ORIGINAL ARTICLE

Hydrodynamic shear-based purification of cancer cells with enhanced tumorigenic potential

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Abstract

Tumor-initiating cells (TICs), a subpopulation of cancerous cells with high tumorigenic potential and stem-cell-like properties, drive tumor progression and are resistant to conventional therapies. Identification and isolation of TICs are limited by their low frequency and lack of robust markers. Here, we characterize the heterogeneous adhesive properties of a panel of human and murine cancer cells and demonstrate differences in adhesion strength among cells, which exhibit TIC properties and those that do not. These differences in adhesion strength were exploited to rapidly (~10 min) and efficiently isolate cancerous cells with increased tumorigenic potential in a label-free manner by use of a microfluidic technology. Isolated murine and human cancer cells gave rise to larger tumors with increased growth rate and higher frequency in both immunocompetent and immunocompromised mice, respectively. This rapid and label-free TIC isolation technology has the potential to be a valuable tool for facilitating research into TIC biology and the development of more efficient diagnostics and cancer therapies.

Key words: cell adhesion; cancer; tumor-initiating cells; hydrodynamic; microfluidic

INSIGHT BOX

Identification and isolation of tumor-initiating cells (TICs), a subpopulation of cancerous cells with high tumorigenic potential and stem-cell-like properties, are limited by their low frequency and lack of robust markers. We demonstrate differences in adhesive signatures among murine and human cells, which exhibit TIC properties and those that do not. These differences in adhesion strength were exploited to efficiently isolate cancerous cells with increased tumorigenic potential in a label-free manner using a microfluidic technology. Isolated murine and human cancer cells gave rise to larger tumors with increased growth rate and higher frequency in immunocompetent and immunocompromised mice. This technology has the potential to facilitate research into TIC biology and the development of more effective diagnostics and therapies.

Received May 1, 2019; revised November 1, 2019; editorial decision November 3, 2019; accepted November 5, 2019

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INTRODUCTION

Tumors are heterogeneous tissues, which contain a subpopulation of cancer cells with high tumorigenic potential and stemness properties [1–8]. This subpopulation of cells has been referred to as “tumor-initiating cells” (TICs), “cancer stem cells” (CSCs), or “cancer stem-like cells” (CSLCs). Although there is debate whether TICs isolated from cancer patients are ‘bona fide’ stem cells [9], accumulating evidence indicates that these malignant cells are exceedingly tumorigenic and resistant to treatment with conventional chemotherapies and radiation [7, 10]. As such, TICs likely persist after chemo/radiotherapy and result in multi-drug resistance as well as disease relapse [7, 10]. TIC-enriched populations have been identified in established cell lines and patient samples using discrete surface markers (e.g. CD44^{hi}/CD24^{lo}, CD133⁺, ALDH⁺, and ESA⁺) and their ability to generate tumor spheres and xenograft tumors [1–7, 11–14]. However, TIC sub-populations from various sources differ greatly in their surface marker expression profile, and, to date, there is no universal marker profile to identify TICs [7, 10, 12]. Furthermore, there is no evidence that any combination of surface markers isolates TICs to a high degree of purity [7]. The inability to effectively purify TIC sub-populations is a profound impediment to characterizing the biology of these cells with precision as well as analyzing patient samples for effective diagnosis or prognosis [7, 10]. Therefore, there is a significant and unmet need for unbiased, efficient, label-free technologies for the identification and purification of cancer cell populations, including TIC sub-populations, from heterogeneous cultures and tumors.

Flow cytometric and magnetic-based selection methods have been developed to enrich for TICs, including surface antigen expression profiles [15–19], aldehyde dehydrogenase (ALDH) [20–22] or reactive oxygen species levels [23], and flow cytometric side population analysis [24]. A key drawback of these label-based methods is the inherent variability among different cancer types, even within tumor samples and cell lines of the same cancer type, causing markers that work for some tissues to be suboptimal for others. Furthermore, these methods rely on defined markers and reagents (e.g. antibodies), are time consuming, require specialized equipment, and are difficult to scale-up. In contrast, label-free biophysical methods based on differential cell adhesion or stiffness have been explored to isolate cancer cells [25]. For example, increased cell elasticity is correlated to increased migratory behavior in cancer cells [26–29] and activation of the EMT program [30]; both of these biological phenomena correlate with TIC phenotype [31]. Selection for highly elastic breast cancer cells showed enrichment for the CD44^{high}/CD24^{low} TIC phenotype and increased mammosphere formation [32]. However, cell elasticity may not be a robust selection marker as direct measurements of stiffness using atomic force microscopy showed that TICs from other sources are softer than non-TIC cells [33]. Selection of cancer cells from non-cancerous cells based on differential adhesion due to dysregulated adhesive mechanisms has also been reported [34–36]. These approaches exploit differences in binding rate or capture efficiency, which are time-, cell type-, and extracellular matrix-dependent. Here, we present a label-free strategy for the separation of cancer cell populations from cell cultures and tumor explants based on hydrodynamic detachment forces using the μ SHEAR (micro-Stem cell High Efficiency Adhesion-based Recovery) microfluidic technology [37]. Advantages of the μ SHEAR technology include its label-free and unbiased basis, facile operation and scale-up, high performance in terms of purification efficiency, yield and cell

viability, flexibility in performing negative or positive selection, and microfluidics format that allows for integration with other analytical platforms such as single-cell surface marker profiling and genomic and proteomic technologies.

RESULTS

Human breast cancer cell lines exhibit diverse adhesion strengths

The adhesion strength, defined as the force required to detach a cell from a surface, of various human breast cancer cell lines (details provided in [Supplementary Table 1](#)) as well as non-cancerous immortalized mammary cells was measured using the spinning disk assay [37–39]. In this assay, cells adhering to an extracellular matrix-coated glass coverslip are exposed to a linear range of hydrodynamic forces ([Fig. 1A](#)). Cell detachment can then be quantified at specific radial locations and fitted to a sigmoid to obtain τ_{50} , the shear stress at which 50% of the cells detach ([Fig. 1B](#)). Fibronectin (FN) was chosen as the ECM protein for coating because it plays a crucial role in breast cancer invasiveness and migration [40]. Non-cancerous immortalized mammary cells (hTERT-HME1) had significantly higher adhesion strength to FN (997 ± 68 dyne/cm²) compared to all cancerous cell lines examined ($P < 0.0001$) ([Fig. 1C](#)). Mean adhesive strength values varied significantly among breast cancer cell lines, ranging from 60 to 700 dyne/cm² ([Supplementary Table 2](#)).

The adhesion strength of a cell to its ECM is dependent on its morphology/size, integrin receptor expression levels, ECM ligand density, integrin-ECM bond numbers and distribution, and the association between ligated integrins and cytoskeletal elements [41–42]. We evaluated integrin expression levels for a subset of these human breast cancer and non-cancerous immortalized mammary cells to determine whether integrin expression levels correlated with adhesion strength ([Fig. S1](#)). This flow cytometry analysis demonstrated significant differences in expression of particular integrin subunits among these cell lines. However, the differences in integrin subunit expression do not account for the differences in adhesion strength among these cell lines.

The adhesion strength of MDA-MB-231, MDA-MB-453, and MCF7 breast cancer cells was also assessed using the μ SHEAR technology [37] ([Fig. 2A](#)). In this microfluidic device, hydrodynamic shear forces proportional to the fluid flow rate are applied to adherent cells [37]. Cells adhering to FN were exposed to predetermined shear forces for 10 min, with different devices being exposed to different amounts of shear force, while cell detachment was visualized with a microscope. In this fashion, a profile for adherent cells vs. surface stress was generated for each cell line ([Fig. 2B](#)). The detachment profiles clearly demonstrate lower adhesion strength values for the cancer cell lines compared to the non-cancerous hTERT-HME1 and a distribution in adhesion strength for each cell line with a fraction of cells exhibiting high adhesion strength.

The ability to separate cancerous cells from non-cancerous hTERT-HME1 cells based on differences in adhesion strength using the μ SHEAR technology was explored. The MDA-MB-231 cell line was chosen for this assay since it was among the cell lines, which exhibited the largest adhesion difference relative to the hTERT-HME1 cells. The cell lines were fluorescently labeled with different dyes, mixed, and cultured in μ SHEAR devices. In a similar fashion to the previously described μ SHEAR experiments, the cell mixture was exposed to a predetermined shear stress of 440 dynes/cm² and the detachment of cells was

Figure 1. Cell adhesion strength to FN for human breast cancer cell lines. (A) Schematic of the spinning disk assay. Cells adhere to a FN-coated circular coverslip, which are spun at a predetermined speed (ω). The rotation applies shear stress (τ) proportional to the radial position (r) within the cover slip and cell detachment can be quantified at predetermined radial positions. (B) Representative detachment profiles for MDA-MB-231, MDA-MB-453, and hTERT-HME1 cell lines. (C) Adhesion strength measurements for the hTERT-HME1 immortalized mammary cell line as well as a panel of breast cancer cell lines (One-way ANOVA **** $P < 0.0001$, detailed statistical comparisons available in [Supplementary Table 2](#), each symbol represents an independent sample).

visualized with a microscope. After 10 min, MDA-MB-231 constituted on average $<10\%$ of the cell population remaining in the device, down from 45 to 85% in the initial mixture of cells, achieving more than a 10-fold reduction in cancer cells within the mixture of cells (Fig. 2C). Notably, the final purity of the cell population was independent from the initial purity. These results demonstrate that the differences in adhesion strength between these cell lines can be exploited to enrich for one cell line.

Murine breast cancer cell line E0771 exhibits an adhesion strength signature distinct from non-cancerous mammary cells

Cell function is highly dependent on the adhesive interactions between cells and their extracellular environment [43–44], and dysregulation of cell adhesion can cause a range of diseases, including cancer [45]. The aforementioned spinning disk results suggest that cancerous cells have adhesion strength signatures, which differ significantly from non-cancerous cells. To further test this, the spinning disk assay was used to measure the

adhesion strength of E0771 murine breast cancer cells as well as cells isolated from the mammary fat pad of C57BL/6 mice (Fig. 3), from which the E0771 line was originally derived [46–47]. Consistent with our observations for human cells, non-cancerous mouse mammary fat pad cells have significantly higher adhesion strength to FN ($289 \pm 92 \text{ dyne/cm}^2$) compared to cancerous E0771 cells ($60.7 \pm 21 \text{ dyne/cm}^2$).

Murine cancer cell sub-populations with different tumorigenic potentials can be isolated by adhesive force

The adhesion strength analyses of human and murine cancer cell lines suggest the presence of sub-populations of cells with differential adhesive properties. As such, whether different adhesive subpopulations of cells within an established cell line have different tumorigenic potentials was explored. We chose to study the E0771 breast cancer cell line [46–47], as this murine line allows evaluation of tumor formation in immunocompetent C57BL/6 mice. E0771 cells were transduced with a lentivirus for eGFP to generate eGFP-expressing E0771 (E0771-GFP+) cells for tracking transplanted cells; eGFP expression did not influence

Figure 2. μ SHEAR microfluidic-based separation of cell mixtures. (A) Schematic of the μ SHEAR microfluidic device. Cells are cultured in FN-coated microfluidic channels. Defined shear-stress levels are applied to enrich for cell populations based on their adhesion properties. (B) Detachment profiles for MDA-MB-231, MDA-MB-453, and MCF7 human breast cancer lines and the hTERT-HME1 immortalized mammary cell line as assessed by μ SHEAR. For each microfluidic device, a pre-determined amount of shear stress was applied and cell detachment quantified by use of a microscope with motorized stage (results from four independent experiments shown). (C) Enrichment of hTERT-HME1 cells from mixed cultures with MDA-MB-231 breast cancer cells by μ SHEAR, achieving hTERT-HME1 purities of 90–95% (Two-way ANOVA * $P < 0.05$, **** $P < 0.0001$).

the adhesion strength of this cell line (Fig. S2). A titration assay was performed with a range of E0771-GFP+ cell doses (125–10,000 cells) injected into the mammary pad of C57BL/6 female mice, and tumor size was determined by caliper measurements of the diameter of the tumor mass at defined time points (Fig. S3). In general, tumor latency and tumor size increased with increasing cell dose. A dose of 250 cells was selected for subsequent experiments as this dose resulted in small-to-medium sized tumors detectable at 7 days post-implantation.

Based on adhesion data collected using the spinning disk technology (Fig. 3A), μ SHEAR-based separation of adhesive and detached fractions of E0771-GFP+ cells was performed for three different shear-stress levels: 110 dynes/cm², 220 dynes/cm², and 330 dynes/cm². FN was chosen as the coating ECM protein because it plays a crucial role in breast cancer invasiveness and migration [40]. Furthermore, mammary epithelial cell interactions with FN have been shown to induce EMT [48],

which in turn is correlated with TIC properties. E0771-GFP+ cells that detached upon application of hydrodynamic shear forces (detached fraction) and cells that remained attached in the device (attached fraction) were collected. In addition, cells in the device that were not exposed to hydrodynamic shear were collected to serve as controls. The collected cell fractions were counted and 250 cells from each fraction were implanted into the mammary pad of C57BL/6 female mice (Fig. 4A). Tumor sizes were measured at 9, 11, 13, 15, and 17 days post-implantation. For cells exposed to 110 dynes/cm², there was a small but significant decrease in tumor size for the attached cell fraction compared to the control group at 15 and 17 days post-implantation (Fig. 4B). Remarkably, the detached cell fraction isolated at 220 dynes/cm² produced significantly larger tumors at 15 and 17 days post-implantation compared to the control unsorted E0771 cells (Fig. 4C). Furthermore, the attached fraction yielded significantly smaller tumors than both the detached fraction and the control

Figure 3. Adhesion strength for E0771 murine cancer and fat pad cells. Adhesion strength of E0771-GFP+ breast cancer cells and fat pad cells isolated from C57BL/6 female mice to FN (Student's t-test **** $P < 0.0001$).

group. No differences in tumor size were detected among any of the groups exposed to 330 dynes/cm² (Fig. 4D). Figure 2E shows tumor sizes at the completion of the experiment on day 17 for both attached and detached cell fractions for all shear-stress isolation conditions, along with average volume for control unsorted E0771-GFP+ cells. The cell fraction detached at 220 dyne/cm² generated larger tumors compared to cell fractions detached at 110 and 330 dyne/cm² as well as the attached fraction exposed to 220 dyne/cm². Furthermore, the tumors generated by the detached cell fraction at 220 dyne/cm² were 50% larger than those formed by unsorted E0771-GFP+ cells. In addition to increased tumor size, the detached cell fraction at 220 dyne/cm² generated tumors faster compared to other groups as indicated by the percentage of mice with detectable tumors as a function of time (Fig. 4F). Histological staining of explanted tumors confirms the growth and size of the implanted E0771-GFP+ cells (Fig. S4). Taken together, these results demonstrate that sub-populations of cells in the E0771 murine cell line with different tumorigenic potential that can be isolated by differences in adhesive force to FN.

Human colon cancer cells separated by hydrodynamic forces have higher tumor formation properties

We next examined the adhesion strength characteristics for CA2 human sporadic TICs established from primary human colonic cancer resections [49–50]. These cells contain a large population of cells (20–30%) with high levels of ALDH activity (Fig. 5A), which has been shown to be a reliable marker for TIC activity in various cell lines, including the CA2 cell line [49–50]. CA2 cells were cultured and expanded in suspension culture as previously described [50]. CA2 cells were plated onto FN- or Matrigel (MT)-coated surfaces for 4 days, stained for ALDH activity, and sorted into ALDH^{LO} and ALDH^{HI} fractions. Transition to adhesion culture on MT- or FN-coated surfaces for 4 days did not alter the frequency of ALDH^{HI} TIC cells in the population, although a reduction in the fraction of ALDH^{HI} cells was observed at 7 days of adhesion culture (Fig. S5). The adhesion strength for ALDH^{LO} and ALDH^{HI} fractions for cells in 4-day adhesion culture was then measured using the spinning disk assay. ALDH^{HI} cells showed

higher adhesion strength to MT-coated surfaces compared to ALDH^{LO} fraction. For FN-coated surfaces, the overall levels of adhesion strength were significantly lower than those observed for MT (Fig. 5B).

We selected MT-coated surfaces for subsequent experiments because this ECM resulted in significant differences in adhesion strength between ALDH^{HI} and ALDH^{LO}. Furthermore, a basement membrane-rich matrix such as Matrigel is a physiologically relevant matrix for this epithelial cell line. μ SHEAR-based separation of the cells at 47.4 dynes/cm² was performed, and detached and attached fractions were collected. This shear-stress value was selected because it led to the detachment of ~50% of the CA2 cells cultured within the μ SHEAR devices. Cells cultured in the microfluidic device but not exposed to hydrodynamic forces were used as unsorted controls. In addition, cells maintained in suspension culture and not plated on MT-coated supports were used as an additional control. Detached and attached cell fractions, unsorted and suspension cells (100 cells) were injected into the subcutaneous space in the flank of NSG immunocompromised mice. Tumor sizes were measured every other day starting 23 days post-implantation by use of calipers, based on previous tumor growth data [49]. The attached fraction of CA2 cells formed ~3-fold larger tumors compared to the detached fraction and unsorted cells (Fig. 5C). Furthermore, the attached cell fraction generated tumors in 100% (5/5) of mice, whereas the detached cell fraction only formed tumors in 60% (3/5) of mice. Immunostaining of explanted tissue demonstrate positive staining for human EpCAM, ALDH1, and E-cadherin (Fig. 5D–F), confirming that the implanted CA2 cells gave rise to these tumors. Collectively, these results demonstrate that a sub-population of human colon cancer cells with higher tumor formation properties can be separated by hydrodynamic forces.

DISCUSSION

The discovery of TICs and characterization of the roles that they play in tumor progression, metastasis, and relapse have revolutionized the field's approach to cancer and its treatment [51–53]. A better understanding of the importance of TICs has led to the development of novel therapeutic agents, which are currently being evaluated in clinical and preclinical studies [54]. Most of these novel therapies could be used in conjunction with traditional therapies, such as chemotherapy, to target both non-TIC and the TIC subpopulations of cells within tumors [54]. Nevertheless, the efficient and reproducible purification of TIC population remains a hurdle for further advancements in the field. In this study, we examined differences in adhesive forces between non-cancerous and cancerous cells and found significant differences among these populations. We also applied a microfluidic method to enrich for mouse and human cancer cells with the ability to form larger tumors with increased frequency and faster growth rates, all of which are hallmarks of TIC phenotype.

Cell adhesion impacts tumorigenesis via various mechanisms, and it is likely that the dominant mechanism(s) depends on many factors including tumor type/origin, microenvironment, and patient. As such, we do not expect that a simple parameter (adhesion strength) would serve as a 'universal' marker of tumorigenesis, so the goal of this study was not to correlate adhesion strength to tumorigenesis. Instead, the objective of this study was to test whether tumorigenic cells could be enriched based on differences in cell-adhesion strength regardless of the underlying mechanisms driving tumorigenesis. We show that breast cancer cells (several human lines, murine

Figure 4. μ SHEAR-mediated enrichment of TIC-like murine breast cancer cells. (A) Schematic of experimental setup. E0771-GFP+ cells were cultured in FN-coated μ SHEAR devices and separated based on pre-determined shear-stress values (110, 220, and 330 dyne/cm²). Adherent and detached cell fractions as well as unsorted cells were injected into mice and tumor formation monitored. (B–D) Tumor growth curves for detached and attached cell fractions and unsorted cells after applying (B) 110 dyne/cm², (C) 220 dyne/cm², or (D) 330 dyne/cm². (E) Tumor volumes for recovered cell fractions and unsorted controls. Results were analyzed using two-way ANOVA (mean $\pm$ standard error of the mean. \$: detached fraction vs. attached fraction, #: detached fraction vs. unsorted control, &: attached fraction vs. unsorted control. One symbol $P < 0.05$; two symbols $P < 0.01$; three symbols $P < 0.001$; four symbols $P < 0.0001$). (F) Percentage of mice with detectable tumors over time post implantation.

E0771 line) exhibit lower adhesion strength to FN compared to normal human mammary epithelial and murine fat pad cells. We also demonstrate differences in adhesion strength to Matrigel among ALDH^{HI} and ALDH^{LO} sub-populations in CA2 human colon cancer TICs. For both E0711 and CA2 cells, we enriched for TIC fractions by exploiting differences in adhesion strength. The less adherent E0711 cell fraction and more adherent CA2 fraction were more tumorigenic than the unsorted parental population. We note important distinctions between these two different

cancer lines. First, E0771 are murine breast cancer cells, whereas CA2 are human colon cancer cells. The tumor source, type, and origin (including species) could impact the adhesivity of the tumorigenic cells. Second, the adhesion strength for these lines was characterized for different matrices—FN was used for E0771 cells, while Matrigel was used for CA2 cells. We note that the adhesion strength to FN of the tumorigenic ALDH^{HI} CA2 cells is lower than ALDH^{LO} CA2 cells. Our study demonstrates the ability to enrich for tumorigenic cell fractions based on differences in

Figure 5. μ SHEAR-mediated enrichment of human cancer CA2 cells with increased tumorigenic potential. (A) ALDH activity levels in CA2 cells measured using the ALDEFUOR assay. (B) CA2 cells were sorted based on their ALDH activity levels and the adhesion strength properties of ALDH^{HI} and ALDH^{LO} cell populations to FN- and MT-coated surfaces were assessed using the spinning disk assay. For MT, ALDH^{HI} exhibited a significantly higher adhesion strength than ALDH^{LO} cells. (C) Tumor volume for μ SHEAR-isolated adherent and detached fractions and control CA cells implanted in NSG mice. Results were analyzed using two-way ANOVA (mean $\pm$ standard error of the mean; \$: attached fraction vs. unsorted control, &: attached fraction vs. suspension control, #: detached fraction vs. attached fraction. One symbol $P < 0.05$; two symbols $P < 0.01$; three symbols $P < 0.001$; four symbols $P < 0.0001$). Immunostaining for (D) EpCAM, (E) ALDH1, and (F) E-cadherin in tissue sections.

adhesion strength using a facile and label-free methodology. This work provides the foundation for expanded and systematic analyses of patient-derived tumor cells based on tumor type, origin, genetics, and patient history. For instance, direct of comparison of the adhesion strength and tumorigenicity for patient-derived cells from tumors of similar origin, for example breast or colon tissue, would provide a platform to evaluate mechanisms connecting cell-matrix adhesion to tumorigenesis. Furthermore, evaluation of tumorigenicity for cell fractions separated by this adhesion strength-based technology for patient-derived tumors of similar origin (e.g. breast, colon, and lung) could be implemented to assess whether a cell fraction with a distinctive adhesion strength signature provides a tool for diagnostic purposes.

ALDHs are a family of enzymes, which play a role in the metabolism of aldehydes [55]. Prior work has established a correlation between high levels of ALDH and stemness and succeeded in isolating hematopoietic stem cells based on ALDH activity [56–58]. More recently, high ALDH levels have been associated with other stem cell types as well as TICs [20–21, 59–61]. Here, we used a human colorectal cancer cell line, CA2, to study the relationship between ALDH expression and adhesion strength, showing significantly higher adhesion strength values in cells with high ALDH activity. Moreover, using the μ SHEAR microfluidic technology, we enriched for CA2 cells that, when implanted into mice, formed larger tumors with higher frequency.

Microfluidic cell isolation methods have emerged as powerful platforms for cell enrichment, particularly for cancer cells (as reviewed in [25, 62–63]). These systems employ differential surface marker expression [64–65] or differences in cell mechanical properties (e.g. elasticity) [66–68]. Selection of cancer cells from non-cancerous cells based on differential adhesion has also been reported [34–36]. The label-free technology μ SHEAR technology used in our study exploits differences in steady state detachment force, which are scalable, robust, and easy to implement. Our strategy for enriching for more tumorigenic cell populations could facilitate research into TIC biology and the development of more efficient cancer therapies. In the long term, the μ SHEAR technology could enable TIC detection in diagnostic settings, allowing physicians to better determine optimal therapeutic regimens.

METHODS

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. All cell lines were sub-cultured at 70–80% confluency. E0771 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C, 5% CO₂. The cell line CA2 established from primary human colonic biopsies was cultured in suspension in ultra-low-attachment 6-well plate (Corning) at a density of 100 000 to 150 000 cells per well as previously described [50]. 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 μ g/mL insulin (Sigma), 13 μ g/mL transferrin (Sigma), 15 nmol/L sodium selenite (Sigma), 4 mg/mL bovine serum albumin (BSA; Sigma), 50 μ mol/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 six wells coated either with FN (10 μ g/mL in phosphate buffered saline solution – PBS, 30 min) and blocked with bovine serum albumin (1% BSA in PBS, 30 min) or coated with Matrigel™ hESC-Qualified Matrix according to the manufacturers guidelines (60 min) and cultured for 4 days.

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 (ThermoFisher) was used as a dead cell stain at a 1:1000 dilution. Cells were sorted using a BD Aria Cell Sorter. CA2 TICs were isolated and propagated under IRBs held at the University of Michigan, University of Florida and the Cleveland Clinic. STR analyses confirm cell uniqueness (Duke University DNA Analysis Facility).

Integrin expression profiling

MDA-MB231, MDA-MB453, MCF7, and hTERT-HME1 cells were thawed, plated, and expanded to reach ~80% confluency. Cells were incubated in primary antibody (see Table S3 for list and dilutions) for 1 h on ice in staining buffer (2% FBS). Cells were washed twice using the staining buffer and incubated in secondary antibody for 50 min on ice in the staining buffer. Integrin expression measured by flow cytometry (BD Accuri C6).

GFP lentiviral transduction of E0771 cells

Stably transduced E0771-eGFP+ cells were generated by transducing E0771 mouse melanoma cells with LV-CMV-GFP lentivirus and sorting for GFP+ cells on a BD Aria sorter. Briefly, cells were seeded at 60% confluency in six-wells and allowed to attach overnight. The cultures were then incubated in 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 sorter.

Isolation of fat pad cells

The tissue in the abdominal mammary glands of female C57BL/6 mice was excised and digested with collagenase D (0.375 U/ml) and hyaluronidase (125 U/ml) for 2–3 h 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, 0.1 mg/ml, room temp). The mixture of cells was filtered through a 40 μ m filter twice, resuspended in the relevant volume of media, and used for the adhesion experiments.

Spinning disk assay

Circular glass cover slips (25 mm diameter) were clean with ethanol, coated with FN (10 μ g/mL) for 30 min, and blocked with 1% BSA for 30 min. Cells were seeded onto 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 h, the coverslips were spun for 5 min in 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 0.05% Triton-X100 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 for GFP for 2 h with a 1:100 dilution of the primary antibody (Abcam #ab290) and for 1 h with a 1:200 dilution of an anti-rabbit 488 secondary antibody with three washes (1% BSA) after incubation with each antibody. 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 τ_{50} (force required to detach 50% of the cells) [42, 69].

Microfluidic device fabrication

Polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning) microfluidic devices were fabricated as reported earlier using a negative photoresist (SU-8 2050, 50- μ m thickness, MicroChem) and UV photolithography [37]. 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 h. 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.

μ SHEAR adhesion experiments

Devices were first washed with ethanol, washed with PBS, either coated with FN (10 μ g/mL in PBS, 45 min) and blocked (1% BSA, 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 $\times$ 10 cells/mL [6] 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 Instruments Pico Plus Elite (#70–4506) syringe pump. Detached cells were collected and treated with trypsin in suspension, and attached cells were trypsinized and collected. Trypsin in both fractions was inactivated by use of serum-containing media. For detachment experiments, cells were labeled with CellTracker™ Red CMTPX Dye (ThermoFisher Scientific #C34552) within the devices according to manufacturer's guidelines prior to force application. 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/or CellTrackerRED+ cells was quantified along eight specific locations at predetermined time points.

E0771 tumor generation and size measurement

eGFP-E0771 cells were injected (125–10 000 cells/injection for tumor titration assays; 250 cells/injection in tumor growth assays in 20 μ L of 1:1 Matrigel to saline solution) through the inguinal mammary gland into the abdominal mammary gland of female C57BL/6 mice to establish tumors. Tumor diameter sizes were measured in 3D at days 9,11,13,15, and 17 using digital calipers.

CA2 tumor generation and size measurement

CA2 cells were injected (100 cells/injection in 100 μ L of 1:1 Matrigel to CA2 media) into the subcutaneous flank of female NSG mice to establish tumors. Tumor diameter sizes were measured in 3D every other day starting 23 days post implantation using digital calipers.

Statistics

Data points were plotted using Prism (GraphPad) with a horizontal line representing the mean and vertical error bars indicating the standard error. Statistical significance ($P < 0.05$) was determined using either, Student's t-test, 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).

SUPPLEMENTARY DATA

Supplementary data are available at INTBIO Journal online.

FUNDING

This work was supported by the National Cancer Institute of the National Institutes of Health under Award Number R21 CA202849 (A.J.G., S.N.T.).

CONFLICT OF INTEREST

This work forms the basis of a patent application filed by the Georgia Tech Research Corp listing E.A.C., S.N.T., and A.J.G. as inventors.

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