ADHESION SIGNATURE-BASED ENRICHMENT OF TUMOR INITIATING CELLS

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To my family and friends

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

AFM	atomic force microscopy
ALDH	aldehyde dehydrogenase
BSA	bovine serum albumin
CCO	cancer cell of origin
CSC	Cancer stem cell
CSLCs	cancer stem-like cells
CTC	Circulating tumor cells
ECM	extracellular matrix
EMT	Epithelial-mesenchymal transition
ESC	human embryonic stem cell
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
FN	fibronectin
hiPSC	human induced pluripotent stem cells
hMSC	human mesenchymal stem cell
MACS	magnetic-activated cell sorting
MOI	multiplicity of infections
MT	Matrigel
PBS	phosphate buffered saline solution buffer
PEM	Polyelectrolyte multilayers
PSC	pluripotent stem cell

ROS	reactive oxygen species
SP	side population
TIC	Tumor initiating cell
μSHEAR	micro-Stem cell High- Efficiency Adhesion based Recovery

SUMMARY

In spite of major therapeutic advances, cancer relapse and low rates of patient response to cancer therapeutics persist. This failure is due in part to a small subpopulation of tumor initiating cells (TICs) with stem cell-like properties that are responsible for the growth of the tumor and the progression of metastasis. These cells are capable of surviving chemotherapy, rendering them highly resistant to conventional cancer therapies. Although the question of whether TICs are stem cells remains a controversial topic in the cancer field, it has become increasingly evident that a better understanding of their biology and function is necessary to effectively treat cancer and eradicate tumors without allowing for relapse to occur.

This project aimed to develop an objective, label-free, fast, and scalable method for cancer cell and TIC enrichment based on the adhesion strength signature of these cells. Currently, no efficient and reliable methods to isolate TICs exist. Although many in the field rely on surface marker expression profiles, these are variable and subjective, which hinders the study of TIC biology. Our lab has developed a technology to isolate cells based on their unique adhesion binding strength to a matrix. The novel technology (micro-Stem cell High- Efficiency Adhesion based Recovery [μSHEAR]) consists of a microfluidic device that applies varying degrees of detachment shear forces to adherent cells. Using this device, human pluripotent stem cells and their progeny have been isolated with high reproducibility, yield (>97%), purity (95-99%), and survival (>95%) rates (Singh et al, Nature Methods 2013). The process is fast (<10 min), label free, and scalable. Our hypothesis was that subtypes of cancer cells will exhibit distinct ‘adhesive

force signatures' that can be exploited to selectively purify TICs with high efficiency using the μSHEAR technology. The significance of this work was the development of a novel platform for objective, reliable, and scalable TIC purification.

CHAPTER 1. INTRODUCTION AND SPECIFIC AIMS

1.1 Introduction

Tumor initiating cells (TICs), a subpopulation of cancerous cells within tumors responsible for their maintenance, present a major hurdle to cancer treatment and recovery because of their resistance to conventional therapies [48-51]. While conventional cancer therapies target and often succeed in killing the bulk of the tumor's cancer cells, TICs, sometimes called cancer stem cells (CSC) or cancer stem-like cells (CSLCs), are resistant to these treatments, surviving hostile microenvironments and driving cancer relapse and metastasis and relapse [52, 53]. Unlike bulk cancer cells, TICs have the ability to self-renew and differentiate into many subtypes of cancer cells. TICs have been identified in a variety of cancer types in both primary tumors and cancer cell lines by use of surface marker expression profiles as well as the ability to form tumorspheres and tumors [54]. Nevertheless, the surface marker expression profiles used to isolate TICs vary widely among cancer types and even within tumor samples and cell lines of the same cancer type (Table 2). The inability to isolate TICs with high purity/efficiency has complicated the development of novel therapies to specifically target these cells and continues to be a major hurdle in the cancer research and diagnostic fields. Our lab has recently developed a technology to isolate cells based on their unique adhesion binding strength to the matrix [30]. This novel technology (micro-Stem cell High- Efficiency Adhesion based Recovery [μSHEAR]) consists of a microfluidic device that applies varying degrees of shear force to adherent cells. Using this device, human pluripotent stem cells (both human induced pluripotent stem cells [hiPSCs] and human

embryonic stem cells [ESCs]) have been isolated from their parental cells, spontaneously differentiated cells, and partially reprogrammed cells with high reproducibility, yield (>97%), purity (95-99%), and survival (>95%) rates. The process is fast (<10 min), label free, and scalable. The ***objective*** of my project was to characterize the adhesion strength properties of cancer cells and TICs and exploit any differences to isolate them from each other and noncancerous somatic cells. I ***hypothesized*** that subtypes of cancer cells will exhibit distinct 'adhesive force signatures' that could be exploited to selectively purify TICs and cancer with using the μSHEAR technology. The objective of the project was achieved through the following specific aims.

1.2 Specific Aim 1

Examine the ability of the μSHEAR microfluidics platform to purify cancer cell populations from tumors.

Tumors of mouse cancer cells (B16, E0771, 4T1) were explanted, dissociated, purified, and profiled using the microfluidic platform. The ability of the microfluidic platform to separate cancer cells from non- cancerous host cells was examined by use of time lapse microscopy with the E0771 cell line. This study established the ability of the integrated microfluidics platform to purify and identify cancer cell populations from tumors.

1.3 Specific Aim 2

Establish the ability of the μSHEAR platform to purify TIC sub-populations from cancer cell lines based on adhesive force signatures.

We studied the adhesion properties of a panel of human breast cancer cell lines, mouse cancer cell lines, and cells established from primary human colonic biopsies (CA2) by use of the hydrodynamic spinning disk technology and the μSHEAR technology. For the xenograft cell line CA2, the spinning disk technology was used to quantify differences in adhesion between cells expressing high levels of established TIC marker aldehyde dehydrogenase (ALDH) and those not expressing ALDH. For the mouse cell line E0771, isolated sub-populations were challenged using assays for tumor spheroid formation and invasiveness. Sub-populations of interest were then implanted into C57BL/6 mice to examine their ability to form tumors. This study demonstrated that TIC sub-populations could be purified by differences in adhesive force signature and the correlation between adhesive force signature and surface marker profile and tumorigenicity.

1.4 Innovation and Significance

TIC-enriched populations have been identified in established cell lines and patient samples using a variety of techniques including discrete surface markers (CD44hi/CD24lo, CD133+, ALDH+, ESA+) and their ability to generate tumorspheres and xenograft tumors. Although surface marker expression is the most widely used method for TIC isolation, the expression profiles vary widely among cancer of different tissue origin and, moreover, among TIC populations of different tumors and cell lines within a specific tissue. To date, there is no universal marker profile to identify TICs. The inability to effectively, scalably, and objectively purify TIC subpopulations is a significant impediment to characterizing the biology of these cells with precision as well as analyzing patient samples for effective diagnosis or prognosis. Therefore, there is a significant and unmet need for unbiased, efficient, label-free technologies for the

identification and purification of various cancer cell populations from heterogeneous cultures and tumors.

The proposed project is **innovative** because it will use state of the art bioengineering technologies develop a novel method of TIC isolation from both cancer cells lines and tumors. Furthermore, this novel technology provides an objective and label-free alternative to current TIC isolation approaches which will be fast, easy to use, and scalable. The method developed will have potential applications in cancer research by facilitating the study of TICs as well as clinically in cancer diagnostics and prognostics.

CHAPTER 2. LITERATURE REVIEW

[Adapted from Cermeño, E. A., & García, A. J. (2016). Tumor-Initiating Cells: Emerging Biophysical Methods of Isolation. *Current stem cell reports*, *2*(1), 21-32.][54]

Increasing numbers of parallels are being drawn between cancer and stem cell research. Until recently, cancer progression was described using mainly the clonal evolution model [52, 55, 56] which postulates that cancers evolve by a repeating process of clonal expansion, mutation, and selection. As cancer progresses, different mutations accumulate in clones within the tumor and selective pressure leads to the survival of some clones and the extinction of others in a manner similar to Darwinian natural selection. Within this model, all cancer cells have the ability to rapidly divide and give rise to a new tumor [56]. A growing body of data supports an alternative view of cancer, dubbed the cancer stem cell (CSC) model. In contrast to the clonal evolution model, the CSC model proposes a hierarchical organization of cells in which a small population of tumor-initiating cells (TICs) are capable of self-renewal into more TICs and 'differentiation' into bulk cancer cells. As the name suggests, TICs are defined by their unique ability to initiate new tumors, whereas other cancer cells cannot, but also display distinct marker expression profiles, chemotherapy/drug resistance, and biophysical properties [57-60] (**Error! Not a valid bookmark self-reference.**).

Table 1. Characteristics of TICs

Property	Description	Reference
Tumor initiation	TICs have the capacity to form tumors that resemble the tumor of origin in immunodeficient hosts	[61-63]
Drug/stress resistance	An increased resistance to stresses including hypoxia, radiation, chemotherapy, treatment with other cancer drugs has been observed in TICs. This has been party attributed to an enhanced DNA damage response as well as more effective clearance of cytotoxic agents from the cell	[64-67]
Surface marker expression	Surface markers expression levels are widely used as tools for TIC purification. The markers vary widely among cancer types.	[61, 68-70]
High ALDH activity	ALDH activity is increased in TICs which results in protection from ROS damage and increased survival	[71, 72]
Sphere formation	TICs have an increased ability to grow and form spheroids in suspension culture	[73, 74]
Pluripotent gene activation	The expression of pluripotent genes such as Oct4 and Nanog is increased.	[75, 76]
Unique metabolic activity	Higher mitochondrial membrane potential, lower quantity of mtDNA, and lower intracellular concentration of ATP and ROS have been observed in TICs	[77]
Changed cell adhesion	The expression of adhesion proteins such as integrins is dysregulated, resulting in a changed cell adhesion profile	[78, 79]
Decreased cell stiffness	Decreased cell stiffness and increased deformability have been observed in TICs	[80]
Differential Hoechst 33342 staining	The increased activity of the ABC transporter results in differential staining of TICs by Hoechst 33342, allowing for isolation by SP staining	[81]

TICs are thought to be responsible for the maintenance, progression, recurrence, and metastasis of cancer [82, 83]. Often, their higher propensity to be drug-resistant allows TICs to survive conventional therapies and leads to drug resistant cancer relapse

and metastasis development [84-86]. It is for this reason that targeting TICs in cancer therapy has attracted such excitement from the field [55, 87, 88]. However, TICs are usually rare populations within a tumor and their purification has proven challenging, even after *in vitro* culture. Efficient isolation and enrichment of TICs would facilitate their study and the development of drugs that selectively target them.

It is important to distinguish between the cancer cell of origin (CCO) that initiates a tumor and the CSCs/TICs that sustain it, as they may not necessarily be related [89]. The CCO is the original cell that accumulates the first genetic mutations that lead to cancer. While the CCO is involved in the initiation of the primary tumor, CSCs/TICs are involved in the maintenance of this tumor and the initiation of secondary ones [90]. The terms CSC and TIC are often used interchangeably to denote cancer cells that can self-renew to make more of themselves as well as 'differentiate' into bulk cancer cells [91]. As mentioned previously, these cells are often referred to as cancer stem cells because of the similarities to somatic stem cells and tumor initiating cells because they are able to initiate tumors in immunocompromised mice [92].

2.1 Controversies and the evolving CSC model

The field has been plagued by controversy surrounding the existence and the properties of TICs, with many still doubting the existence of these cells [60, 93]. This debate has been caused in part by the use of the term 'cancer stem cells', which suggests that they are derived from somatic stem cells. TICs can develop from normal somatic stem cells as well as progenitors and perhaps even terminally differentiated cells [57, 94-96]. Furthermore, TICs are referred to as cancer stem cells because the definition of a

stem cell is a cell that can both self-renew and differentiate, both of which a TIC can do [93, 97].

Other controversies stem from reports that the percentage of TICs within a tumor varies widely, sometimes accounting for a small fraction whereas other times the vast majority of cancer cells have the ability to reinitiate tumors [60, 98]. Several studies have suggested that the melanoma TIC frequency varies from around 2% to greater than 40% [52, 99, 100]. In addition to demonstrating the vast variability in TIC frequency, these studies challenge the idea that only a small population of cells within a tumor are TICs. Regardless, populations of cells with TIC properties have been identified in a variety of cancers including those of lung [101], ovarian [102], brain [61], breast [62], colon[103], and prostate [104] origins which have the signature TIC characteristics outlined above.

Although useful, the original CSC model for cancer progression has evolved over the years. Current evidence suggests an intermediary progenitor state in between the TICs and the differentiated cancer cells. While TICs are quiescent and self-renewing, progenitors, sometimes called transit-amplifying cells, rapidly proliferate and have a limited self-renewal capability [59, 105]. Notably, the CSC model and the clonal evolution models are not mutually exclusive, but rather extremes in a spectrum into which most tumors fall. While there is a hierarchy of cancer cell phenotypes, there is also clonal selection within the TIC population, with different clones evolving in parallel and experiencing selection [59, 106]. Furthermore, it seems that differentiated cancer cells can dedifferentiate and go back to a TIC state, although how often this happens is not known [55, 106, 107]. A new report also suggest the existence of several TIC states in breast cancer, including mesenchymal quiescent TICs that are $CD44^{high}/CD24^{low}$, a

phenotype associated with TIC phenotype [108]; epithelial proliferative TICs that are positive for the TIC marker aldehyde dehydrogenase (ALDH); and a double positive TIC population that is even more tumorigenic [55, 109].

2.2 Relationship between EMT and TICs

Epithelial-mesenchymal transition (EMT) is an important process during embryogenesis which allows polarized epithelial cells to transdifferentiate into migratory and invasive mesenchymal cells [110, 111]. This process is also activated during cancer progression and is believed to be crucial driver of metastasis, enabling cells to migrate away from the primary tumor to secondary sites [112, 113]. EMT has been correlated with the acquisition of TIC properties [114-116]. Activation of EMT leads to the expression of TIC markers, increased ability to grow in suspension, and higher tumorigenesis *in vivo* [115]. However, recent studies have challenged the perception that EMT is crucial for cancer progression and TIC phenotype: one study showed that only some TICs undergo EMT, while others retain their epithelial phenotype [109], while another study showed that EMT inhibition does not affect lung metastasis [117]. The exact relationship between EMT and TICs, whether EMT activation results in TIC phenotype or just promotes it, and whether all TICs undergo EMT remains to be elucidated. What has been shown is that drivers of EMT such as Slug, Snail, and the Wnt pathway are implicated in the acquisition of TIC characteristics [47, 118]. Conversely, forced expression of pluripotency genes such as Oct4 and Nanog in breast cancer cells leads to upregulation of Snail, Slug, and mesenchymal markers such as N-cadherin, whereas $CD44^{high}/CD24^{low}$ cancer cells have activated EMT markers and a fibroblast-like morphology [112]. It may be that EMT facilitates TIC phenotype acquisition but is not

necessary for it. A more in depth discussion of this relationship can be found elsewhere [47, 114].

2.3 Relationship between CTCs and TICs

Circulating tumor cells (CTC) are a population of cancerous cells that have been shed into the vascular or lymphatic systems [119, 120]. A significant amount of research has been done on CTCs since they can be used as a tool for cancer prognostic and other clinical applications [120-122]. Although they have been reported to be more aggressive than other cancer cells [123, 124], it is important to note that not all CTCs are TICs, but rather a fraction of them are [119, 122, 125, 126]. The CTCs that do not have the plasticity characteristic of the TIC phenotype are not capable of forming secondary tumors [122], suggesting that a cell needs to have properties of both CTCs and TICs in order to successfully metastasize [119]. A significant number of systems for CTC detection and isolation have been developed as discussed elsewhere [119, 122]. However, many of them rely on specific CTC markers, such as EpCAM, which may not be expressed in all CTCs [55, 119]. Therefore, there is still a significant need for the development of robust platforms to purify TICs.

2.4 Methods of TIC purification

Many different methods of TIC purification have been developed to exploit unique attributes in these cells. Common methods of enrichment include surface marker-based purification and isolation based on TIC intrinsic functional markers, such as ALDH expression, reactive oxygen species (ROS) levels, flow cytometric side population (SP) analysis, and mitochondrial membrane potential differences. Many of these purification

platforms rely on probes such as antibodies and separation technologies such as flow cytometry and magnetic beads. Although popular, these methods have several drawbacks including high price, non-specificity, inability to scale-up, and lack of robustness, which have led to the recent development of biophysical methods of TIC purification based on differences in adhesion, stiffness, and niche/scaffold affinity. The methods of TIC enrichment are summarized on Figure 1.

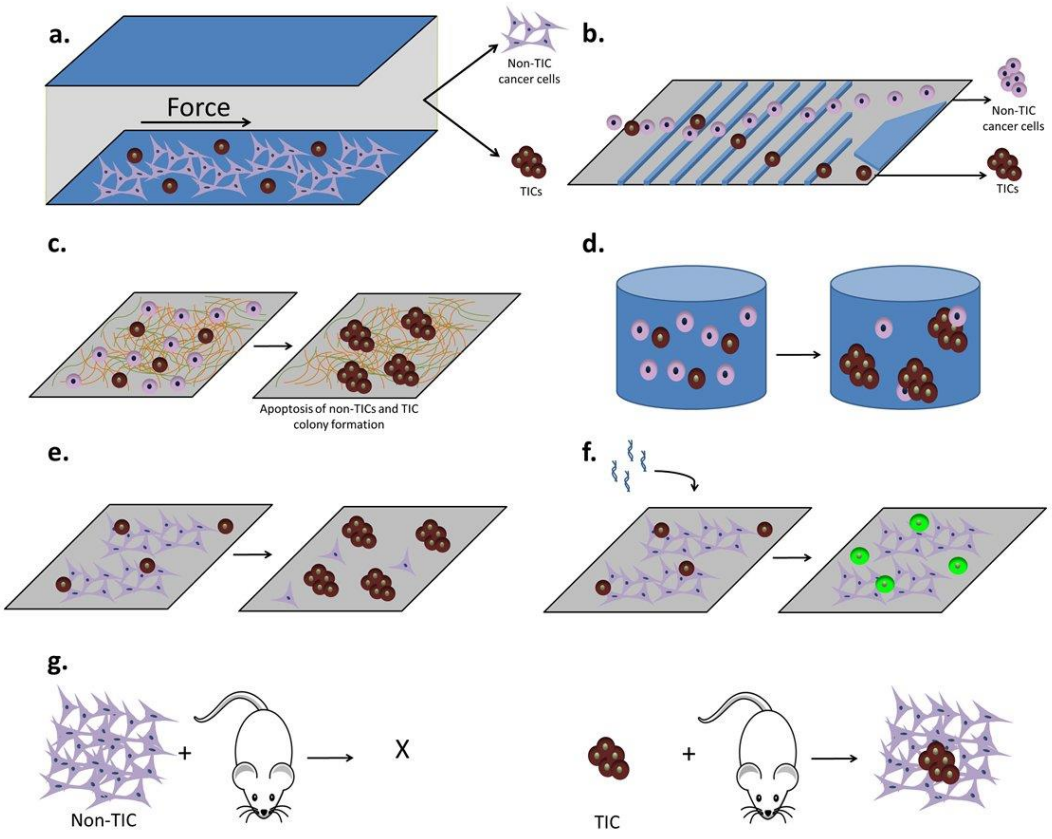

Figure 1 Methods of TIC purification, isolation, and enrichment. (a) Differential adhesion: Cells can be separated from surfaces based on their adhesion strength by applying defined amounts of force. (b) Differential stiffness: Cells are flowed through a microfluidic channel with diagonal ridges that interact with flowing cells. More deformable cells travel perpendicular to the ridges while stiffer cells attempt to travel parallel to them. (c) Niche mimicking/biomaterials: Cells are introduced into a biomaterial or plated on a coated surface that mimics some property of the TIC niche

which leads to enrichment of TICs and/or apoptosis of non-TICs. (d) Suspension culture: Cells are grown in suspension culture. TICs have an enhanced ability to grow in these conditions resulting in their enrichment. (e) Drug resistance: Culture is treated with a drug, which results in the apoptosis of non-TIC cancer cells. (f) Reporter-based selection: A reporter line is made with a fluorescent protein under the control of a TIC marker (e.g. pOct4-GFP). (g) In vivo tumorigenesis: Cells are introduced into an immunocompromised mouse. Only TICs have the ability to form tumors at low doses.

2.5 Marker-based purification

2.5.1 Surface markers

Table 2. Common TIC surface markers

Tumor type	Markers	Reference
Breast	$CD44^+/CD24^{low}$, $CD133^+$, $CD44^+/CD176^+$, ESA^+ ($EpCam^+$), $CD24^+/CD29^+$, $CD24^+/CD49f^+$	[62, 127-129]
Colorectal	$EpCAM^{high}/CD44^+$, $CD133^+$	[130, 131]
Liver	$CD90^+$, $CD44^+/CD176^+$, $CD133^+$, $CD13^+$	[128, 132-134]
Pancreatic	$CD44^+CD24^+ESA^+$, $CD133^+$ $CXCR4^+$	[86, 135]
Ovarian	CD133+, $CD44^+CD117^+$, $CD24^+$	[136-138]
Prostate	$CD44^+/\alpha_2\beta_1^{hi}/CD133^+$,	[104]
Bladder	$CD44^+CK5^+CK20^-$	[139]
Lung	$CD176^+$, $CD133^+$, $CD44^+$	[128, 140, 141]
Brain	$CD133^+$, SSEA-1^+	[61, 142]
Melanoma	$CD20^+$, $CD166^+$, $CD133^+$, $ABCB5^+$	[100, 143-145]
Gastric	$CD44^+$, $CD133^+$	[70, 146]
Osteosarcoma	$CD133^+$,$CD117^+$,Stro-1^+	[147, 148]

TICs have been identified in many types of solid tumors based on their expression of surface markers (Table 2). Various surface markers continue to be identified; however, no universal marker exists. Instead, TIC surface markers appear to be tissue specific and may vary among different tumors requiring extensive validation. Moreover, even well validated markers such as CD133 seem to fail to specifically identify TICs in certain applications [149, 150]. In spite of their limitations, surface markers are widely used for TIC purification, with some groups developing non-antibody based aptamer probes [151, 152]. Many of the developed markers are conjugated with fluorescent labels and used in combination with techniques such as fluorescence-activated cell sorting (FACS) [153] and magnetic-activated cell sorting (MACS) [86] for isolation.

2.5.2 Intrinsic functional markers - ALDH

ALDHs are a family of enzymes that play a role in the metabolism of aldehydes [154]. Studies dating more than a decade have described a correlation between high levels of ALDH and stemness and succeeded in isolating hematopoietic stem cells based on ALDH activity [155-157]. More recently, high ALDH levels have been associated with other stem cell types and with TICs [71, 72, 158-160]. Importantly, overexpression of some members of the ALDH family has been shown to be predictive of poor clinical outcome [161, 162]. This might be in part due to the role ALDH plays in drug resistance development, as these enzymes have been shown to help protect cells from reactive oxygen species (ROS) damage [163]. It seems that ALDH overexpression is a characteristic that TICs from different tissues share [159], making ALDH detection kits extremely useful for TIC isolation. Nevertheless, ALDH-based isolation is not capable of detecting all TICs, as even $ALDH^{low}$ fractions of cancer cells can lead to the formation of

tumors *in vivo* [164]. In fact, recent studies suggest that tumors may have a subpopulation of TICs that are not characterized by the $ALDH^{high}$ phenotype [109].

Nevertheless, screening for cells expressing high levels of ALDH remains a powerful tool for research into TIC biology and behavior. Xenograft lines with high percentages of $ALDH^{Hi}$ cells have been developed and used to study the initiation and maintenance of cancer [165-168]. Their ability to maintain a large population of cells in a TIC-like state facilitates the research into the role TICs play in cancer progression. In particular, these lines have been a powerful tool for elucidating the role of TICs in the transition from colitis to colon cancer [165, 166] as well as the interactions of TICs with other important cell types in colon cancer[167, 168].

2.5.3 Other functional markers

Other functional markers for TICs include ROS levels [169], SP analysis by Hoechst 33342 staining [170], and differences in mitochondrial membrane potential [77]. Of these three methods, the SP exclusion is the most widely used. Although easy and simple to perform, SP exclusion can be culture condition-dependent, since the staining conditions influence the number positive cells and have low specificity [171].

2.6 Biophysical methods of purification

2.6.1 Adhesion-based purification

The interaction between cells and their extracellular environment is of critical importance to normal development and function [172, 173]. Integrin receptors mediate this interaction by mechanically coupling to an extracellular matrix (ECM) ligand,

associating with the actin cytoskeleton and clustering together [174]. These interactions may strengthen to give rise to focal adhesions, which function as structural links between the cell's cytoskeleton and the surrounding ECM [175]. Abnormal integrin function can lead to a variety of diseases, including cancer [176]. Other proteins involved in cell adhesion and focal adhesion complex formation such as focal adhesion kinase (FAK) [177, 178] are also dysregulated in cancer cells and, along with integrins, contribute to disease progression and metastasis. Integrins in particular are often upregulated in many types of cancer and the levels of several integrin subtypes are prognostic of disease severity [176]. In addition to having important roles in cancer cell survival, migration, and invasion, integrins have been shown to cooperate with oncogenes to increase tumorigenesis [176].

Given that integrins and other important cell adhesion proteins are upregulated in cancer, it follows that cancer cells might bind to the ECM with a different amount of force than normal cells. A study by Kwon *et al* supports this premise, as they were able to separate breast cancer cells from normal mammary cells based on adhesion forces. MCF7 breast cancer cells and MCF10A human breast epithelial cells were introduced into microfluidic channels, allowed to attach to gelatin-coated surface, and differentially detached using shear fluid forces [179]. A limitation of this study, however, is that only cell lines are used which might not recapitulate the behavior of primary cancer cells.

Cell adhesion proteins are also important for normal development and embryogenesis [180]. Integrins are essential for stem cell homing to their niche during embryogenesis and development [181]. Several studies have shown the feasibility of separating stem cells based on their adhesion levels and integrin expression profiles [30,

182, 183]. For example, our group recently demonstrated different levels of integrin expression in pluripotent stem cells (PSCs) and their differentiated progeny [30]. These differences resulted in differential ECM adhesion strengths that could be exploited to isolate PSCs from a mixture of differentiated and partially reprogrammed cells. Furthermore, these results suggest that upon differentiation, integrin expression levels change resulting in varying adhesion strengths to ECM proteins.

Given the vital role that adhesion proteins play in normal stem cell function, it is expected that they also play important roles in TICs. In fact, many adhesion proteins have been shown to promote TIC stemness. FAK ablation results in a depletion of the TIC population [184], integrin αvβ3 regulates expression of TIC marker CD44 [185] and is necessary to drive stemness and EGFR inhibitor resistance for epithelial cancers [186], and integrin α6β1 knockdown results in loss of stemness in TICs [187, 188]. In addition to simply being overexpressed in TICs, adhesion proteins potentiate TIC function and enable tumor propagation and drug resistance [189].

Abnormal expression and function of the cell-ECM adhesion apparatus in TICs have allowed groups to isolate them based on their adhesion dynamics. Bansal *et al* isolated prostate TICs from other cancer cells by allowing them to bind to collagen-coated surfaces for a short period of time. They found that TICs bound faster, and by washing away other cells, cancer cells with stem-like properties could be enriched [78]. Zhang *et al* applied the same idea in a more controlled manner to isolate breast cancer TICs. They used a microfluidic platform to slowly flow cells through a channel coated with selected ECM proteins. Since the TICs interact more rapidly with the ECM, they became trapped in the channel, while other cells flowed through [79].

It is important to note that adhesion strength separation can be done by exploiting differences in the rates at which cells bind to the substrate or differences in the amount of force required to detach adherent cells. When exploiting differences in binding rate, the cells are slowly rolled over a coated surface and some will bind faster that others allowing for separation. On the other hand, when exploiting differences in detachment force, cells are allowed to reach some level of adhesion strength with the ECM and then forces are applied to separate them. Although this might not seem like a major difference, the two assays measure different cell properties which may vary independently: the first how fast cells can form adhesion complexes and the second how strongly these adhesion complexes bind to the ECM. Thus far, only differences in binding speed have been exploited to isolate TICs. Separation based on detachment force might prove more useful since it is generally more scalable and robust.

2.6.2 Stiffness-based purification

Cell elasticity (i.e. stiffness) is another important mechanical property of cells, and it is modulated by the cytoskeleton which is comprised of actin filaments, microtubules, and intermediate filaments [190]. Cells are capable of regulating their elasticity by regulating the composition and organization of their cytoskeleton in response to internal and external cues [190, 191].

Cell elasticity has been shown to be increased in cancer cells from a variety of tissues including those from breast, pancreatic, and ovarian origin [190, 192, 193]. Other studies have shown that the trend of decreased stiffness for cancer cells might not hold for cells from different tissues. In contrast to other cancer cells, brain cancer cells appear

to be less mechanically compliant than non-cancerous cells [194]. In spite of this, the differences in cell stiffness between cancer cells and benign cells present the opportunity for stiffness-based separation of cancer cells. Several microfluidic platforms have been developed for this application. The platforms use diagonal ridges [195] or triangular posts [196] to force cells to deform as the flow through the channel. The cells deform to varying degrees depending on their elastic properties and travel differentially through the channels to different outlets.

Cell elasticity has been correlated to the metastatic potential of cells, with cells that are more elastic having higher invasive and migratory behaviors [193, 197-199]. It is speculated that the lower stiffness allows cells to squeeze through and migrate to other parts of the body. The activation of the EMT program in tumor cells would also make them more elastic, since the loss of adhesion and stiffness are hallmarks of EMT [200]. Interestingly, both the activation of the EMT program as well as metastatic potential correlate closely with TIC phenotype [201], suggesting some of the highly metastatic elastic cells studied above might have been TICs. Babahosseini *et al* used atomic force microscopy (AFM) to directly show that an enriched TIC population is at least 45% softer than other cancer cells [80]. Isolation of TICs should thus become feasible by using one of the stiffness fractioning microfluidic platforms mentioned earlier. One study suggests the feasibility of this approach. Highly elastic breast cancer cells were isolated and shown to be enriched for the $CD44^{high}/CD24^{low}$ TIC phenotype. They were also tested for mammosphere formation, an ability unique to TICs [202]. Although encouraging, the study has severe limitation, since it does not include any primary tissue nor does it test the cells for tumor formation capacity *in vivo*.

2.6.3 Niche/biomaterial interaction based purification

Similar to somatic stem cells, TICs have a niche which regulates their differentiation and self-renewal and protects them from the host's immune system [203-205]. Under certain conditions, TICs will home to normal stem cell niches and hijack them [206, 207]. A variety of methods for TIC enrichment have been developed which take advantage of their preferential adhesion or homing into specific scaffolds or topographies. A study by Tan *et al* demonstrated that breast TICs preferentially bind to a specific nanotopography, characterized by thinner grating. A significant enrichment was seen in the $CD44^{high}/CD24^{low}/ESA^{+}$ phenotype when MCF7 cells were cultured in a nanopatterned surface with thin gratings as opposed to unpatterned surfaces and other patterned designs [208]. However, only a slight difference was seen in the $CD44^{high}/CD24^{low}$ population. It is difficult to determine which TIC phenotype was enriched for, since no functional testing was done on the cells and only low levels of ESA expression are associated with TICs, whereas ESA^{hi} is seen in luminal breast cancer cells [209]. More testing is therefore needed to assess the efficacy of nanopatterned surfaces for TIC enrichment.

Another strategy involves culture within three-dimensional scaffolds to enrich for TIC populations. Different scaffolds have been used in this context including chitosan-alginate scaffolds for glioblastoma [210] and hepatocellular carcinoma [211] TIC enrichment and collagen scaffolds for breast [212] and liver [213] TIC enrichment. In these examples, cancer cells infiltrate the scaffold and are maintained within them. The scaffold modulates cancer cell behaviors, resulting in an enrichment of cells with TIC markers, more resistance to chemotherapy/cancer drugs, and higher tumorigenesis *in*

vivo. Since this approach results in both enrichment of TICs and mimicking of *in vivo* environment of cancer cells, it might prove to be a useful tool for modeling *in vivo* behavior and screening new therapies.

Other groups have gone a step further and attempted to model the TIC niche in order to isolate this population of cancer cells [213, 214]. Polyelectrolyte multilayers (PEM) nanofilms were used to fabricate microenvironments that simulated hepatocellular carcinoma TIC niches. Cells were then seeded on top of the PEM layer. After being cultured for 7 days, 70% of the cells were positive for CD44/CD133 TIC markers. This enrichment was due to cell death of non-TICs, presumably because the PEM/hyaluronic acid based environment mimics a TIC-niche topographical cue which is not conductive for their survival, and resulted in a population that was more resistant to chemotherapy agents. Further *in vivo* testing of the enriched populations is critical to validate this enrichment strategy.

An exciting development is a platform for *in vivo* capture of early metastatic cells or CTCs [215]. Poly(lactide-co-glycolide) scaffolds were implanted in immunodeficient NSG mice that had undergone tumor inoculation 7 days beforehand with a highly metastatic variant of the MDA-MB-231 breast cancer cell lines. Cells not only homed to the scaffold, but the tumor burden on common metastatic sites was decreased five-fold, suggesting that cancer cells and TICs were being trapped in the scaffold and prevented from migrating elsewhere in the mouse. A light scattering technique could then be used to detect the cells in the scaffold in a label-free manner. Although this approach does not directly recruit TICs, one could envision modifying the scaffold to facilitate the homing of TICs. Therapeutically, this strategy could serve to deplete the remaining TIC

population after initial treatment and reduce the probability of cancer metastasis and relapse.

All these approaches appear to enrich the TIC population by providing topological or adhesive cues that either decrease the survival of non-TICs or increase amount of cells that remain in the TIC state. The label-free, reproducibility, and relative ease of use are major advantages of these systems. Furthermore, most of them mimic elements of the *in vivo* environment of cancer cells, which can be useful for other applications such as disease modeling. Nevertheless, a major disadvantage might be the broad applicability of these platforms to different types of cancer. If they indeed work by mimicking the TIC niche, those niches might vary among cancers of different tissues.

In summary, biophysical methods for TIC isolation and enrichment offer several advantages over marker based approaches. The label-free nature of biophysical methods leads to more objectivity as there is no bias towards a marker, less manipulation of the cells, and potentially lower costs. Furthermore, some of these methods are more easily scalable since they can be performed in large groups of cells instead of requiring each cell to be passed through a flow cytometer one at a time. Nevertheless, the biophysical properties of cells, including adhesion and stiffness, change when EMT is activated. If there indeed are both epithelial and mesenchymal TIC states [55, 109], then these approaches might not be able to isolate them both at the same time.

2.7 Other methods of purification

2.7.1 Suspension culture

It has become increasingly common to use suspension culture to isolate TICs. The spheres that form in these conditions express many of the TIC surface markers, have higher ALDH expression, and are drug resistant [73, 74, 216]. Although widely used, this system has several drawbacks. First, whereas the spheres do have some true TICs, many of the cells are differentiated progeny or progenitor cells, which are also able to form spheres, and contain a necrotic core [217-219]. There is also a large variation in the percentage of TICs within a sphere, partly caused by the variation in methodology that is employed, which may cause conflicting results and make cross comparison difficult among labs [218, 220, 221]. Some groups have attempted to encapsulate the spheres in a liquid core hydrogel, but the TIC enrichment remains fairly low at 25-30% [222]. Finally, suspension culture might not be able to detect quiescent TICs [220]. Some groups have even reported better results at isolating pure TIC cell lines by using adherent culture with defined media [219]. While useful in some situations, suspension culture has major drawbacks and attention should be paid to the methodology that is used.

2.7.2 Drug selection

In cancers that do not go into remission or which relapse after what appears to have been successful treatment, TICs have been shown to be chemoresistant [47, 223-225] as well as resistant to other cancer drugs [65, 226] and even radiotherapy [66]. Moreover, treatment with drugs such as tamoxifen [65], temozolomide [227], and gemcitabine [228], trastuzumab [229], and staurosporine [230] results in the enrichment of cancer cells with TIC properties [64]. A growing body of literature suggests that this drug resistance allows TICs to survive conventional cancer treatments and leads to relapse and metastasis [47, 224]. The exact mechanism of drug resistance is not known,

but it probably involves a combination of ABC transporter expression, increased ALDH activity, enhanced DNA damage response, quiescence, and activation of other key signaling pathways [225]. The EMT process has also been associated with drug resistance acquisition [47, 231]. Several groups have taken advantage of this property to enrich TIC populations by treating the whole population of cells with chemotherapy or other cancer drugs [232-234]. This leads to the death of normal cancer cells and enrichment of the TIC population. However, there have been reports of cancer drug treatments changing the gene expression profiles of cancer cells [229, 230]. The changes in expression profiles could be due to changes in the relative amounts of TICs to non-TICs as enrichment proceeds, but it could also be due to the process altering the cells themselves. If the cells are changing their gene expression in response to drug selection, this would present a major limitation since it would mean this method could not be used to isolate naïve TICs that have not been altered by drug selection.

2.7.3 Reporter genes

Pluripotency genes such as Oct4 and Nanog are expressed in TICs [75, 76]. This has led to the development of TIC reporter systems in which cancer cells expressing Oct4 [235] or Nanog [236] express fluorescent proteins such as GFP. Interestingly, some of the procedures for reporter line derivation have caused the cells to get stuck in a TIC-like state in which the cells express TIC surface markers, have higher tumorigenesis and cancer drug resistance, and cannot exit the TIC state [235]. Although these reporter lines are useful *in vitro* systems, developing these lines is time intensive and genetically modifies the cells, which may impact other cells functions and behaviors. Furthermore,

this technique is not applicable when attempting to purify or enrich cells from primary human tumors.

2.7.4 In vivo tumorigenesis

By definition, TICs are characterized by their ability to form *de novo* tumors that resemble the primary tumor following transplantation [58, 61, 62]. A gold standard for the characterization and identification of TIC populations is their ability to form tumors in serial immunodeficient hosts. In contrast to TICs, differentiated cancer cells cannot give rise to a tumor and progenitor cells are not able to form tumors in serial hosts. Although it has not been shown that a single TIC can give rise to an entire tumor, significantly lower numbers of TICs are required to do so relative to bulk cancer cells. This limitation, however, might result from our technical inability to get completely pure populations of TICs and not a functional inability of single TICs to give rise to tumors. Although a gold standard, protocols and animal models for *in vivo* tumorigenic evaluation of TICs vary in the literature and a consensus has not yet been reached [60].

Moreover, by using mouse cancer lines derived from specific mouse strains, such as the E0771 mouse breast cancer cell line derived from the C57BL/6 mouse line, the ability of TICs to form tumors can be examined in immunocompetent mice. Similar tumor formation models have been developed in the cancer field [237-241] and utilized to study a variety of cancer related fields including cancer drug development [238, 240], the role of metalloproteases in cancer progression [239], and processes involved in the progression of metastasis [241].

2.8 Future directions

TICs are a unique population of cells within tumors capable of establishing new tumors which drive cancer relapse and metastasis. While usually rare, TICs are resistant to chemotherapy and hypoxic environment. *In vitro,* they are detected by their expression of defined surface markers, ALDH activity, growth in suspension culture, drug resistance, and tumorigenesis in immunocompromised mice. Efficiently isolating TICs will be will be essential in order to diffuse the debate surrounding the existence and importance of TICs as well as to better understand their biology and develop new drugs that selectively target them.

Much effort has gone into identifying appropriate surface markers for TICs, which vary widely according to tissue of origin. It is important that other methods, such as the ones described here, are studied as well in order to develop more reproducible and robust methods for TIC purification.

In summary, the development of the CSC model has revolutionized the cancer research field. Although the model continues to evolve, an impressive amount of data supports the existence of TICs in many cancers. Isolating them will be of pivotal importance to effectively treat and eradicate cancer.

CHAPTER 3. μSHEAR MEDIATED PURIFICATION OF CANCER CELL POPULATIONS FROM TUMORS

3.1 Abstract

It is estimated that 1,735,350 new cancer cases and 609,640 cancer deaths will occur in the US alone in 2018. Conventional treatment of cancer relies on standardized and predetermined therapeutic protocols determined by tumor histological analysis and organ of origin, which often center on systemic administration of cytotoxic chemotherapy. Over the last decades, deeper understanding of the molecular pathways that lead to cancer progression have allowed researchers to develop more targeted cancer therapies that take aim at the specific aberrations that have driven each particular cancer subtype. The targeted nature of these new therapies makes it necessary to identify which patient populations carry the genetic abnormalities targeted by each drug and what the optimal biomarkers are for the identification of said populations. We developed a method for isolating cancer cells based on their unique adhesion binding strength to extracellular matrix (ECM) proteins by using a adhesion based sorting technology micro-Stem cell High- Efficiency Adhesion based Recovery [μSHEAR]) consisting of a microfluidic device that applies varying degrees of shear force to adherent cells. This novel purification method is able to increase the frequency of cancer cells to ~60%, achieving a fourfold increase in cancer cell purification in mixed populations of cells.

3.2 Introduction

Cancer continues to be a major public health problem both worldwide and in the United States, where it is one of the leading causes of death, second only to heart disease, and accounted for 22% of total deaths in 2015 [1]. It is estimated that 1,735,350 new cancer cases and 609,640 cancer deaths will occur in the US alone in 2018 [1]. Although cancer incidence and death rates have both declined over the last decade (2005-2014), by 9% and 15% respectively [1], the overall 5-year survival still remains low at around 61-68% [2]. However, this decrease in cancer mortality has stemmed primarily from lifestyle changes and earlier detection and treatment of cancer cases, not necessarily from more advanced or efficient therapies [1, 3, 4]. Moreover, although overall cancer mortality rates have declined by about 1.5% per year since 1990, mortality rates have increased for some specific cancer types, including liver and pancreatic cancers [1].

Conventional treatment of cancer relies on standardized and predetermined therapeutic protocols determined by tumor histological analysis and organ of origin, which often center on systemic administration of cytotoxic chemotherapy [5, 6]. Although successful, these treatments have a high variability in response and success rate [7] and sometimes lead to severe side effects [6]. Over the last decades, deeper understanding of the molecular pathways that lead to cancer progression have allowed researchers to develop more targeted cancer therapies that take aim at the specific aberrations that have driven each particular cancer subtype [6, 8]. In fact, a new study performed an in depth analysis of the PanCancer Atlas, clustering tumors based on molecular alterations and not site of origin with the hope that the traditional method of organ of origin tumor classification can be supplemented with this novel clustering and

that this may lead to improved clinical trials and patient treatment responses [9]. This shift to a more personalized approach to cancer treatment is evident in the drugs approved by the FDA since 2000, with 15 being targeted therapies and only 5 being traditional chemotherapy agents [6]. Furthermore, cancer immunotherapies, specifically checkpoint inhibitors for solid tumors and adoptive T-cell therapy with chimeric antigen receptor T cells against B-cell–derived leukemia and lymphomas, are disrupting the therapeutic options available to patients. The excitement in the field is evident given that there are currently 200+ and 300+ clinical trials with 6 and 2 FDA approved drugs respectively for these two cancer therapy approaches. In essence, these immunotherapies rely on leveraging immune pathways, either through antibodies or genetically modified T cells, to inhibit the growth of established tumors and recruit the patient's own immune system to attack and eradicate them [10, 11].

Unlike conventional therapies, the targeted and personalized nature of these new therapies means that they will work optimally only on the cancer subtypes that have the genetic abnormalities that they target, while not necessarily providing a benefit in other cancer subtypes [12, 13]. This differential response makes it necessary to identify which patient populations carry the genetic abnormalities targeted by each drug and what the optimal biomarkers are for the identification of said populations [6, 8, 14, 15].

Another hurdle in the cancer field is the high propensity of failure of drug candidates undergoing clinical trials [16, 17]. Approximately 62% of clinical trials involving cancer drugs from 2005 to 2009 did not achieve statistical significance [18]. Furthermore, the estimated approval success rate for cancer drugs between 1993 and 2004 was 13.4% [19]. This high failure rate might be due in part to the limited

availability of preclinical models in which to study prospective drugs, which often rely heavily on cancer cell lines [17]. Cancer cell lines accumulate a large amount of aberrations during their prolonged *in vitro* expansion [20, 21], which cause them to not be representative of clinical scenarios. Furthermore, the limited number of cell lines also fails to recapitulate the diversity of genetic and non-genetic aberrations present the large number of patients suffering from cancer [17, 22, 23].

Because of the need for better validation tools for preclinical testing and the need to identify novel biomarkers of the different cancer subtypes, novel methods for generating and culturing primary tumor cells from patients are important [17, 24-26]. Nevertheless, it is nontrivial to develop said lines successfully and the contamination of non-tumor cells in the cultures as well as normal stromal cells outcompeting cancer cells in long term culture remain hurdles that need to be overcome [27-29].

Here, we characterized the adhesion properties of cancerous and noncancerous cells by use of the hydrodynamic spinning disk technology. Furthermore, we developed a method for isolating cancer cells based on their unique adhesion binding strength to the matrix by using a adhesion-based sorting technology micro-Stem cell High- Efficiency Adhesion based Recovery [μSHEAR]) [30] consisting of a microfluidic device that applies varying degrees of shear force to adherent cells.

3.3 Materials and methods

3.3.1 Cell culture

All human breast cancer cell lines as well as the hTERT-HME1 cell line were acquired from ATCC and cultured according to their protocols. E0771 cells were cultured in RPMI 1640 supplemented with 10%FBS and 1% penicillin, and 1% Streptomycin at 37 ºC, 5% CO2. All cell lines were sub-cu ltured at 70-80% confluency.

3.3.2 Spinning disk Assay

Circular cover slips (25 mm diameter) were sterilized with ethanol, coated with fibronectin (10 μg/mL) for 30 min, and blocked with a 1% solution of bovine serum albumin (BSA) for 30min. Cells were seeded onto fibronectin-coated circular coverslips and cultured overnight at concentrations of 50,000-200,000 cells/mL depending on the cell line in order to achieve 40-50% confluency. After 24 hrs, the coverslips were spun for 5 min in phosphate buffered saline solution buffer (PBS), thus applying a range of forces to the cells proportional to the cell's radial position in the cover slip. The cells were fixed with 4% paraformaldehyde for 15 min, permeabilized in a 00.05% triton solution for 40 min, stained with DAPI for 30 min, washed three times with PBS, and mounted into slides for imaging. For E0771-GFP+ experiments, the cells were also stained against GFP for two hours with a 1:100 dilution of the primary antibody (Abcam #ab290) and for one hour with a 1:200 dilution of an anti-rabbit 488 secondary antibody with three washes (1% bovine serum albumin [BSA]) after incubation with each antibody all at 37C. The number of cells at defined radial positions was quantified by use of a fluorescence microscope with a mechanical stage. A Matlab program was used to fit the data into sigmoidal and calculate the T_{50} (force required to detach 50% of the cells). [31, 32]

3.3.3 Microfluidic Device Fabrication

PDMS (Sylgard 184, Dow Corning) microfluidic devices were fabricated as reported earlier using a negative photoresist (SU-8 2050, 50-μm thickness, MicroChem) and UV photolithography [30]. Patterned negative molds were then exposed to vapor-phase tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (United Chemical Technologies) in a vacuum desiccator to prevent adhesion of PDMS. A 5-mm-thick layer of degassed PDMS mixture (10:1) was cast onto the mold and cured at 110°C for 20 hrs. Cast PDMS devices were peeled off and then punctured for inlet-outlet holes and bonded to glass coverslips by exposure to oxygen plasma for 15 s and allowed to bond at 110°C for 30min.

3.3.4 GFP lentiviral transduction of E0771 cells

Stably transduced eGFP-E0771 cells were generated by infecting B16 mouse melanoma cells with LV-CMV-GFP lentivirus and sorting for GFP+ cells on an Aria sorter. Briefly, cells were seeded at 60% confluency in 6-wells and allowed to attach overnight. The cultures were then lentivirally infected at several multiplicity of infections (MOIs) ranging from 10 to 50. Four days post-infection, the cells were trypsinized and the GFP expression levels assessed in a flow cytometer. The MOI that gave the best efficiency of infection, MOI=50, was selected for further use. Two rounds of sorting were performed in order to achieve a >99% GFP+ population of cells by use of the BD Aria sorting machine.

3.3.5 Tumor generation and digestion

eGFP-E0771 cells were injected (20,000 cells in 20 μL of 1:1 Matrigel to saline solution) through he inguinal mammary gland into the abdominal mammary gland of female C57BL/6 mice to establish tumors. Tumors were excised after 7 days and digested with collagenase D (0.375 U/mL) and hyalurodinase (125 U/mL) for 2-3 hrs at 37 °C with mechanical agitation every 30 min, 0.25% trypsin-EDTA (5 min, 37 °C), red blood cell lysis buffer (5 min, room temperature), and DNAse (10 min, 0.1mg/mL, room temp). The mixture of cells was filtered through a 40 μm filter twice, resuspended in the relevant volume of media, and used for the adhesion experiments.

3.3.6 μSHEAR experiments

Devices were first washed with ethanol, washed with PBS, either coated with fibronectin (10 μg/mL in PBS, 45 min) and blocked with bovine serum albumin (1% BSA in PBS, 45 min) or coated with Corning Matrigel™ hESC-Qualified Matrix according to the manufacturers guidelines (90 min), and washed with complete media. Cells were then introduced at a concentration of 4-10 x10^6 cells/mL and cultured at 37 °C, 5% CO2 overnight. For digested tumor isolates, a gravity driven media wash was performed 2.5hrs post-seeding. The following day, cells were labeled with CellTracker™ Red CMTPX Dye (ThermoFisher Scientific #C34552) within the devices according to manufacturer's guidelines. CellTracker™ Red Dye diluted in RPMI1640 was introduced into devices by gravity driven flow and stained for 30 min, after which a gravity driven media wash was performed. Devices were loaded into a Nikon TE300 microscope equipped with a Ludl motorized stage, Spot-RT camera, and Image Pro analysis system and the number of GFP+ and CellTrackerRED+ cells was quantified along 8 specific locations. Predetermined amounts of force were applied to the cells for a 10 min period by flowing

PBS at well-defined flow rates controlled by a Harvard syringe pump. The number of GFP+ and CellTrackerRED+ cells was quantified in those same 8 locations at predetermined timepoints.

3.3.7 Statistics

Data points were plotted using Prism (GraphPad) with a horizontal line representing the mean and vertical error bars indicating the standard deviation. Statistical significance ($p<0.05$) was determined using either one way ANOVA with Tukey post hoc test or two way ANOVA with Tukey post hoc test as noted with Prism (GraphPad). Sigmoidal non-linear regression was also used in some parts of the analysis (GraphPad).

3.4 Results

3.4.1 Breast cancer cell lines have diverse adhesion properties

The adhesion signature force of a panel of breast cancer cell lines was measured using the spinning disk technology, which applies a linear range of forces to cell attached to a fibronectin-coated glass coverslip. Shear flow-mediated detachment of the cells can then be quantified at specific radial locations and fitted into a curve to acquire the τ_{50}, the shear stress at which 50% of the cells detach. Genetic and cell line information on each of the cell lines is provided in Table 3, which is adapted from Dai A *et al* [33]. MCF7-TS and MCF7-TR5 are cell lines derived from the MCF7 cell line, which are tamoxifen sensitive and resistant, respectively. Representative detachment profiles and curve fits for the non-cancerous mammary immortalized hTERT-HME1 cell line and the MDA-MB-231 and MDA-MD-453 breast cancer cell lines are shown (Figure 2A). After fitting the

data into sigmoidal curves, the τ_{50} was calculated (Figure 2B). Non-cancerous immortalized mammary cells (hTERT-HME1) had a significantly higher adhesion strength signature than all cancerous cell lines ($p<0.0001$). Furthermore, adhesive strength values were significantly different among the majority of the breast cancer cell lines tested, suggesting a large degree of variation in adhesive properties among them.

Table 3. Characteristics of breast cancer cell lines tested.

Cell line	ER	PR	HER2	BRCA1 Mutation	Subtype	Tumor type
MDAMB453	-	-	+	WT	HER2 positive	adenocarcinoma
MDAMB231	-	-	-	WT	Triple negative B	adenocarcinoma
MCF7	+	+	-	WT	Luminal A	invasive ductal carcinoma
ZR751	+	+/-	-	WT	Luminal A	invasive ductal carcinoma
MDAMB468	-	-	-	WT	Triple negative A	adenocarcinoma
HCC1937	-	-	-	Mutated	Triple negative A	ductal carcinoma

The detachment profile of MDA-MB-231 breast cancer cells was also assessed by use of the μSHEAR technology, a microfluidic device that applies varying degrees of shear force to adherent cells proportional to the fluid flow through the channel. It is important to note that this approach allows for the live monitoring of the detachment of the cells, something that is not possible with the spinning disk technology. Figure 2C show the sigmoidal fit detachment profiles for the MDA-MB-231 breast cancer cell line. While >80% of the cells detach with 200 dynes/cm^2 of shear force, some remain attached

in spite of much higher forces, suggesting the existence of a subpopulation of strongly adherent cells within the cell lines.

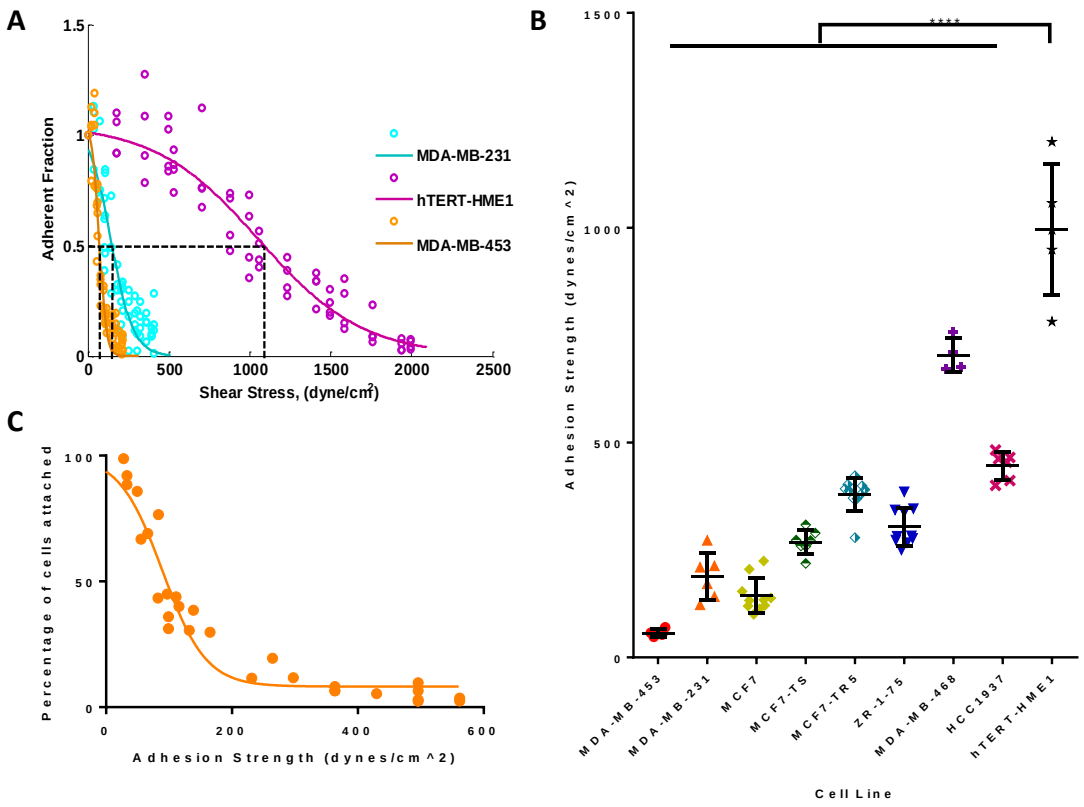

Figure 2 The adhesive strength value of breast cancer cells varies significantly not only among cell lines, but also within them. (A) Representative spinning disk detachment curves for non-cancerous immortalized mammary cell line hTERT-HME1 as well as breast cancer cell lines MDA-MB-231 and MDA-MD-453. The T_{50} is indicated with dashed black lines for each cell line. (B) Spinning disk adhesive force measurements of a panel of breast cancer cell lines and the hTERT-HME1 immortalized mammary cell line. Statistical analysis was performed using one way ANOVA. A non-cancerous hTERT-HME1 cells were significantly more adhesive to fibronectin than all the breast cancer cell lines tested ($p<0.0001$) for detailed significant differences among cell lines see

Table 4. (C) μSHEAR detachment profiles of MDA-MB-231 cells.

Table 4 Tukey's multiple comparisons test results for Figure 2B

Tukey's multiple comparisons test	Significant?	Summary
MDA-MB-453 vs. MDA-MB-231	Yes	*
MDA-MB-453 vs. MCF7	No	ns
MDA-MB-453 vs. MCF7-TS	Yes	****
MDA-MB-453 vs. MCF7-TR5	Yes	****
MDA-MB-453 vs. ZR-1-75	Yes	****
MDA-MB-453 vs. MDA-MB-468	Yes	****
MDA-MB-453 vs. HCC1937	Yes	****
MDA-MB-453 vs. hTERT-HME1	Yes	****
MDA-MB-231 vs. MCF7	No	ns
MDA-MB-231 vs. MCF7-TS	No	ns
MDA-MB-231 vs. MCF7-TR5	Yes	****
MDA-MB-231 vs. ZR-1-75	Yes	**
MDA-MB-231 vs. MDA-MB-468	Yes	****
MDA-MB-231 vs. HCC1937	Yes	****
MDA-MB-231 vs. hTERT-HME1	Yes	****
MCF7 vs. MCF7-TS	Yes	**
MCF7 vs. MCF7-TR5	Yes	****
MCF7 vs. ZR-1-75	Yes	****
MCF7 vs. MDA-MB-468	Yes	****
MCF7 vs. HCC1937	Yes	****
MCF7 vs. hTERT-HME1	Yes	****
MCF7-TS vs. MCF7-TR5	Yes	**
MCF7-TS vs. ZR-1-75	No	ns
MCF7-TS vs. MDA-MB-468	Yes	****
MCF7-TS vs. HCC1937	Yes	****
MCF7-TS vs. hTERT-HME1	Yes	****
MCF7-TR5 vs. ZR-1-75	No	ns
MCF7-TR5 vs. MDA-MB-468	Yes	****
MCF7-TR5 vs. HCC1937	No	ns
MCF7-TR5 vs. hTERT-HME1	Yes	****
ZR-1-75 vs. MDA-MB-468	Yes	****
ZR-1-75 vs. HCC1937	Yes	***
ZR-1-75 vs. hTERT-HME1	Yes	****
MDA-MB-468 vs. HCC1937	Yes	****
MDA-MB-468 vs. hTERT-HME1	Yes	****
HCC1937 vs. hTERT-HME1	Yes	****

3.4.2 Cancer cells adhere less strongly than noncancerous cells

Given that the immune system plays an important role in tumor formation and microenvironment [34, 35], we wanted to use immunocompetent mice to study the formation of tumors . Murine C57BL/6-derived E0771 breast cancer cells were used. E0771 murine breast cancer cells were infected with a GFP+ lentivirus and selected for GFP expression. The genetic modification for GFP expression was done to facilitate the isolation of the E0771 cancerous cells from other non-cancerous host cells post-explantation. E0771-GFP+ cells were then implanted in the mammary fat pad of female C57BL/6 mice and allowed to form tumors. After 7 days, tumors were then explanted, digested, and sorted into GFP+ E0771 and GFP- host cells. Two cell doses were tested for tumor formation (10k and 20k) and their adhesion properties post-explantation were analyzed (Figure 3). The E0771-GFP+ mouse cancer cells adhered significantly less strongly than their GFP- counterparts ($p<0.01$), which is consistent with the results acquired when measuring the adhesive properties of human breast cancerous and non-cancerous cell lines (Figure 2B). The cell dose of 20k resulted in a significant difference ($p<0.05$) between the GFP+ and GFP- cells and was thus selected for all further studies.

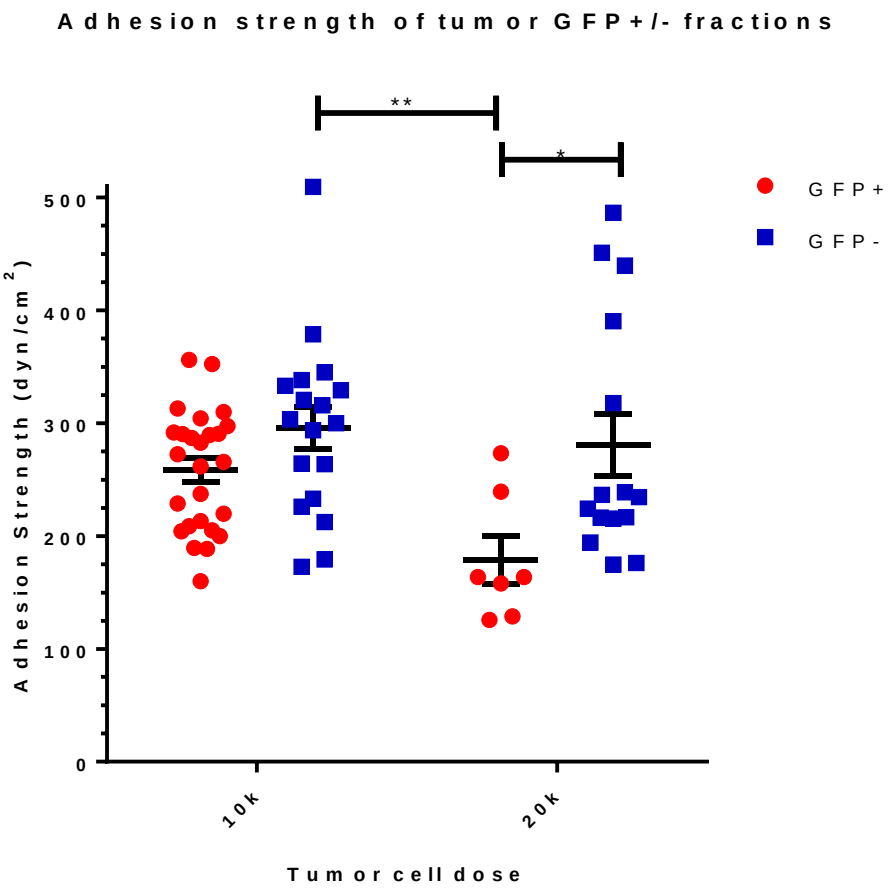

Figure 3 Differences in adhesive forces between mouse breast cancer cells and non-cancerous mouse cells are significant. Adhesive properties of GFP+ E0771 and GFP- host cells for two doses of E0771 post tumor formation. Statistical analysis was performed using a two way ANOVA. Differences were detected for both tumor implantation cell dose ($p<0.05$) and cell type ($p<0.01$). For the 20k cell dose, a significant difference was detected between the GFP+ and GFP- cell fractions ($p<0.05$; lines denote mean and standard error of the mean).

3.4.3 Culture of cancer cells with non-transformed host cells alters their adhesive properties

Cancer cell lines are often cultured by themselves, which does not recapitulate the conditions in which these cells grow *in vivo.* We were therefore interested in what effects co-culture of cancerous cells with non-cancerous host cells would have on the adhesion

properties of the cancer cells. In order to assess any changes that occurred to the E0771 cells during tumor formation, the adhesion properties of E0771 cells before and after tumor implantation into C57BL/6 mice were assessed (Figure 4). There is a significant ($p<0.0001$) increase in adhesion strength after tumor formation in a mouse for 7 days. Once the GFP+ E0771 breast cancer cells were sorted from the C57BL/6 host cells, a significant decrease in adhesion is detected ($p<0.0001$) suggesting that continued co-culture with the host cells is required to retain the changes in adhesive properties acquired during tumor formation in host mice. Nevertheless, sorted tumor explanted E0771 cells continue to have increased adhesion as compared to E0771 in standard culture conditions even 24 hrs after sorting ($p<0.001$), suggesting that the increased adhesiveness of the cells persists for some time after withdrawal of the host cells. E0771 cells were also co-cultured for 24 hrs with C57BL/6 host cells of mice that had not been implanted with tumors. An increase in adhesion strength ($p<0.001$) was observed, although not as pronounced as the one detected when the cells were implanted into mice and allowed to form tumors. The data suggests that simple co-culture with host cells changes the adhesive properties of cancer cells.

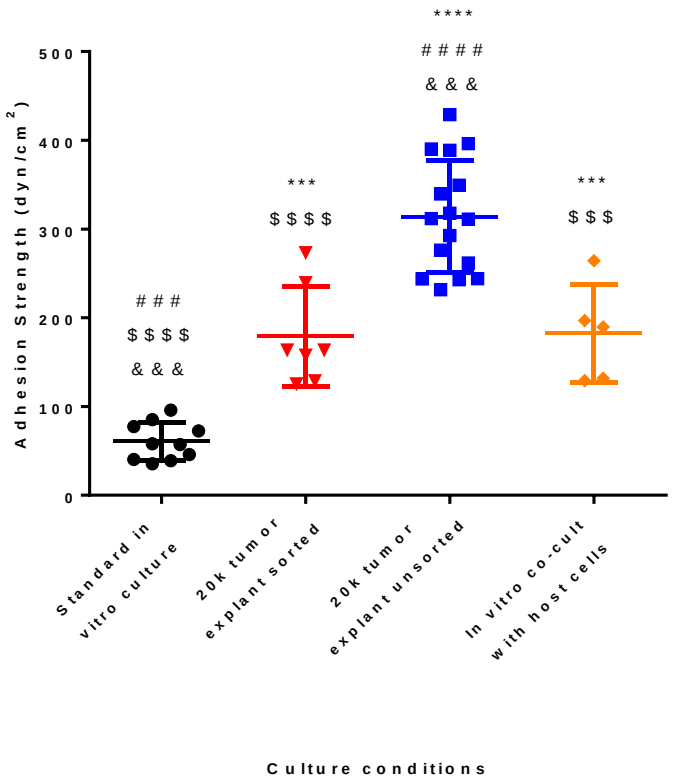

Figure 4 Implantation into mice or co-culture with mouse host cells causes an increase in the adhesion strength of E0771. Spinning disk measured adhesive properties of E0771 in standard in vitro culture (black data) and after implantation into C57BL/6 mice and tumor formation. Post tumor formation adhesive properties were assessed either in co-culture with host cells (blue data) or post-sorting with an Aria cell sorter (red data). The same measurements were performed on E0771 cells that had never been implanted into mice, but were co-cultured with C57BL/6 isolated cells (orange data). Data was analyzed using a one way ANOVA, which detected differences among all groups except between the 20k tumor explant sorted and the in vitro co-cult with host cells groups (* -significant difference when compared to standard in vitro culture, # - significant difference when compared to 20k explant sorted, $ - significant difference when compared to 20k explant unsorted, & - significant difference when compared to *in vitro* co-cult with host cells. *,#,$,& $p<0.05$; **,##,$$,&& $p<0.01$; ***,###,$$$,&&& $p<0.001$; ****,####,$$$$,&&&& $p<0.0001$).

Moreover, we were interested in whether some of the changes in adhesion seen in the mouse cancer cells upon co-culture with non-cancerous cells would also be seen in human cancer cells. In order to explore this, the effects of co-culture of human mesenchymal stem cells (hMSCs) on the adhesive properties of human breast cancer

MCF7 cells were studied by means of both the spinning disk and μSHEAR technologies. The adhesion strength of MCF7 cells was measured in the spinning disk assay for fibronectin (FN)-coated coverslips for three conditions: MCF7 cells cultured in fresh media, MCF7 cells cultured in 24hr hMSCs-conditioned media, and MCF7 cells co-cultured with hMSCs for 24hrs. Although no significant differences in adhesion were seen between MCF7 cells cultured in fresh media and conditioned media, a significant increase ($p<0.0001$) of ~36% in adhesion strength was observed when the MCF7 cells were cultured with hMSCs (Figure 5A). Similar increases in the adhesion strength of MCF7 cells was observed when using the μSHEAR technology in both Matrigel (MT)- and FN- coated microfluidic devices (Figure 5B, C). The τ_{50} for each condition was calculated. For FN coated surfaces, the τ_{50} of standard cultured MCF7 was 60.3 ± 4.2 dynes/cm^2, while 167.4 ± 13.3 dynes/cm^{2}for co-cultured MCF7 and 391.5 ± 24.2 dynes/cm^{2}for hMSCs. For MT coated surfaces, the τ_{50} of standard cultured MCF7 was 67.4 ± 26.0 dynes/cm^2 and 440.7 ± 24.1 dynes/cm^2 for co-cultured MCF7. For MT-coated surfaces, detachment of hMSCs was minimal even at the highest shear stresses tested and it was therefore impossible to calculate a τ_{50}. The force required to detach 50% of MCF7 cells when co-cultured with hMSCs increased threefold and sevenfold as compared to MCF7 cells in standard ulture for FN- and MT-coated surfaces respectively, matrices chosen because of their abundance in the tumor microenvironment. Taken together, this data indicate that stromal cells, either hMSCs in culture or murine tissue cells, have an effect on the adhesion properties of cancerous MCF7 cells only when in direct contact with them.

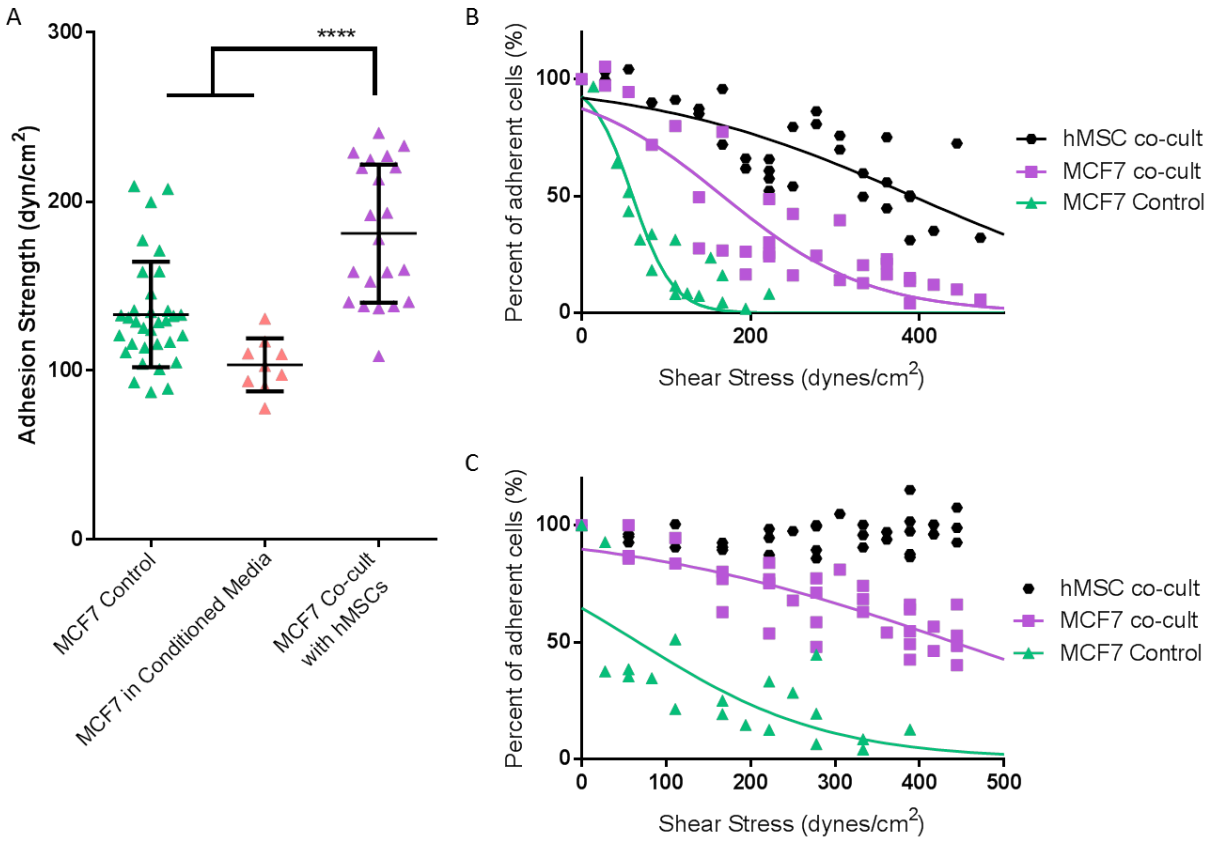

Figure 5 Direct co-culture with hMSCs significantly increases the adhesion strength of MCF7 breast cancer cells to both MT and FN coated surfaces. (A) Spinning disk measured adhesion strength to FN coated surfaces for MCF7 cells culture in fresh media, MCF7 cells culture in hMSC condition media, and MCF7 cells co-cultured with hMSCs. (B-C) µSHEAR measured adhesion properties to FN (B) and MT (C) coated microfluidic devices of MCF7 human breast cancer cells with cultured by themselves and in co-culture with hMSCs. Significant ($p<0.0001$) differences exist in the adhesion properties of all groups except for the hMSCs in MT conditions where the data did not converge to a sigmoidal curve and was therefore excluded from the analysis. A one way ANOVA was performed for the spinning disk data and a sigmoidal linear regression for the µSHEAR data.

3.4.4 µSHEAR-mediated separation of cancerous and non-cancerous cells

The adhesive properties of the explanted cells were also analyzed using the µSHEAR technology. E0771-GFP+ derived tumors were explanted and dissociated, and cells were introduced in microfluidic devices coated with either Matrigel (MT) (Figure 6)

or fibronectin (FN) (Figure 7). After overnight culture within the devices, the cells were stained with CellTrackerRed and exposed to varying amounts of shear stress. Each microfluidic device was mounted into a mechanical stage where seven fields of cells were identified and a single shear stress was applied for 10 min. At predetermined time points, the number of GFP+ and CerllTrackerRed+ cells were quantified at the seven predetermined fields of view. The detachment of the two populations of cells, GFP+/CellTrackerRed+ E0771 breast cancer murine cells (Figure 6A for MT and Figure 7A for FN) and GFP-/CellTrackerRed+ host cells (Figure 6B for MT and Figure 7B for FN), was assessed using fluorescence microscope and motorized stage, which enabled the quantification of cell detachment at specific locations within the devices over time. The average detachment for each cell population over time is plotted in Figure 6A-B for MT coated surfaces and Figure 7A-B for FN coated surfaces. The collected data allows for the calculation of the depletion of GFP+ cells within the devices (Figure 6C for MT and Figure 7C for FN) as well as the enrichment of GFP+ outside the devices (Figure 6D for MT and Figure 7D for FN). There is a tradeoff between yield (the number of GFP+ cells that detach over the original number of GFP+ cells) and purity (the frequency of GFP+ cells in the detached fraction of cells), where MT-coated plates enabled a higher yield of GFP+ cancer cells to be detached, but at a lower purity. In this condition, around 65% of the GFP+ cells were detached at the higher shear stress conditions, but less than a two-fold enrichment of these cells was achieved. On the other hand, FN-coated devices allow for lower yields of detached cancer cells at higher purities. In this condition, only around half of the GFP+ cells were detached from the devices, but more than three-fold increases in GFP+ E0771 purity was achieved.

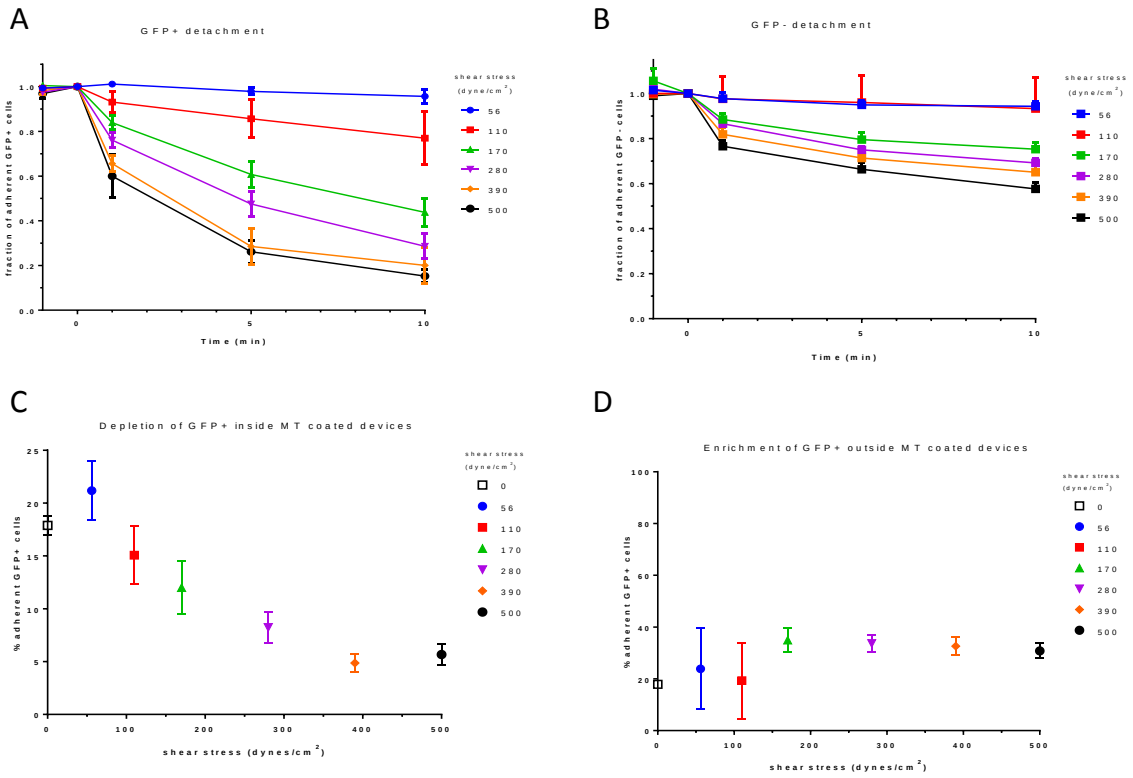

Figure 6 µSHEAR mediated enrichment and depletion of E0771-CFP+ cells from tumor explants in MT coated surfaces. Digested E0771 tumors and surrounding cells were introduced into uSHEAR devices coated with Matrigel and exposed to varying degrees of shear forces. Detachment of E0771 GFP+ cells (A) and GFP- host cells (B) was quantified by use of fluorescence microscopy. Depletion (C) and enrichment (D) of GFP+ cells within MT coated uSHEAR devices.

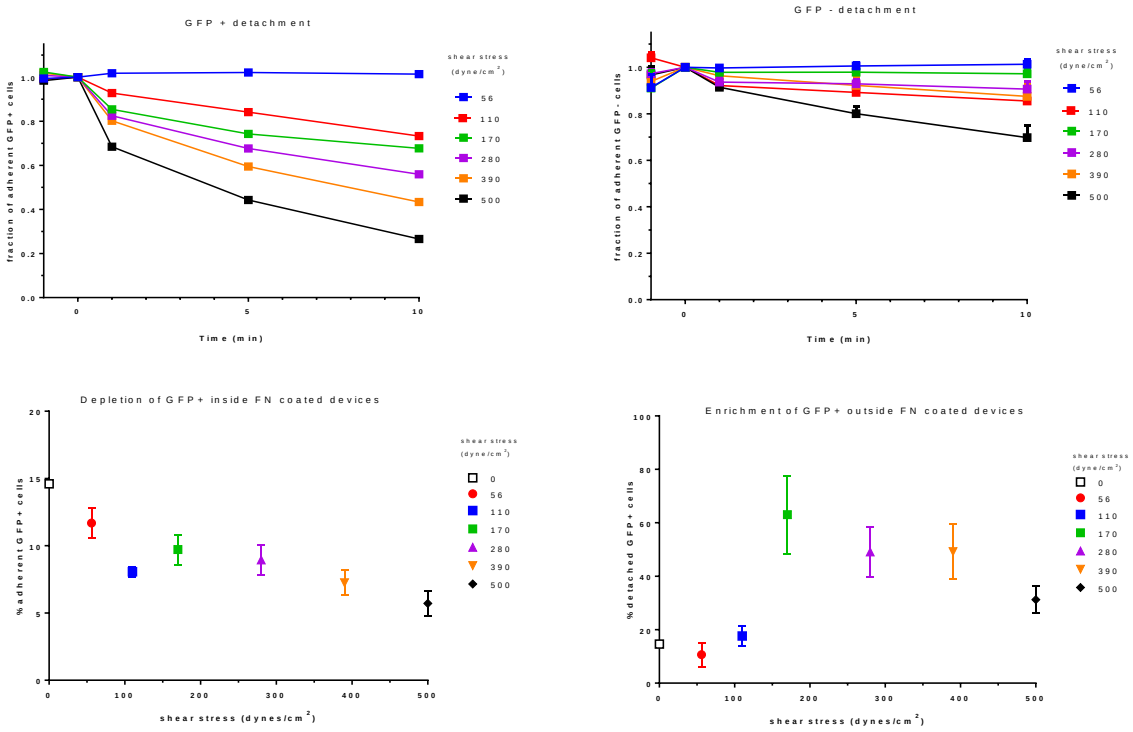

Figure 7 µSHEAR mediated enrichment and depletion of E0771-CFP+ cells from tumor explants in FN coated surfaces. Digested E0771 tumors and surrounding cells were introduced into uSHEAR devices coated with fibronectin and exposed to varying degrees of shear forces. Detachment of E0771 GFP+ cells (A) and GFP- host cells (B) was quantified by use of fluorescence microscopy. Depletion (C) and enrichment (D) of GFP+ cells within FN coated uSHEAR devices.

We next tested the ability of the µSHEAR technology to separate human breast cancer cells (MDA-MB-231) from non-cancerous immortalized hTERT-HME1. A mixture of these cells was inserted into the microfluidic devices and a predetermined shear stress was applied. We found that it was possible to enrich for the hTERT-HME1 inside the device and to consistently detach 90-99% of cancerous MDA-MB-231 (Figure 8).

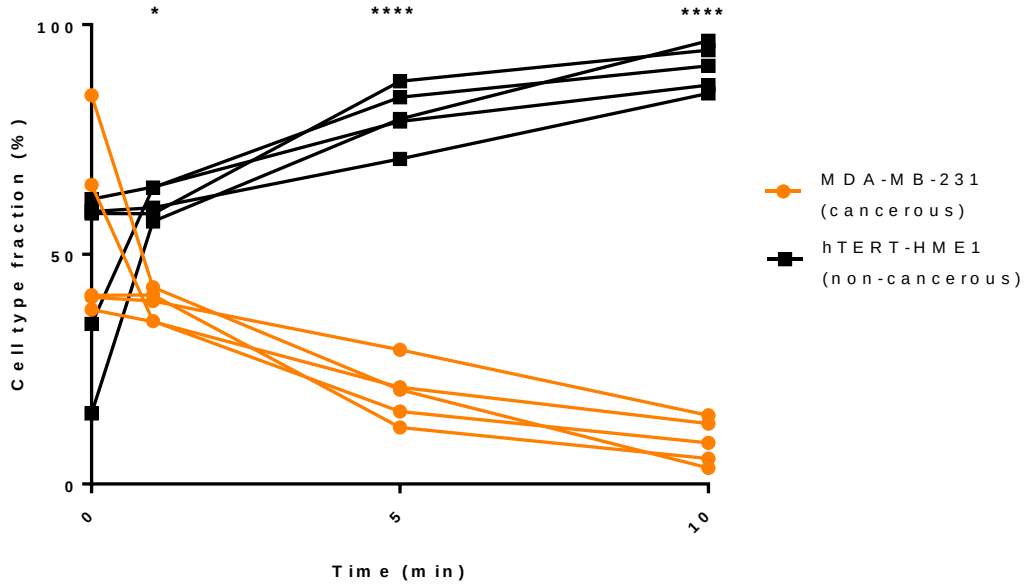

Figure 8 The µSHEAR technology can separate cancerous from non-cancerous cell lines based on adhesion. Fluorescently labeled hTERT-HME1 and MDA-MB-231 were introduced into microfluidic devices and exposed to 437dynes/cm^2 of shear stress. After 10min, MDA-MB-231 constituted less than 10% of the cell population remaining in the device, down from 40-80% in the initial mixture of cells, achieving more than a tenfold reduction in cancer cells within the mixture of cells. A two way ANOVA was performed (T=1 min $p<0.05$, T= 5,10 min $p<0.0001$).

3.5 Discussion

We have assessed the adhesion properties of a panel of breast cancer lines. There were large variations among cell lines in how strongly these cells adhered to FN, which is not surprising since many of these cells were derived from different patients, types of breast tumors, and by different research groups. Furthermore, other groups have shown that there are large variations in integrin expression and their capacity to form focal adhesions among breast cancer lines [36], factors that are directly related to the strength with which cells adhere to the ECM coated surfaces studied in this aim. The large

variation in adhesion properties highlights the variability in behavior between different cancerous tissues and provides further support for a more mechanistic approach to cancer therapies and research where organ of origin takes a backseat to the specific genetic changes that drove cancer progression. Other studies have also shed light on the importance of taking this new approach to cancer classification and treatment [9].

Immortalized cancer cell lines continue to be used as preclinical models to study cancer progression and test potential therapies in spite of numerous studies suggesting that they have limited clinical relevance and provide a poor representation of in-patient tumor development [20, 21, 37]. Moreover, a wide range of studies have shown that cancer cells undergo changes in proliferation and protein expression [38-42]. Here we show that implantation of mouse breast cancer cells into mice, as well as at least 24hrs *in vitro* co-culture with mouse primary cells, significantly increases their adhesion. Moreover, these changes persisted for at least 24 hrs after removal of the mice primary cells supporting published evidence that there is crosstalk between the two cell groups and suggesting that these effects are prolonged.

We corroborated these results by co-culturing human breast cancer cells with hMSCs and assessing their adhesion properties. After co-culture with healthy hMSCs, cancer cells adhered more strongly to FN and MT, displaying a change in adhesion properties reminiscent of those seen in the experiments with mouse cells. Co-culture with hMSCs was done because of the crucial role these cells play in cancer progression[43, 44], however, moving forward it would be interesting to assess whether similar behavios are observed with other non-cancerous cells such as breast fibroblasts or cancer associated fibroblasts These findings provide additional insights into the crosstalk of

cancer cells and other relevant cell types in the tumor microenvironment and highlight the need for more complex culture systems than the standard 2D culture widely used today.

Furthermore, our results show that for both mouse and human breast cancer, cancerous cell lines adhere less strongly to fibronectin than non-cancerous cell lines and healthy primary cells. Although this has been known for other tumors since the 1940s [45], our results suggest that isolating cancer cells based on their adhesion properties could be possible.

We pursued this goal and enriched both human and mouse breast cancer cells from co-cultures with either non-cancerous mammary cell lines or primary healthy cells. Our approach was highly reproducible and, depending on the conditions, achieved a 10-fold depletion of cancer cells from the co-cultures for a 3-fold enrichment. The ease of use, short time requirement, and reproducibility of our approach makes it a valuable tool for enriching cancer cell populations when developing new cancer specific cell lines for cancer progression studies and drug testing/validation. Furthermore, the microfluidic setup allows for new compartments to be added in the future, which would enable more complex co-cultures that recapitulate the *in vivo* tumor environment more accurately. A platform such as this would also allow scientist to test drugs in a well-controlled manner and to identify potential biomarkers that correlate with patient treatment responsiveness, a major requirement for the progress of personalized cancer treatments to continue to advance.

In this aim, we have developed a microfluidic technique for the enrichment and separation of cancer cells from cell mixtures containing non-cancerous cells. We have also shown that large variations exist in the adhesion properties of breast cancer cell line, but that these all adhere less strongly than healthy non-cancerous cells. Our results highlight the deficiencies of cell lines, particularly when studied in isolation, as non-cancerous cells are required to recapitulate the in vivo conditions and behaviors of cancerous cells. The procedures developed in this aim can also be applied in a modular fashion to current cancer organ on a chip systems to allow for the rapid and repeatable collection and isolation of cancer cells from these systems.

Nevertheless, the system can certainly be improved. Enrichment rates achieved are around 65%. Improving these should be a priority, perhaps by micro-patterning the surface that the cells adhere to. Other, non ECM, ligands could also be used for separation. Changes in cadherin expression have been observed in some forms of cancer [46, 47]; perhaps enrichment based on cadherin mediated adhesion would yield better results than integrin mediated adhesion. Moreover, the method was only tested in one cell type in these studies. Validating the technology in other cell lines, human ones in particular, should certainly be the next step. Finally, further characterization of the cell populations used would be useful. Although we are certain that the GFP- mouse cells isolated for the cancer isolation experiments are non-cancerous and can hypothesize that they are mostly fibroblasts and adipose cells, determining the exact composition of tis population would shed more light on the purification process.

CHAPTER 4. μSHEAR MEDIATED PURIFICATION OF TIC SUB-POPULATIONS FROM CANCER CELL LINES BASED ON ADHESIVE FORCE SIGNATURES

4.1 Abstract

Tumor initiating cells (TICs), a subpopulation of cancerous cells within tumors responsible for their maintenance, present a major hurdle to cancer treatment and recovery because of their resistance to conventional therapies. However, because of their low frequency and lack of good markers, TIC purification has proven challenging, even in *in vitro* settings. The interaction between cells and their extracellular environment is of critical importance to normal development and function. Integrin receptors mediate this interaction by mechanically coupling to an ECM ligand. Many adhesion proteins, including integrins, or pathways activated by these proteins have been shown to promote TIC stemness. We found that there were significant differences in adhesion strength between $ALDH^{HI}$ TIC-like cells and $ALDH^{LO}$ non-TIC cancer cell populations. Furthermore, we developed a method for the isolation of TIC populations based on their detachment forces which allowed for the enrichment of E0771 cells that formed larger (4X volume) tumors faster and higher frequency when implanted into the mammary fatpad of female C57BL/6 mice.

4.2 Introduction

Tumor initiating cells (TICs), a subpopulation of cancerous cells within tumors responsible for their maintenance, present a major hurdle to cancer treatment and recovery because of their resistance to conventional therapies [48-51]. This resistance frequently leads to drug resistant cancer relapse and metastasis development [84-86]. It is for this reason that targeting TICs in cancer therapy has attracted such excitement from the field [55, 87, 88, 242]. However, because of their low frequency and the lack of good markers, TIC purification has proven challenging, even in *in vitro* settings.

Many different methods of TIC purification have been developed to exploit unique attributes in these cells. Common methods of enrichment include surface marker-based purification and isolation based on TIC intrinsic functional markers, such as ALDH expression, reactive oxygen species (ROS) levels, flow cytometric side population (SP) analysis, and mitochondrial membrane potential differences. Many of these purification platforms rely on probes such as antibodies and separation technologies such as flow cytometry and magnetic beads. Although popular, these methods have several drawbacks including high price, non-specificity, inability to scale-up, and lack of robustness, which have led to the recent development of biophysical methods of TIC purification based on differences in adhesion, stiffness, and niche/scaffold affinity.

The interaction between cells and their extracellular environment is of critical importance to normal development and function [172, 173]. Integrin receptors mediate this interaction by mechanically coupling to an ECM ligand, associating with the actin cytoskeleton and clustering together [174]. These interactions may strengthen to give rise

to focal adhesions, which function as structural links between the cell's cytoskeleton and the surrounding ECM [175]. Abnormal integrin function can lead to a variety of diseases, including cancer [176]. Other proteins involved in cell adhesion and focal adhesion complex formation such as focal adhesion kinase (FAK) [177, 178] are also dysregulated in cancer cells and, along with integrins, contribute to disease progression and metastasis. Integrins in particular are often upregulated in many types of cancer and the levels of several integrin subtypes are prognostic of disease severity [176]. In addition to having important roles in cancer cell survival, migration, and invasion, integrins have been shown to cooperate with oncogenes to increase tumorigenesis [176].

Cell adhesion proteins are also important for normal development and embryogenesis [180]. Integrins are essential for stem cell homing to their niche during embryogenesis and development [181]. Several studies have shown the feasibility of separating stem cells based on their adhesion levels and integrin expression profiles [30, 182, 183].

Given the vital role that adhesion proteins play in normal stem cell function, it is expected that they also play important roles in TICs. In fact, many adhesion proteins or pathways activated by these proteins have been shown to promote TIC stemness [189, 243-245]. FAK ablation results in a depletion of the TIC population [184], integrin αvβ3 regulates expression of TIC marker CD44 [185] and is necessary to drive stemness and EGFR inhibitor resistance for epithelial cancers [186], and integrin α6β1 knockdown results in loss of stemness in TICs [187, 188]. In addition to simply being overexpressed in TICs, adhesion proteins potentiate TIC function and enable tumor propagation and drug resistance [189].

Abnormal expression and function of the cell-ECM adhesion apparatus in TICs have allowed groups to isolate them based on their adhesion dynamics. Bansal *et al* isolated prostate TICs from other cancer cells by allowing them to bind to collagen-coated surfaces for a short period of time. They found that TICs bound faster, and by washing away other cells, cancer cells with stem-like properties could be enriched [78]. Zhang *et al* applied the same idea in a more controlled manner to isolate breast cancer TICs. They used a microfluidic platform to slowly flow cells through a channel coated with selected ECM proteins. Since the TICs interact more rapidly with the ECM, they became trapped in the channel, while other cells flowed through [79].

It is important to note that adhesion strength separation can be done by exploiting differences in the rates at which cells bind to the substrate or differences in the amount of force required to detach adherent cells. When exploiting differences in binding rate, the cells are slowly rolled over a coated surface and some will bind faster that others allowing for separation. On the other hand, when exploiting differences in detachment force, cells are allowed to reach some level of adhesion strength with the ECM and then forces are applied to separate them. Although this might not seem like a major difference, the two assays measure different cell properties which may vary independently: the first how fast cells can form adhesion complexes and the second how strongly these adhesion complexes bind to the ECM [54].

Thus far, only differences in binding rate have been exploited to isolate TICs. Here, we aim to develop a method for separation of TIC populations based on their detachment forces, which might prove more useful, that currently available methods since it is generally more scalable and robust. In this study, we measured the adhesion

differences between cancer cells expressing TIC markers and non-TIC cancer cells. Furthermore, we used the same μSHEAR technology as aim 1 to purify populations of cells with higher tumor formation capabilities and validate this by implantation into mice.

4.3 Materials and methods

4.3.1 Cell culture

E0771 cells were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin, and 1% streptomycin at 37 ºC, 5% CO2 and sub-cultured at 70-80% confluency. The xenograft cell line CA2 established from primary human colonic biopsies was cultured in suspension in ultra-low-attachment 6-well plate at a density of 100,000 to 150,000 cells per well (Corning) as previously described [246]. Briefly, CA2 cells were suspended in a serum-free medium containing DMEM/F12 (Life Technologies), 6 mg/mL glucose (Sigma), 10 nmol/L progesterone (Sigma), glutamine (Life Technologies), 10 μg/mL insulin (Sigma), 13 μg/mL transferrin (Sigma), 15 nmol/L sodium selenite (Sigma), 4 mg/mL bovine serum albumin (BSA; Sigma), 50 μmol/L putrescine (Sigma), and 15 mmol/L HEPES (Life Technologies). Cells were supplemented with 10 ng/mL fibroblast growth factor and 20 ng/mL epidermal growth factor (Sigma) every other day. Cells were sub-cultured every 4-6 days. For adhesion studies, single CA2 cells were plated in tissue culture treated 6 wells coated either with fibronectin (10 μg/mL in PBS, 30 min) and blocked with bovine serum albumin (1% BSA in PBS, 30 min) or coated with Corning™ Matrigel™ hESC-Qualified Matrix according to the manufacturers guidelines (60 min) and cultured for 4 days.

4.3.2 Spinning disk assay

Circular cover slips (25 mm diameter) were washed with ethanol, coated with fibronectin (10 μg/mL) for 30 min, and blocked with a 1% solution of bovine serum albumin (BSA) for 30 min. Cells were seeded onto fibronectin-coated circular coverslips and cultured overnight at concentrations of 100,000-200,000 cells/mL in order to achieve 30-40% confluency. After 24 hrs, the coverslips were mounted on the spinning disk device and spun for 5 min in phosphate buffered saline solution buffer (PBS), thus applying a range of forces to the cells proportional to the cell's radial position in the cover slip. The cells were fixed with 4% paraformaldehyde for 15 min, permeabilized in a 0.05% Triton-X solution for 40 min, stained with DAPI for 30 min, washed three times with PBS, and mounted into slides for imaging. The number of cells at defined radial positions was quantified by use of a fluorescence microscope with a mechanical stage. A Matlab program was used to fit the data into sigmoidal and calculate the T_{50} (force required to detach 50% of the cells). [31, 32]

4.3.3 ALDH staining and sorting of CA2 cells

CA2 cells were trypsinized and stained for ALDH activity using the ALDEFLUOR assay (StemCell Technologies) according to the manufacturer's guidelines. Propidium Iodine was used as a dead stain (ThermoFisher) at a 1:1000 dilution. Cells were sorted using a BD Aria Cell Sorter.

4.3.4 Microfluidic device fabrication

PDMS (Sylgard 184, Dow Corning) microfluidic devices were fabricated as reported earlier using a negative photoresist (SU-8 2050, 50-μm thickness, MicroChem) and UV photolithography [30]. Patterned negative molds were then exposed to vapor-phase

tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (United Chemical Technologies) in a vacuum desiccator to prevent adhesion of PDMS. A 5-mm-thick layer of degassed PDMS mixture (10:1) was cast onto the mold and cured at 110°C for 20 hrs. Cast PDMS devices were peeled off and then punctured for inlet-outlet holes and bonded to glass coverslips by exposure to oxygen plasma for 15 s and allowed to bond at 110°C for 30 min.

4.3.5 GFP lentiviral transduction of E0771 cells

Stably transduced eGFP-E0771 cells were generated by infecting B16 mouse melanoma cells with LV-CMV-GFP lentivirus and sorting for GFP+ cells on an Aria sorter. Briefly, cells were seeded at 60% confluency in 6-well plates and allowed to attach overnight. The cultures were then infected with lentivirus at several multiplicity of infections (MOIs) ranging from 10 to 50. Four days post-infection, the cells were trypsinized and the GFP expression levels assessed in a flow cytometer. The MOI that gave the best efficiency of infection, MOI=50, was selected for further use. Two rounds of sorting were performed in order to achieve a >99% GFP+ population of cells by use of the BD Aria sorting machine.

4.3.6 Tumor generation and size measurement

eGFP-E0771 cells were injected (125-10,000 cells/injection for tumor titration assays and 250 cells/ injection in tumor growth assays in 20 μL of 1:1 Matrigel to saline solution) through he inguinal mammary gland into the abdominal mammary gland of female C57BL/6 mice to establish tumors. Tumors diameter sizes were measured in three directions at days 9,11,13,15, and 17 using digital calipers.

4.3.7 μSHEAR experiments

Devices were first sterilized with ethanol, washed with PBS, coated with fibronectin (10 μg/mL in PBS, 45 min) and blocked with bovine serum albumin (1% BSA in PBS, 45 min), and washed with complete media. Cells were then introduced at a concentration of 4 x10^6 cells/mL and cultured at 37 °C, 5% CO2 overnight. Predetermined amounts of force were applied to the cells for a 10 min period by flowing PBS at well-defined flow rates controlled by a Harvard syringe pump. Detached cells were collected and attached cells were trypsinized and collected as well. Cells were centrifuged, re-suspended in saline solution, counted, and 250 cells/dose were injected into C57BL/6 mice.

4.3.8 Statistics

Data points were plotted using Prism (GraphPad) with a horizontal line representing the mean and vertical error bars indicating the standard deviation or standard error of the mean as indicated. Statistical significance ($p<0.05$) was determined using either one way ANOVA with Tukey post hoc test or two way ANOVA with Tukey post hoc test as noted with Prism (GraphPad).

4.4 Results

4.4.1 Higher levels of TIC marker ALDH activity are correlated with changed adhesion strength to ECM coated surfaces

In order to assess whether TIC phenotype was associated with changed adhesion binding strength to ECM protein coated surfaces, a xenograft line established from

primary human colonic biopsies, CA2, with a large population of cells having high activity for TIC marker ALDH was used in conjunction with the spinning disk assay. The frequency of CA2 cells with TIC marker $ALDH^{HI}$ activity was determined to be 20-30% using the StemCell Technologies ALDEFLUOR assay and a BD Aria Cell Sorter (Figure 9A,B). These cells were cultured and expanded in suspension culture. It was therefore necessary to verify that transition into the adhesion culture required to perform the spinning disk assay did not decrease the frequency of $ALDH^{HI}$ TIC cells. CA2 cells were cultured in FN and MT coated surfaces for 4 or 7 days and the activity levels of ALDH assessed. Although 4 days in adhesive culture did not decrease the $ALDH^{HI}$ TIC fraction in either matrix culture condition, culture for additional three days did result in large decreases in TIC fraction for both matrices (Figure 9C,D).

Once it was confirmed that adhesive culture for short periods of time (4 days) did not affect the frequency of $ALDH^{HI}$ TICs in CA2 culture, the cells were transferred into culture on MT and FN coated surfaces for 4 days, stained for ALDH activity, and sorted into $ALDH^{LO}$ and $ALDH^{HI}$ subpopulations. The spinning disk analysis was performed on these subpopulations of cells and the τ_{50} calculated for each of them (**Error! Reference source not found.**E,F). Interestingly, although $ALDH^{HI}$ cells had higher adhesion binding strengths in MT coated surfaces, in FN coated surfaces the opposite was observed, suggesting that TICs may bind differentially to ECM proteins.

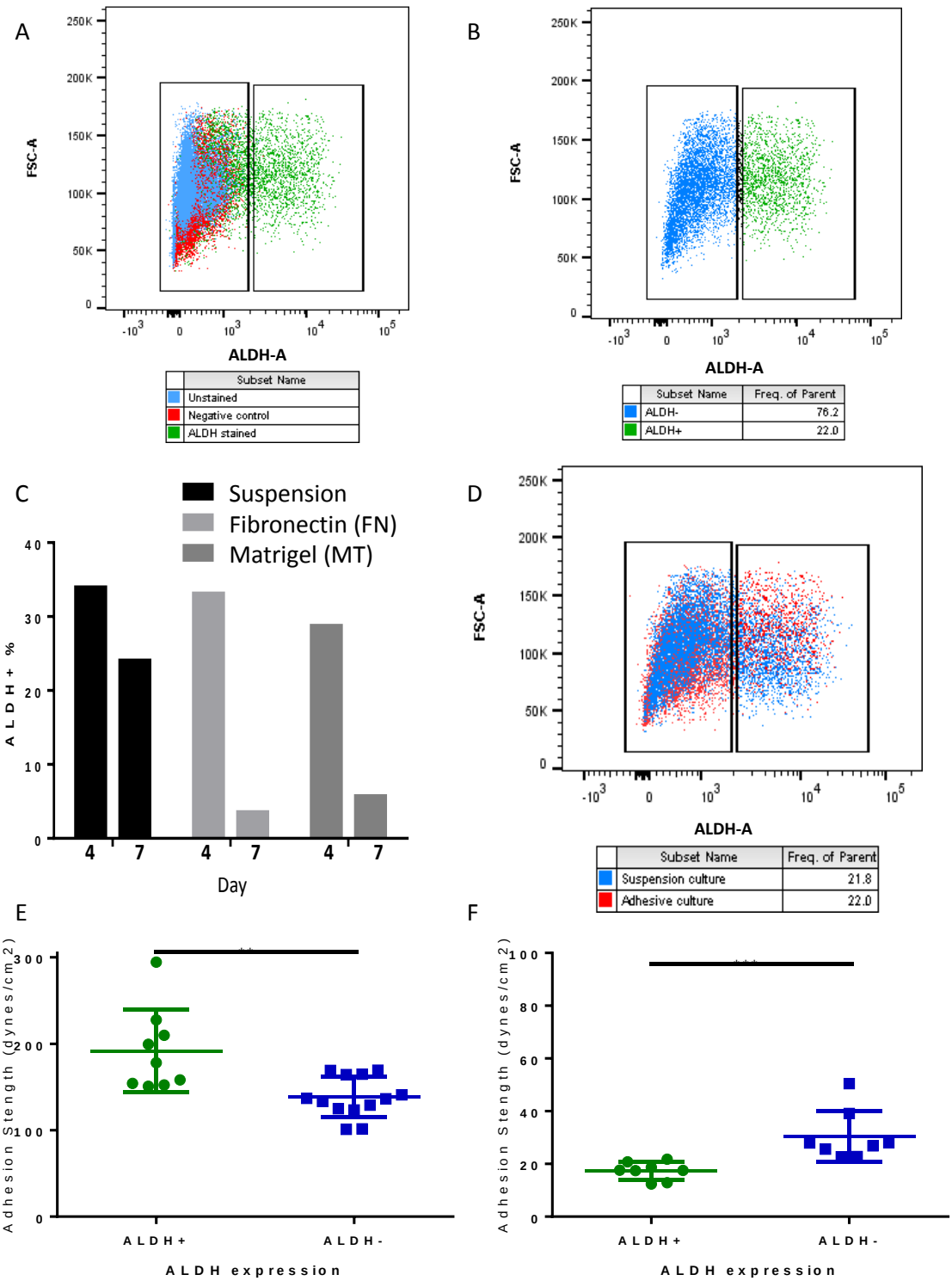

Figure 9. Populations of ALDH^{HI} CA2 TICs have unique adhesion strength signatures. CA2 cells were stained for ALDH activity by means of the ALDEFLUOR assay (A,B). ALDH activity levels were assessed in adhesion culture conditions and no

decrease in activity was seen after 4 days. Quantified data (C) as well as a representative staining in the Aria sorter (D) are shown. The spinning disk technology was used to quantify the adhesion binding strength of both $ALDH^{LO}$ and $ALDH^{HI}$ subpopulations in MT (E) and FN (F) coated surfaces. A non-parametric Mann-Whitney test was used (** $p<0.01$, *** $p<0.001$)

4.4.2 E0771 cells with a specific adhesive signature have higher tumour formation capabilities

To better assess the differences in adhesion strength of TICs vs non-TICs, the E0771 mouse breast cell line was chosen, which allowed for immune competent C57BL/6 mice to be used for implantation studies. A tumor titration assay was performed over a range of E0771-GFP+ cell dose (125-10,000 cells) injections into the mammary pad of C57BL/6 female mice and the tumor size were determined by calliper measurements of the diameter at predetermined time points (Figure 10A). Generally, as cell dose was decreased, the speed at which tumors formed decreased and the size of the resulting tumors decreased. However, for doses lower than 250 cells/injection, this trend was not as marked. For further experimentation, a dose at that caused a significant lag time for tumor formation and formed small to medium sized tumors that could be measured for a 5-7 day range would be ideal, which is why an injection dose of 250 cells/injection was chosen.

For these assays, FN was chosen as the ECM protein to coat the μSHEAR devices because of its crucial role in breast cancer invasiveness and migration [247]. Furthermore, mammary epithelial cell interactions with fibronectin have been shown to induce EMT [248], which in turn is correlated with TIC properties.

After identifying the optimal E0771 injection dose, μSHEAR-based separation of the cells was performed. Cells were introduced into the microfluidic devices and each device was exposed to one of three predetermined amounts of shear stress; 110 dynes/cm^2 (Figure 10B), 220 dynes/cm^2 (Figure 10C), or 330 dynes/cm^2 (Figure 10D). The cells that detached upon applying the shear stress (detached fraction in Figure 10) and the cells that remained attached the device (attached fraction in Figure 10) were collected. For some devices, the entire population of cells inside the device was trypsinized and collected to serve as controls. The different cell fractions were counted and the correct cell dose was injected into the mammary pad of C57BL/6 female mice. Tumor sizes were measured at 9, 11, 13, 15, and 17 days post injection by use of callipers. Interestingly, the cells detached at 220 dynes/cm^2 produced significantly larger tumors after 15 and 17 days relative to the control group, unsorted E0771 cells. Furthermore, the remaining fraction of adherent cells at 220 dynes/cm^2 yielded significantly smaller tumours than both the detached fraction and the control group. For cells exposed to 110 dynes/cm^2, there was a slight, but significant decrease in the tumor volumes for the attached fraction as compared to the control group after 15 days post injections. No differences were detected for any of the groups exposed to 330 dynes/cm^2. Figure 10E shows the final tumor volumes for both the attached and detached fractions of cells for all shear stress conditions. The green line represents the average volume for the control group, mice that were injected with unsorted E0771-GFP+ cells.

In addition to measuring tumor sizes, the lag time for sizeable tumor formation was measured (Figure 10F). In addition to the detached fraction of cells exposed to 220 dynes/cm^2 forming larger tumors, these cells also formed tumors faster than the unsorted

control group and at a higher frequency. The attached fraction exposed to 220 dynes/cm^2, on the other hand, took longer than both the control group and the detached fraction to form tumors and did not form as frequently.

Taken together, these results suggest that most of the TIC population within the E0771 cell line require shear stresses that are higher that 110 dynes/cm^2 and around 220 dynes/cm^2 to detach from FN coated surfaces. This unique adhesion strength signature allows them to be enriched with shear stresses of 220 dynes/cm^2. These cells yield tumors with more frequency that grow faster and reach larger sizes relative to the cells that remain attached to the FN coated surfaces when exposed to this level of shear force as well as compared to non-sorted E0771.

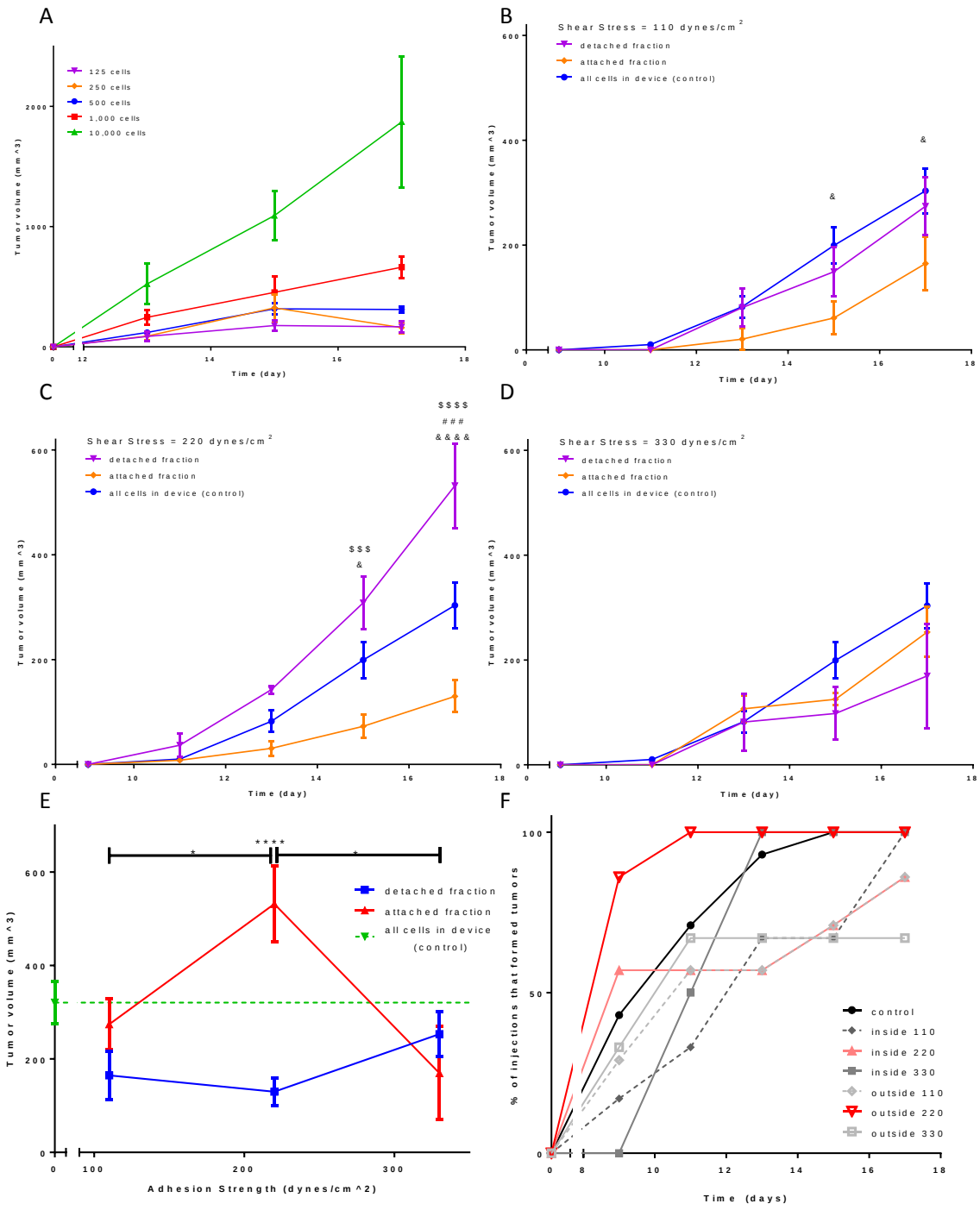

Figure 10 Adhesion mediated purification of E0771 TICs. A tumor titration was performed by injecting different doses of E0771 cells into the mammary fat pad of C57BL/6 female mice (A). E0771 cells were exposed to either 110dynes/cm^2 (), 220dynes/cm^2 (C), or 330dynes/cm^2 (D) of shear stress and sorted based on adhesion strength. Unsorted cells from devices not exposed to shear stress were used as controls. The cell fractions as well as controls were injected into the mammary fat pad of C57BL/6

female mice using a dose of 250 cells/injection and the growth of the tumors was measured. Detached cells exposed to 220dynes/cm^2 formed significantly larger tumors as compared to any of the other cell fractions (E) and they formed them faster and with higher frequency (F). Panels B-E were analyzed using two way ANOVAS (Graphs show means and standard error of the mean. Symbols denote: $ - significant difference between detached fraction and attached fraction, # - significant difference between detached fraction and control group, & - significant difference between control group and attached fraction. *,#,$,% $p<0.05$; **,##,$$,%% $p<0.01$; ***,###,$$$,%%% $p<0.001$; ****,####,$$$$,%%%% $p<0.0001$)

4.5 Discussion

The discovery of tumor initiating cells and the characterization of the role that they play in tumor progression, metastasis, and relapse have revolutionized our approach to cancer and its treatment [249-251]. In fact, because of our better understanding of the importance of TICs, novel therapeutic agents have been developed and are currently being evaluated in clinical and preclinical studies [252]. Most of these novel therapies could be used in conjunction with traditional therapies, such as chemotherapy, to target both the non-TIC and the TIC subpopulations of cells within tumors [252].

In order to efficiently develop novel TIC targeting therapies, it is necessary to develop robust, straightforward, reproducible, and rapid methods for TIC enrichment that will enable their enrichment for research and drug testing purposes as well as, later on, for diagnostic purposes when deciding the optimal therapeutic regimes for patients. Here, we examined the potential differences in adhesion between the non-TIC and TIC populations of cancer cells. Moreover, we developed a microfluidic method to enrich for cancer cells with the ability to form more and larger tumors faster, all of which are hallmarks of TIC phenotype.

In order to study the potential differences in adhesion signatures between non-TIC cancer cells and TICs, a xenograft line established from primary human colonic biopsies, CA2, with a large population of cells having high activity for ALDH was used. ALDHs are a family of enzymes that play a role in the metabolism of aldehydes [154]. Studies dating more than a decade have described a correlation between high levels of ALDH and stemness and succeeded in isolating hematopoietic stem cells based on ALDH activity [155-157]. More recently, high ALDH levels have been associated with other stem cell types and with TICs [71, 72, 158-160]. The large percentage of cells with high ALDH activity in the CA2 cell line facilitated their isolation by means of a cell sorter and the measurements in adhesion strength by use of a spinning disk, which requires relatively large numbers of cells. Furthermore, the suspension culture conditions the cells are expanded in, its xenographt nature, and the short time since derivation all should make this cell line more representative of *in vivo* cancer cells relative to standard cell lines derived decades ago.

We found that were significant differences in adhesion signature between $ALDH^{HI}$ TICs and $ALDH^{LO}$ non-TIC cancer cells in both MT and FN coated surfaces. Interestingly, $ALDH^{HI}$ cells bound more strongly in MT coated surfaces and less strongly in FN coated surfaces, suggesting that TICs may bind preferentially to some ECM proteins. The lowered adherence of the $ALDH^{HI}$ TICs to FN coated surfaces was also observed in mouse E0771 cells with tumor formation capabilities. The shared decrease in adhesion strength for the TIC-like subpopulations of both E0771 and CA2 cells, from mouse mammary and human colonic origins respectively, suggests that perhaps this lowered adhesive signature to FN coated surface could be a characteristic of all TICs.

Nevertheless, the data presented here is not sufficient to ascertain whether this is a commonality across tumor types and more studies with a variety of other cancer cell lines will be necessary to reach that conclusion.

Once we had characterized the differences in adhesion properties between $ALDH^{HI}$ and $ALDH^{LO}$ populations of CA2 cancer cells, we examined whether differences in adhesive signatures could be exploited to enrich for TICs by using a functional tumor formation assay. To this end, and because the immune system is known to play a key role in tumor formation and microenvironment, it was determined that a murine cancer cell line that allowed the use of an immunocompetent mouse model for tumor formation should be used. Several cell lines were tested and the E0771 cell line was selected because of its ability for form tumors in a timely manner and their high survival within microfluidic channels.

We found that it was possible to enrich for cancer cells that formed significantly larger tumors, faster, and with a higher frequency of tumor formation by enriching for cells that detached from FN coated surfaces when 220 dynes/cm^2 of shear stress was applied. When applying a lower shear stress of 110 dynes/cm^2, the enrichment for cancer cells with these tumor formation was not seen, perhaps because enough enrichment was not being achieved to detect a difference between the detached fraction and the control unsorted population of E0771 cancer cells. Surprisingly, a significant difference was not detected when a higher amount of shear stress of 330 dynes/cm^2 was applied. This may be because the larger shear stresses affect the health of the cell population leading to larger amounts of cell death and lower tumor formation capabilities. Although exciting, it will be necessary to examine whether we are able to repeat the process with other cancer

cell lines, human cancer cell lines, and even primary tumors. It will be necessary to run further controls in this assay to determine if the exposure to flow negatively impacts the health of the cancer cells. One way to do this is to mix the attached and detached cell populations for each shear stress condition and determine the tumor formation capabilities of the whole cell population post exposure to shear stress based sorting.

In this aim, we studied the differences in adhesion strength between TIC and non-TIC cancer cell populations and exploited these differences in order to develop a microfluidic technique for the enrichment and separation of TICS from cancer cell populations. Our results serve as a proof of principle for adhesion based TIC enrichment which could have powerful applications for studies into the biology of these cells, the search for effective therapeutic targets in them, and potentially their detection in diagnostic settings for the determination of the optimal therapeutic regime.

Further work on this aim should focus of the *in vitro* characterization of the enriched TIC-like cells and reproducing the enrichment process with other mouse cancer cells as well as human cancer cells. In terms of *in vitro* characterization, extreme limiting dilution assays, surface marker expression profiles, and single cell sequencing could further establish the enriched cells as TICs. Moreover, it wold be interesting to look at the expression levels of different integrins, and other cell adhesion molecules such as focal adhesion kinase (FAK), in the different subpopulations of cancer cells.

CHAPTER 5. FUTURE CONSIDERATIONS

The objective of this thesis was to examine the adhesive properties of cancer cells and any differences that might exist both between them and non-tumorigenic cells as well as among the different cancer cell subpopulations. We have demonstrated that these differences do exist, with cancer cells being less adhesive than their non-transformed counterparts and TICs having unique adhesive signatures. Furthermore, we developed a microfluidic technology that isolates cancer cells and enriches for TICs based on the differences in adhesive properties that were identified

In Aim 1, we studied the adhesive properties of a panel of breast cancer cells and found that there were large degrees of variability among them, in part due to the differences in sourcing of these cell lines. Nevertheless, we found that all cancer cell lines adhered significantly less strongly to ECM proteins than non-tumorigenic cells. While performing the adhesion studies, we found that the adhesive properties of cancer cells, of both human and mouse origin, significantly change when allowed to interact with other non-transformed host cells, confirming reports in the literature that studying cancer cells in isolation does not accurately recapitulate the properties or conditions that these cells exhibit and are exposed to *in vivo*. Moreover, we were able to enrich mouse cancer cells from a mixture of tumorigenic and non-transformed host mouse cells derived from tumors.

In Aim 2, we studied the adhesive properties of TIC populations and any differences that might exist between them and their non-TIC cancer cell counterparts. We found that colonic cancer cells expressing high levels of TIC marker ALDH had

significantly different adhesion properties when compared to cells with low ALDH activity. Interestingly, $ALDH^{HI}$ cells bound more strongly to MT, but less strongly to FN, suggesting that they preferentially bind to certain ECM properties perhaps because of different integrin expression profiles in TICs. TICs were also isolated from the E0771 mouse breast cancer cell lines based on their adhesive properties and these cells were capable of forming more and larger tumors faster when implanted into in the mammary fatpad of female C57BL/6 mice, key properties of TICs.

Futures studies should focus on verifying some of the results we have presented here in other mouse cell lines, human cell lines, and ultimately primary human tumors. In terms of the adhesion based enrichment of cancer cells, the studies presented here were done with tumors created by injecting cancer cells into mice mammary pads. It would be interesting to see if tumors isolated from a spontaneous tumor generating mouse model also display the same adhesion differences between cancerous and non-cancerous cells. Ultimately, performing these studies with primary human tumors would make the technology much more powerful and applicable in the cancer field. Adding micro patterns to the surface of the microfluidic channels could also be tested in order to increase the purity of the isolated cells.

The modular aspect of the microfluidic technology used here could allow for other compartments to be added such that cancer cells could be co-cultured with other cell types to better recapitulate *in vivo* conditions. This would create a full and closed cancer cell culture and purification system with powerful uses in future cancer research.

Future studies in the TIC enrichment portion of the studies should also focus on translating the technology from mouse cells to human cells. Studying whether human TICs also display an adhesive signature would certainly be useful given that one of the main limitations of the results shown here is that the work was done in mouse cells and only with one cell line. It would also be interesting to investigate whether the purified TICs have higher metastatic potential in addition to the increased tumor formation capabilities observed here.

Overall, the studies presented in this thesis begin to address the potential of adhesion based enrichment of cancer cells and TIC subpopulations. Although more work is needed, the ability to enrich TICs specifically without the use of markers and with a technology as cheap, easy to use, and fast as the one developed here could provide researcher with a powerful tool to study TIC biology.

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