig. 3.6, cross-talk of cells mediated by RAGE-S100A8 interaction was observed in tumor cell clusters.
Invasion, Metastasis

Figure 3.5 Illustration of hypothesized CTC-MDSC interactions. CTC express RAGE receptor and MDSC expression S100A8/A9 ligand. The interaction between these two cell types will promote invasiveness and metastatic potential of CTC.

Figure 3.6 CTC-MDSC in 4T1 mouse model. 4T1 tumor cells were xenografted into NOD/SCID mice. Mice were sacrificed at week 6. Blood sample was obtained via intracardiac puncture, and analyzed for CTC using microfilter technology. Filter was stained for DAPI, S100A8 and RAGE analysis.
Figure 3.7 Illustration of experimental plan to interrogate CTC-MDSC interactions in MDA-MB-231 xenograft model

Reassured with positive results from 4T1 mouse model, we proceeded and interrogated CTC numbers, CTC-MDSC cluster numbers and RAGE expression on CTCs in MDA-MB-231 xenograft model. Respectively, we established xenograft tumor mouse models as following: MDA-MB-231 shRAGE model v.s. scramble control, 4175 (lung tropic MDA-MB-231 cells) shRAGE model v.s. scramble control, and MDA-MB-231 RAGE overexpression model v.s. vector control. Blood samples were taken at week 6 via intracardiac puncture. Blood samples from the same group of mice were divided into 2 sets, one set was fixed and analyzed for CTC-MDSC interactions by immuno-fluorescent phenotyping, and the other set was processed for CTC culture using slot filter. As seen in Fig 3.8. CTC-MDSC clusters were seen captured on filter from these mice models. We also managed to culture CTC from MDA-231 vector, MDA-231 RAGE overexpression
Figure 3.8 CTC-MDSC interactions observed in MDA-MB-231 xenograft models. Tumor cells were expressing GFP (Green), and we stained the sample with DAPI (Blue), S100A8 (Red) and RAGE (White). and 4175 Scramble mice. [Fig. 3.9] Unexpectedly, in contrast to our hypothesis, CTC and CTC-MDSC clusters are detected at a higher rate in shRAGE mice vs. scramble mice, and higher in vector control mice vs. RAGE overexpression mice. [Data not shown] CTC retrieved from mice and expanded in culture also showed a higher proliferation rate in shRAGE mice. However, these studies are inconclusive because the CTC failed to establish culture from scramble controls. Additionally, RAGE antibody employed in this
study was raised in-house and is shown to have off-target affinity, resulting in non-specific staining.

Due to the non-specific binding of RAGE antibody, we then tested just for CTC numbers and cluster numbers in AT-3 and E-0771 syngeneic mouse models. Briefly, AT-3 or E-0771 tumor cells were injected into the mammary fat pad of the C57BL/6 wild type (WT) mice, with heterozygous knockout of S100A9 gene or RAGE gene and homozygous knockout of the S100A9 gene or RAGE gene. Blood samples were taken at week 4 or week 6 post injection. Samples were then processed for CTC enumeration and stained for Pan-cytokeratin and CD45 markers. The data from CTC enumeration in this group indicated that, in general, the WT mice with tumor yield more CTC and CTC-leukocyte pairs/clusters than the heterozygous knockout. And heterozygous knockout yield more CTC and CTC-leukocyte pairs/clusters than the heterogenous knockout. CTC (Pan-cytokeratin as marker)-leukocyte (CD45 as marker) interactions were clearly more seen in WT than in the knockouts. [Table. 3.3]
Day 4-5 in culture

Figure 3.9 CTC in culture from MDA-MB-231 xenograft model at Day 4-Day 5. CTC from MDA-231 and RAGE mice expanded rapidly in culture on filter.
<table>
<thead>
<tr>
<th>Cell Line Injected</th>
<th>Mouse Type</th>
<th>CTC No. (Total)</th>
<th>CTC-Leukocyte interactions No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT-3</td>
<td>Wild Type</td>
<td>48</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>S100A9 (-/-)</td>
<td>17</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E0771</td>
<td>Wild Type</td>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>S100A9 (-/-)</td>
<td>13</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td>(-) CTRL</td>
<td>Wild Type</td>
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<td>0</td>
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<tr>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>S100A9 KO (-/-)</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td></td>
<td></td>
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<tr>
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<td>55</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>160</td>
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<td></td>
<td>RAGE (+/-)</td>
<td>29</td>
<td>1</td>
</tr>
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<td></td>
<td>RAGE (-/-)</td>
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</tr>
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<td></td>
<td></td>
<td>26</td>
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<tr>
<td>E0771</td>
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<td></td>
<td></td>
<td>69</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>RAGE (-/-)</td>
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<td>0</td>
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<td></td>
<td>9</td>
<td>0</td>
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<td></td>
<td></td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>(-) CTRL</td>
<td>Wild Type &amp; RAGE (-/-) &amp; S100A9 (-/-)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.3 CTC and CTC-leukocyte interaction events enumeration in AT-3 and E-0771 syngeneic model.

Concluding Remarks:

Our conclusion from this preliminary analysis is that both RAGE and S100A9 KO whether partial or complete, decrease CTC number and CTC leukocyte interactions. This indicated that CTC could be potentially interacting with MDSC through this RAGE-S100A9 interaction. More mouse model studies are underway to directly interrogate such ligand-receptor expressions on CTC and MDSC in clusters in blood.
Chapter 4 Conclusion

In summary, the field of CTC analysis has grown exponentially in the past decade. As more and more researches have looked into the biological aspects of CTC, the field is going in divergence into 2 separate while equally important directions: studies focusing on clinical utility of CTC and studies focusing on biological nature of CTC. For clinical applications, the CTC assay needs to be standardized, reliable and robust. Thus, the clinical utility of CTC is still largely bound to be based on CTC enumeration since the CellSearch™ system is still the only FDA-cleared technology and it is so far only cleared for CTC enumeration, although a fourth channel on the device can be used for molecular characterization of CTC (Raimondi, Gradilone, Naso, Cortesi, & Gazzaniga, 2014). By utilizing CellSearch system, clinical trials, as described above, have looked extensively into clinical utility of CTC count beyond prognosis, and have begun to address various clinical applications including using CTC to monitor treatment efficacy, using CTC as surrogate endpoint for clinical trials and using CTC for early detection of solid tumor.

5. This chapter is based on previous publication: Datar, R., Ao, Z., Cote, R. (2015). Significance of Studying Circulating Tumor Cells.: Springer.
In my thesis, in Chapter 2.1 and Chapter 2.2, I have discussed how we employed CTC molecular characterization in the Clinical settings. Such studies include interrogating gene expression signatures on CTC as “liquid biopsy” in the hope of substituting the current invasive methods of diagnostics in uveal melanoma; interrogating drug target gene expression dynamic changes during chemotherapy to gather information on treatment effectiveness in the HOG trial; and interrogating markers for chemo-resistance during treatment to study molecular characterization of CTC as surrogate endpoint in the LCI trial. These studies open a door for in depth characterization of CTC as more comprehensive “liquid biopsy” to guide cancer treatment. This will eventually lead to a better understanding on CTC as clinical biomarkers and broaden its application in the clinical settings.

Meanwhile, other studies are also investigating the biological features of CTC by attempting to answer questions about subpopulations of CTC (EMT, stem cell, clusters, tumor tropism etc.), the fate of CTC, and how molecular and functional assays of CTC could benefit patients from the clinical perspective. These questions, if answered, can then both feed back into the understanding of CTC biology and expand or improve the clinical utility of CTC. For example, studying tumor tropism of CTC may answer questions such as the fate of CTC and provide more insight on the “seed and soil” hypothesis (Paget, 1889). Meanwhile it may also provide critical clinical information such as which site needs to be actively monitored and which therapy should be implemented in the adjuvant setting.
In Chapter 2.1.2, I discussed in detail how we designed a study to interrogate “tumor tropism” signature in CTC population, and how this study will reveal CTC heterogeneity and better our understanding of the “seed-and-soil” hypothesis. Such studies, previously limited by methodology for single-cell-analysis, are now made feasible with advanced platforms designed for single cell level applications, such as CellCelector\textsuperscript{TM}, or Nanoliter droplet PCR. With such workflow established, including an effective method to identify CTC and an effective method to isolate such cells, we are now able to interrogate multiple gene expression panels, which will allow us to study the biology nature of CTC in a much more comprehensive manner as compared to before.

Although it is a rapidly advancing field, there is still a lot to learn about CTCs. One “hotbed” for CTC applications is CTC culture. Culture of CTC will not only provide abundant material for genomic analysis of CTC but will also provide a robust \textit{in vitro} model system for drug sensitivity test. In Chapter 2.3, I described in details in a project where I coated a thermo-sensitive polymer layer onto our microfilter platform to effectively release CTC onto the ideal culture substrate. This technology, in combination with CRC technology from GUMC, could be an effective method to establish CTC culture from cancer patient blood. Such methodology, if established, can greatly benefit CTC research by providing abundant material for biology study and clinical drug testing.

Another important aspect in cancer research is tumor microenvironment. There is substantial evidence highlighting the role of all types of stromal cells in tumor progression and metastasis. And recently, CTC has been shown to travel with tumor
microenvironment components as clusters. We are interested in 2 particular cellular components here: CAF and MDSC. In Chapter 3.1, we have described how we successfully identified, for the first time, circulating cancer associated fibroblasts (cCAFs) in metastatic breast, colorectal cancer patients and localized prostate cancer patients’ peripheral blood samples. We also discussed how cCAFs can serve as a potentially superior biomarker for metastasis as compared with CTC. In Chapter 3.2, we have discussed how CTC can interact with MDSC in circulation and how genetic knock-out of these two genes affect both CTC numbers and their interactions with leukocytes in these murine models. Both these two studies have highlighted the importance of studying circulating tumor microenvironment components, and how these studies might reveal novel drug targets and novel biomarkers.

More ongoing research will likely be expanding the definition of “liquid biopsy”, and we believe the ultimate goal will be developing a “universal test” that allows us to not only look at CTC counts but also phenotypic, genotypic and functional features of CTC, as well as possibly other circulating blood-based biomarkers including associated cells, cell-free DNA and microRNA, etc., all studied in one or serial simple and minimally invasive blood draw(s).
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