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A Dendrimer-based Platform for Effective Capture of Tumor Cells after TGF β_1 -induced Epithelial-Mesenchymal Transition

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Abstract

Detection of circulating tumor cells (CTCs) relying on their expression of epithelial cell markers, such as epithelial cell adhesion molecule (EpCAM), has been commonly used. However, this approach unlikely captures CTCs that have undergone the process of epithelial-mesenchymal transition (EMT). In this study, we have induced EMT of *in vitro* prostate (PCa) and breast cancer (BCa) cell lines by treatment of transforming growth factor beta 1 (TGF β_1), a pleiotropic cytokine with transition-regulating activities. We found that the TGF β_1 -treated, post-EMT cells exhibited up to a 45% reduction in binding affinity to antibodies against EpCAM (aEpCAM). To overcome this limitation, we designed our capture platform that integrates a unique combination of biomimetic cell rolling, dendrimer-mediated multivalent binding, and antibody cocktails of aEpCAM/aEGFR/aHER-2. Our capture surfaces resulted in up to 98% capture efficiency of post-EMT cells from mixtures of TGF β_1 -treated and untreated cancer cells spiked in culture media and

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Author Contributions

J.H.M. and S.H. were involved in research study design and concept. J.H.M., A.C., K.A.T. and S.H. were involved in the research performance using *in vitro* cancer cell lines. J.H.M., R.E.M. and S.H. were involved in healthy donor enrollment. A.B., B.S. and G.V. were involved in patient enrollment and clinical investigation. Data analysis, interpretation and statistics was conducted by J.H.M., A.C., K.A.T. and S.H. J.H.M., A.C., K.A.T., M.P. and S.H. drafted and revised the manuscript.

Supporting Information

The Supporting Information for additional electronic information (i.e., Materials, cell culture, confirmation of TGF β_1 -induced EMT, observation of cellular responses using flow chamber, clinical study design, and immunostaining for CTC Confirmation as pointed in the text) is available free of charge on the ACS publications website (<http://pubs.acs.org>).

Disclosure of Potential Conflicts of Interest

S.H. is a co-founder of Capiro Biosciences, a biotech startup that is commercializing CapiroCyte™ CTC technology.

human blood. In a clinical pilot study, our CTC device was also able to capture rare CTCs from PCa patients with significantly enhanced capture sensitivity and purity compared to the control surface with aEpCAM only, demonstrating its potential to provide a reliable detection solution for CTCs regardless of their EMT status.

Graphical Abstract

To simulate the epithelial-mesenchymal transition (EMT) of circulating tumor cells (CTCs) *in vitro*, breast and prostate cancer cells were treated with transforming growth factor beta1 (TGFβ₁). The TGFβ₁-treated, post-EMT cells exhibited reduction in binding affinity to single antibodies against cancer markers. Our capture platform that integrates a unique combination of biomimetic cell rolling, dendrimer-mediated multivalent binding, and antibody cocktails, shows significantly enhanced sensitivity of post-EMT cell detection and clinical CTCs from prostate cancer patients, demonstrating its potential as a reliable detection solution for CTCs regardless of their EMT status.

Keywords

Multivalent binding; Biomimetic cell rolling; Circulating tumor cells (CTCs); Epithelial-Mesenchymal Transition (EMT); Transforming Growth Factor beta 1 (TGFβ₁)

Most antibody-based CTC enumeration techniques to monitor cancer progress and metastasis rely on the assumption that CTCs in blood express epithelial markers, such as epithelial cell adhesion molecule (EpCAM), whereas normal hematologic cells do not.^{1–3} Among these EpCAM-targeted CTC detection techniques, CellSearch® system and Gilupi were cleared by the US FDA and the EU, respectively, for monitoring treatment effectiveness in metastatic prostate (PCa) and breast cancer (BCa) patients. However, in the blood-borne dissemination of human cancers, CTCs that have undergone epithelial-to-mesenchymal transition (EMT) frequently attain ‘cancer stem-like cell’ characteristics. The process greatly augments CTC invasiveness and metastatic potential,^{4–7} while diminishing epithelial properties and surface markers, such as EpCAM^{8,9} and E-cadherin.^{10,11} Thus, EpCAM-targeted platforms are less effective in capturing post-EMT disseminating carcinoma cells.^{5,8,12–14} A series of *in vivo* post-capture analysis revealed that EpCAM-negative CTCs, which are not captured by CellSearch®, showed typical characteristics of

BCa cells that metastasize to brain.¹³ The development of an EMT-independent detection method is thus imperative to accurately diagnose the metastatic potential of CTCs.

The lack of *in vitro* mesenchymal tumor cell lines presents an obstacle to validate post-EMT capture methods at a pre-clinical level. Despite their epithelial origins, CTCs behave differently from available *in vitro* epithelial cell lines due to the frequently-observed phenotypic changes induced by EMT.¹⁵ Attempts to establish cell lines from purified CTCs collected from metastatic cancer patients have been partially successful,¹³ but these efforts have not translated to commercially available monoclonal lines. Thus, modification of well-established cell lines to mimic the mesenchymal properties of CTCs is necessary for validation and optimization of an EMT-independent method of CTC capture. Transforming growth factor beta 1 (TGF β ₁) is a pleiotropic cytokine with multiple cell signaling pathways including the regulation of cell proliferation, functional differentiation, extracellular matrix (ECM) production, cell motility, and apoptosis.¹⁶ It has been reported that TGF β ₁ treatment induces EMT in PCa¹⁷ and BCa^{18,19} cells, resulting in the expression of mesenchymal stem-cell like characteristics, carcinogenesis, and tumor progression. In this study, TGF β ₁ was therefore employed to induce EMT in cell lines to simulate post-EMT CTCs *in vitro*.

Our group previously developed a multifunctional CTC capture platform using a combination of cell rolling, dendrimer-mediated multivalent binding, and multiple antibodies.^{3,20} Compared to linear polymer controls, the increased surface area of dendrimers allowed for a greater density of capture antibodies to be surface immobilized, and the flexible nature of the polymer facilitated multivalent binding.^{20–22} This combination has demonstrated significant enhancement in sensitivity and specificity towards *in vitro* tumor cells,^{21,23,24} *in vivo* CTCs in an animal model,²⁵ and clinical CTCs from cancer patients.²⁶ In this work, we have tested our CTC capture platform to evaluate its detection sensitivity towards post-EMT tumor cells and clinical CTCs. We exploit EMT induction by TGF β ₁ treatment of human epithelial tumor cells to demonstrate the efficiency of our surface in capturing CTCs with mesenchymal stem-cell like characteristics, i.e., post-EMT CTCs (Fig. 1). We optimized and confirmed the TGF β ₁ treatment for EMT induction in human PCa and BCa cells using a series of assays including wound-healing, spheroid formation, and Western blotting. The cellular binding of PCa and BCa cells before and after TGF β ₁ treatment were observed via immunopanning assays using the surface functionalized with a single antibody against tumor cell markers (EpCAM, HER-2, epidermal growth factor receptor (EGFR), or prostate stem cell marker (PSCA)). Based on the surface binding behaviors of the TGF β ₁-treated tumor cells, novel triple-antibody cocktail-coated capture surfaces with the three antibodies, combined with PAMAM dendrimers and E-selectin, were developed to capture PCa and BCa cells. The novel surfaces were further tested using the post-EMT cells (TGF β ₁-treated) after being spiked in human blood. In addition, CTC levels were compared in clinical samples collected from PCa patients.

METHODS

The descriptions of “Materials”, “Cell Culture”, “Confirmation of TGF β ₁-induced EMT”, “Clinical Study Design”, and “Immunostaining for CTC Confirmation” are described in the Supporting Information.

TGF β ₁ Treatment

All BCa and PCa cells at a concentration of 1×10^5 cells/mL (5 mL) were seeded onto a 25 cm² T flask in the 10% FBS-media two days before TGF β ₁ treatment. For TGF β ₁ treatment, the seeded cells except LNCaP were starved in serum-free medium overnight, and then treated at a concentration of 10 ng/mL of TGF β ₁ in basal media for 72 hrs.²⁷ In the case of LNCaP, the serum-starved LNCaP cells were treated with 20 ng/mL of TGF β ₁ in basal media. TGF β ₁-resistant LNCaP cells were treated with 20 ng/mL due to their TGF β ₁ resistance.²⁸ The TGF β ₁-induced EMT was confirmed by western blotting, wound-healing assay, and 3D spheroid formation assay (see the Supporting Information). The changes in expression level of E-cadherin and vimentin proteins from the various cells with and without TGF β 1 treatment were quantified from the western blotting using ImageJ software. To label the viable cells with fluorescence, TGF β ₁-untreated cells were labeled with 4 μ M Calcein AM at 37°C in dark for 30 min. The labeled cells were trypsinized to make their suspensions at a pre-determined concentration in FBS-supplemented cell culture media or whole blood withdrawn from healthy donors. The prepared cell suspensions were kept on ice throughout the subsequent experiments.

Tumor Cell-spiked or Clinical PCa Patient Blood Specimens

Whole blood drawn from healthy donors was collected in heparin-treated tubes and kept at ambient temperature. Studies using human blood were reviewed and approved by UIC institutional review board (IRB) (protocols #2012–0139 for tumor cell-spiked blood specimens and #2013–1033 for clinical PCa patient blood specimens). Fluorescence-labeled BCa or PCa cells were spiked into 3 mL of whole blood at a final concentration of 1×10^5 tumor cells/mL blood. Mononuclear cells including tumor cells in buffy coat were separated from whole blood using Ficoll-Paque Plus (Stemcell Technologies Inc., Vancouver, Canada) as described in our previous publication.²¹ After washing the buffy coat twice with the 2% FBS-containing PBS, the recovered cells were suspended in 3 mL of the complete cell culture media and used for subsequent experiments. For the clinical samples, 12 mL of the blood specimen obtained from prostate cancer patients were used for separation and resuspended with 12 mL of complete media. The experiments were performed within 48 hrs after blood drawing.

Surface Functionalization by Adhesive Protein Immobilization

A polydimethylsiloxane (PDMS) gasket with well-defined micropatterning was used to define the area of an epoxy functionalized glass slide in accordance with our previous publications. Following procedure from our prior publication, the surface was functionalized by PEG, followed by Generation 7 (G7) PAMAM dendrimers, and consequently conjugated with indicated antibodies.²³ For antibody conjugation, all antibody solutions of aEpCAM, aHER-2, aPSCA, and aEGFR were used at a concentration of 5 μ g/mL for single antibody-surfaces in PBS. For antibody cocktails, a fixed concentration of antibodies at 15 μ g/mL was used for 1:1:1 (v/v/v) mixture to make the final concentration of each antibody to 5 μ g/mL. In the case of flow chamber-based experiments, the antibody-stripes on micropatterned surfaces were treated with FITC-conjugated BSA (0.1 mg/mL in PBS buffer) for 1 hr to define the dendrimer-antibody-coated regions, and incubated with 0.2 mL of E-selectin at a

concentration of 5 $\mu\text{g/mL}$ after switching to the suitable PDMS gasket. The volumes of all reagent solutions except E-selectin were fixed at 20 μL for immunopanning assays and 10 $\mu\text{g/mL}$ for micropatterned surfaces. All incubation processes were carried at ambient temperature with constant gentle shaking, and between all preparation steps, the surfaces were washed with DDI water and PBS three times to remove residual chemicals from the surfaces. Potential non-specific binding of both protein-coated and uncoated regions was blocked by a final incubation with 1% (w/v) BSA or 1 $\mu\text{g/mL}$ methoxy PEG-NH₂ (Nektar Therapeutics, Huntsville, AL) solution. The functionalized surfaces were kept at 4°C, and the experiments using the surfaces were performed within 24 hrs after the surface preparation.

Immunopanning Assay

After treatment with bovine serum albumin (1 (w/v)% in PBS) to prevent the non-specific binding, each PEG/Dendrimer-functionalized surface with antibodies was incubated with 250 μL of cancer cell suspensions (total 1×10^5 cells per surface) for 10 min at 37°C.²³ The enhanced binding strength and stability through dendrimer-mediated multivalent binding effect for antibodies were confirmed by observing the change in the number of bound cells on the surfaces after agitation using a plate shaker at 600 rpm at room temperature. The unbound cells were removed from the surface by washing with PBS buffer. Based on the loading cell number, the recovery yield (%) was calculated.

Observation of Cellular Responses using Flow Chamber

Flow chamber experiments were performed as previously reported.²⁴ Suspensions of HL-60 and fluorescence-labeled cancer cells (MDA-PCa-2b, MDA-MB-361, MCF-7) were injected into a proprietary flow chamber using a syringe pump (New Era pump Systems Inc., Farmingdale, NY). The images of captured cells on each of the antibody-immobilized stripes were taken after one cycle consisting of forward flow (pushing) for 10 min and backward flow (withdrawing) for 10 min at 25 $\mu\text{L/min}$ (0.22 dyn/cm^2). The surface was then washed using PBS for 20 min at 100 $\mu\text{L/min}$ (0.88 dyn/cm^2). To remove the leukocyte cells from the E-selectin-regions, the surface was washed using EGTA/Mg²⁺ in PBS buffer for 3 min at 100 $\mu\text{L/min}$ (0.88 dyn/cm^2). Based on the known number of cancer cells perfused into the flow chamber, the numbers of captured cells per antibody-stripe were calculated and converted into capture efficiency (%).

All cells on the surface for both immunopanning assay and flow chamber-based assay were monitored using an Olympus IX70 inverted microscope (IX 70-S1F2, Olympus America, Inc., Center Valley, PA) with fluorescence light, and images were recorded using a 10x objective and a CCD camera (QImaging Retiga 1300B, Olympus America, Inc.). The number of cells on the surfaces was counted, based on the images taken in independent observations/measurements using ImageJ (NIH).

RESULTS

TGF β_1 -induced EMT Confirmation

TGF β_1 -induced EMT in the six tumor cell lines was first confirmed using Western blotting of epithelial and mesenchymal markers. The effect of invasive nature of the six cell lines was compared by employing more invasive (MDA-PCa-2b and PC-3 for PCa and MDA-MB-231 for BCa) and less invasive (LNCaP for PCa and MDA-MB-361 and MCF-7 for BCa) tumor cells.

Lysates of the tumor cells before and after the TGF β_1 treatments were analyzed in terms of downregulation and upregulation of an epithelial marker (E-cadherin) and a mesenchymal marker (vimentin), respectively (Fig. 2a for PCa cells and Supporting Information, Fig. S1 for BCa cells). Downregulation of E-cadherin is a hallmark of EMT and was thus employed for EMT confirmation.^{10,11} E-cadherin expression decreased in all tumor cell lines, but was more pronounced in less invasive cell lines (LNCaP for PCa cell lines and MDA-MB-361 and MCF-7 for BCa cell lines). Vimentin expression, activated upon EMT, consequently increased in all tumor cell lines as well, greater increases observed in the less invasive cell lines. The metastatic cell lines for PCa cells, such as MDA-PCa-2b and PC-3, and for BCa cells, such as MDA-MB-231, showed relatively low E-cadherin and activated vimentin expression prior to TGF β_1 treatment, but differences were still detected upon TGF β_1 -induced EMT.

The mobility changes of the EMT-induced tumor cells were also assessed using a wound-healing assay (Fig. 2b for PCa and Supporting Information, Fig. S1 for BCa). The *in vitro* scratch assay models the migration of cells across a wound while preserving most cell-cell interactions.²⁹ The assay is particularly suitable for studying the effect of TGF β_1 on the enhancement of cell migration by observing the migration rate into the scratched region.^{29,30} This wound-healing rate increased with TGF β_1 concentration, independent of cell type (Fig. 2b for PCa and Supporting Information, Fig. S1 for BCa), although cytotoxicity was observed at 20 ng/mL.³¹ Due to their resistance against TGF β_1 ,²⁸ TGF β_1 -treated LNCaP cells showed relatively less enhancement in migration and higher cell viability than other cells at 20 ng/mL of TGF β_1 (Fig. 2b).

EMT induced by TGF β_1 produced dense spheroid morphologies in three-dimensional, anchor-independent growing conditions (Fig. 2c for PCa and Supporting Information, Fig. S1 for BCa). The formation of spheroids from suspended tumor cells on soft agar can be used to semi-quantitatively measure phenotypic changes and anchorage independent growth.¹⁹ EMT via TGF β_1 stimulation results in large, undifferentiated masses, linked to increased survival in circulation and eventual metastatic tumor formation.¹⁸ The number of such colonies increased between 30% and 125% following TGF β_1 treatment (Fig. 2c for PCa and Supporting Information, Fig. S1 for BCa). Overall, spheres of TGF β_1 -treated PCa and BCa cells showed larger diameters and higher colony counts than those of untreated cells. Based on Western blotting, wound-healing assay, and spheroid formation assay, the concentration of TGF β_1 for EMT induction of PCa and BCa cells was fixed at 10 ng/mL for subsequent experiments, with the exception of LNCaP cells at 20 ng/mL.

The Binding Affinity Changes upon EMT Induction

Immunopanning assays of the six cell lines employed in this study were quantified in terms of recovery yield (defined by number of captured cells per the number of injected cells) using antibodies (aEpCAM/aEGFR/aPSCA for PCa and aEpCAM/aEGFR/aHER-2 for BCa). A human leukemia cell line, HL-60, and IgG were used as a leukocyte model and as a mock antibody control, respectively. An antibody against the epithelial cell marker, aEpCAM, was used to monitor changes in the binding behaviors of the tumor cells upon TGF β ₁ treatment. Antibodies such as aEGFR,¹⁸ aPSCA,³² and aHER-2,^{33,34} recognize specific surface markers with elevated expression among EMT-induced, stem-like tumor cells, were also evaluated.

All less invasive cancer cells tested herein showed highly specific cellular binding to aEpCAM (Supporting Information, Fig. S2). Given that all cell lines showed a negligible capture (recovery yield) on the algG-coated control surface (0.2–1.9%), this level was used as a baseline for subsequent observations on the binding behaviors of the cells to the antibodies tested. The aEpCAM-coated surface captured the pre-EMT PCa (LNCaP) and BCa (MDA-MB-361 and MCF-7) cells at a relatively high efficiency, but did not effectively capture the metastatic cell lines (~20% vs. ~6%). Large numbers of the metastatic PCa (PC-3 and MDA-PCa-2b) and BCa (MDA-MB-231) cells were bound to aEGFR-coated surfaces, although the same surface did not accommodate significant binding of other cell lines. Binding of PCa cell lines on aPSCA and BCa cell lines on aHER2 was cell line-dependent, i.e., PSCA⁺ cells such as PC-3 and MDA-PCa-2b and HER-2⁺ cells such as MDA-MB-361 bound to their respective surfaces in high numbers. All positive and negative binding results of the cell lines used in this study were in agreement with the expression of their surface markers reported in the literature.^{32,35–39}

Binding affinity of PCa and BCa cells significantly changed after EMT induction by TGF β ₁. The binding differences in Fig. 2d and Supporting Information, Fig. S1 were calculated by subtracting the capture efficiencies of TGF β ₁-treated PCa and BCa cells from those without the treatment. The binding of PCa cells changed after TGF β ₁ treatment; the numbers of the bound cells on aEGFR and aPSCA increased by up to 18% and 16%, respectively, whereas aEpCAM exhibited a 45% reduction (Fig. 2d for PCa). As summarized in Supporting Information, Fig. S1, the binding of BCa cell lines was increased on aEGFR (by 8–40%) but decreased on both aHER-2 (by up to 7%) and aEpCAM (by up to 23%) after TGF β ₁ treatment. The binding behaviors of the cells on aEGFR and aPSCA were directly affected by the changed surface expression of EGFR and PSCA upon TGF β ₁ treatment. Although the levels of changes in binding upon TGF β ₁ treatment varied across the cell lines, we confirmed that the aEpCAM-based tumor cell detection could miss the stem-cell-like, low-EpCAM-expressing tumor cells (Fig. 2d and Supporting Information, Fig. S1). For these reasons, we prepared antibody cocktails tailored for PCa and BCa cells to capture cancer cells both pre- and post-EMT. The antibody cocktails selected to prove our concept in this investigation were composed of aEpCAM/aEGFR/aPSCA for PCa and aEpCAM/aEGFR/aHER-2 for BCa cells.

CTC Capture with Antibody Cocktails

Dendrimer-mediated, multivalent binding of TGF β ₁-treated PCa and BCa cells was confirmed by comparing the binding results to linear counter polymer, poly(ethyleneglycol) (PEG) functionalized with the same antibody cocktails. The dendrimer-immobilized surface platforms were composed of bifunctional PEG and PAMAM dendrimers, prepared in the same fashion as our previous publication.²³ To monitor the binding and dissociation of both treated and untreated cells simultaneously, both TGF β ₁-treated and untreated cells were loaded on the surface together after Calcein-AM labeling of TGF β ₁-untreated cells (Fig. 3). Regardless of surface platform composition (PEG only or dendrimers), antibody cocktails showed the enhanced binding for both TGF β ₁-treated and untreated cells (Fig. 3a for PCa and Fig. 3b for BCa), compared to a single antibody (Supporting Information, Fig. S2, $p < 0.0001$, two-way ANOVA). Overall, the recovery yields of the antibody mixtures were highly dependent on the expression of cell surface markers, and higher yields of BCa cells were observed than those of PCa cells. The recovery yields of the TGF β ₁-treated PCa and BCa cells on triple antibody cocktail-immobilized surfaces were as high as the TGF β ₁-untreated PCa and BCa cells (Fig. 3a for PCa and Fig. 3b for BCa, not statistically different, two-way ANOVA). Moreover, dendrimer-platforms with antibody cocktails showed higher recovery yields of both TGF β ₁-treated and untreated PCa cells (15 – 55% recovery) and BCa (50 – 84% recovery), compared to the PEG controls (7 – 27% recovery for PCa in Fig. 3a and 16 – 32% recovery for BCa in Fig. 3b). Based on these enhanced recovery yields and binding stability, we conclude that the stable and sensitive binding between antibody cocktails and cancer cells was augmented by dendrimer-mediated multivalent binding, consistent with our previous publication.²³

To quantitatively analyze the combination effect of antibody cocktails in the cell recovery experiments, the combination index (CI) of each cancer cell line before and after TGF treatment was assessed using two following Equations (1, Bliss Independent Model) and (2, Highest Single Agent Approach).⁴⁰

$$CI = \frac{E_A + E_B + E_C - E_A \times E_B - E_A \times E_C - E_B \times E_C - E_A \times E_B \times E_C}{E_{Total}} \quad \text{Eq. (1)}$$

$$CI = \frac{\max(E_A, E_B, E_C)}{E_{Total}} \quad \text{Eq. (2)}$$

where E_A , E_B , E_C , and E_{Total} are the mean recovery yields using the G7-platforms functionalized with each antibody, such as aEpCAM, aHER-2, aPSCA, and aEGFR, or triple antibody cocktails, respectively. These equations were originally derived to evaluate the combination effect of multiple drug cocktails in Pharmacology. Since the E values in equations are defined as the fraction of drug efficacy ($0 \leq E \leq 1$), the recovery yields we obtained were transformed as fraction form for the assessments. Although vary between the cell types or before/after TGF β ₁ treatment, the CI values obtained from two different equations were in similar trends. It is obvious that the CI values for all cancer cells exhibit near 1.0 or less, which indicates the combination effect is additive ($CI = 1$) or synergetic ($CI < 1.0$) (Table 1). However, the CI values of PC-3 cells (between 1.9 and 3.6) were

significantly higher than 1.0 (Table 1), reflecting a negative effect in combination. Interestingly, when aPSCA was replaced with aHER-2 in the antibody cocktails, the *CI* values of PC-3 fell to 1.3 regardless of TGF β ₁ treatment (Table 1).

CTC Capture with Antibody Cocktails and E-selectin under flow

With the decrease in epithelial marker expression after EMT, TGF β ₁-induced EMT may have altered the other cellular interactions such as E-selectin-mediated cell rolling. By inducing dynamic molecular interactions with ligands expressed on metastatic cancer cells, E-selectin recruits fast moving leukocytes and CTCs from bulk flow to the surface and enhances antibody-based CTC capture efficiency.²⁴ Several ligands against E-selectin have been identified: E-selectin ligand-1 (ESL-1), P-selectin glycoprotein ligand-1 (PSGL-1), sialyl Lewis X (sLe^x), CD24, and CD44. Variable expression levels^{23,41} affect the efficiency of E-selectin-recruitment of different cancer cell types. Additionally, expression may change upon TGF β ₁-induced EMT.⁴² The rolling velocities of TGF β ₁-treated and untreated PCa and BCa cells were measured on E-selectin-functionalized surfaces using a proprietary flow chamber with a width of 5 mm, height of 125 μ m, and length of 6 cm. The net rolling velocities of TGF β ₁-treated PCa and BCa cells on E-selectin were slightly, but not significantly, higher than those of untreated cells in the same analytical conditions (Supporting Information, Fig. S3). Although we did not quantify the expression of E-selectin ligands after TGF β ₁ treatment, the negligible difference in rolling velocity suggests that E-selectin is capable of recruiting both pre- and post-EMT CTCs from bulk flow. Consistent with our previous work with pre-EMT CTCs,²⁴ capture efficiency of TGF β ₁-treated cancer cell lines was significantly enhanced on surfaces containing E-selectin (Fig. 3c for PCa and Fig. 3d for BCa). Capture of all BCa cells and certain PCa cells on E-selectin and antibody cocktail-dendrimer approached 100%.

Post-EMT Cancer Cell-spiked Blood and PCa Patients Specimens

Experiments with clinically relevant human blood samples demonstrated that our platform could both sensitively and efficiently capture target cancer cells. Suspensions of TGF β ₁-untreated and TGF β ₁-treated cells for both PCa and BCa cell lines were spiked in human blood samples drawn from healthy donors after fluorescent labeling (green for TGF β ₁-untreated and red for TGF β ₁-treated, Fig. 4a and 4b). When functionalized with E-selectin, the capture efficiencies were significantly enhanced (Fig. 4a and 4b). Our surface was able to efficiently capture cancer cells from background human hematological cells under flow in an EMT-independent way (Fig. 4c for PCa and Fig. 4d for BCa). The capture efficiencies from the blood samples were comparable to those from cell suspensions. The combination of triple antibody cocktails, dendrimers, and E-selectin on the surface induced the high recovery yield and sensitive binding of TGF β ₁-treated cells across the all cell types we tested. This observation is consistent with the low false-positive detection using the control blood specimens from healthy donors, and enhanced sensitivity (71–99% recovery yields) and specificity (9–74% capture purity) using the spiked cancer cells captured from human blood in our previous publications.^{21,26} However, the capture efficiencies of all PCa cell lines except MDA-PCa-2b from the blood samples were lower than 80%, similar to their capture efficiencies from the cell suspension.

Blood samples from four PCa patients were evaluated in our pilot study. Note that an antibody mixture of aHER-2/aEGFR/aEpCAM was used for clinical blood samples withdrawn from PCa patients, instead of aPSCA/aEGFR/aEpCAM. Literature has shown that PCa cells also overexpress HER-2 and, in our pilot study comparing two mixtures, the aHER-2 mixture outperformed the aPSCA mixture (Table 1 and Supporting Information, Fig. S4). The clinical and demographic information regarding the PCa patient in our pilot study was summarized in Supporting Information, Table S1. Immunostaining against an epithelial marker, cytokeratin (CK), a leukocyte marker, CD45, and a nuclei marker, DAPI, were performed to distinguish CTCs among captured cells. CK-positive, CD45-negative, and DAPI-positive cells were defined as CTCs, while CK-negative, CD45-positive, DAPI-positive cells are as leukocytes (Fig. 5a). Although the numbers of CTCs per patient varied across patients, CTCs were detected in all samples, and the capture surface with antibody cocktail captured a greater number of CTCs that aEpCAM immobilized to PEG alone (Fig. 5b) with a 2.6–6.8 fold increase in purity (Fig. 5c).

DISCUSSIONS

The TGF β ₁ treatment induced EMT *in vitro* PCa and BCa cells to mimic post-EMT CTCs in the pathological conditions for effective detection of CTCs. Tumor cells during metastasis undergo EMT in response to cell type- or tissue type-specific extracellular signals, such as TGF β ₁ from the tumor microenvironment or circulatory system.^{6,43–45} The cooperation of major developmental signaling pathways, including TGF β , Wnt, Notch, and growth factor receptor signaling cascades, affect the EMT of tumor cells.⁴⁶ Other environmental factors, such as hypoxia, inflammatory cytokines from the microenvironment, and mechanical shear stresses in blood vessels, are also involved in the EMT induction of cancer cells.⁴⁶ Most notably, TGF β pathway appears to be a primary inducer of EMT.^{44,46} One of the major sources of TGF β for tumor cells is the stromal fibroblast cells in the tumor microenvironment.⁴⁷ Tumor cells in circulation are also exposed to TGF β ₁-induced EMT. When attached to tumor cells, platelets release TGF β ₁ into circulation,⁴⁵ and strong TGF β ₁ signatures are observed in platelets attached to mesenchymal CTC clusters.⁵ Based on these literatures, we selected TGF β ₁ to induce EMT *in vitro* PCa and BCa cells for EMT-independent CTC capture platform.

The decreased expression of epithelial cell markers, EpCAM in particular, observed in post-EMT tumor cells in the blood imposes limitations on the capture efficiency of aEpCAM-based capture platforms. Recent publications reported that EpCAM-based detection are unable to detect post-EMT CTCs which often have more invasive and aggressive properties that facilitate initiate cancer metastasis in xenograft models.^{5,8,12–14,48} Our findings through immunopanning assays (Fig. 2d and Supporting Information, Fig. 1d) further demonstrate limitations of single aEpCAM-based detection. By simply treating *in vitro* cell lines with TGF β ₁, post-EMT models of CTCs can be produced to better evaluate CTC detection methods.

Dendrimer-mediated multivalent binding is effective in capturing tumor cells, especially EMT-induced cells. We have observed the effect of multivalent binding in aEpCAM-dendrimer conjugates on surface for capturing EpCAM^{low}-MDA-MB-231 cells in our

previous publication.²³ When switched with novel triple antibody cocktails, the initial binding and binding stability of both TGF β ₁-untreated and TGF β ₁-treated tumor cells significantly increased on dendrimer-immobilized platforms compared to linear, PEG-platforms. This enhancement of capture efficiency using antibody cocktails was effective in our clinical pilot study for PCa patients. The enhancements observed were due to higher density of ligand immobilized on the dendrimer surface in the same loading conditions with the control PEG surface²³ and the simultaneous, multivalent binding of heterogeneous ligands with target cells. Moreover, other unique physio-chemical properties, such as polymer backbone topology, flexibility, and hydration of PAMAM dendrimers,^{49–51} may also enhance cellular binding on the dendrimer-nanotopography matrix.^{3,25,52}

E-selectin treatment of the surface was efficient in recruiting tumor cells, including pre- and post-EMT cells, from bulk flow. The rolling velocity of TGF β ₁-treated BCa cells on E-selectin was slightly, but not significantly, higher than that of untreated cells in this study by 10–40% (except 327% increased rolling velocity of LNCaP cells). However, metastatic BCa (MDA-MB-231) and PCa (PC-3) cell lines have shown significantly lower rolling populations and faster rolling velocity on E-selectin surface under flow in this study and in previous publications.^{53,54} Several cell surface markers, ESL-1, sLe^x, PSGL-1, CD44, and CD24,²³ have been identified as ligands for E-selectin. The change in expression of these ligands after EMT induction was not fully analyzed in this study, but previous publications have reported upregulated expression of sLe^x, shifted CD44 expression from the standard isoform (CD44s) to variant isoforms (CD44v),⁵⁵ and downregulated expression of CD24 upon EMT.^{54,56}

Compared to the BCa lines, the selected PCa cell lines for this study generally showed lower capture efficiency on a novel triple antibody cocktail surface in immunopanning and flow chamber-based assays. One possible explanation is that although PSCA mRNA expression increased with tumorigenic potential, it does not directly translate to equivalent amounts of the membrane-bound glycoprotein.³² Additionally, the low capture efficiency of PCa cells may be due to the decreased expression of E-selectin ligands, diminishing the recruitment effect of human recombinant E-selectin.⁴¹ When incorporating aHER-2 instead of aPSCA, the recovery yields of tumor cells in PCa cell lines and CTCs in clinical samples were increased by 2.3 ± 0.1 folds (Supporting Information, Fig. S4). Besides of the increased recovery yields of PCa tumor cells, we also observed that the combination index values for PC-3 cells were changed from negative to near additive effect upon replacing aPSCA with aHER-2 in antibody cocktails (Table 1). These showed the potential for a platform with the antibody mixture of aEpCAM/aEGFR/aHER-2 to capture rare CTCs from broad types of cancers including PCa and BCa. However, further screening for PCa surface markers is necessary to find more suitable antibodies for PCa-originating CTC capture.

CONCLUSIONS

In this study, we have developed and validated an EMT-independent CTC detection technique using TGF β ₁-treated PCa and BCa cell lines. Using a pleiotropic cytokine, TGF β ₁, the EMT of *in vitro* PCa and BCa cell lines were induced and confirmed via multiple molecular and functional assays. The versatility and modularity of PAMAM

dendrimer-immobilized platform functionalized with antibody cocktails resulted in enhanced detection sensitivity (up to 98% capture efficiency) of pre- and post-EMT BCa and PCa cells in culture media and human blood, compared to the surfaces coated with aEpCAM only. Enhanced capture rates were further demonstrated in clinical samples drawn from PCa patients. The exceptional sensitivity and specificity of the results shown here demonstrates the promise of our platform as a diagnostic and prognostic tool for monitoring cancer progression and therapeutic response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic representation of EMT induction using $TGF\beta_1$, its confirmation, and subsequent CTC enumeration for both pre- and post-EMT PCa and BCa cells. a) EMT of *in vitro* PCa and BCa cells were induced by $TGF\beta_1$ treatment. b) To confirm $TGF\beta_1$ -induced EMT, immunoblotting and other functional assays were performed. c) Both pre- and post-EMT PCa and BCa cells were efficiently enumerated on our biomimetic platform with G7 PAMAM dendrimers and two novel triple antibody cocktails.

Figure 2.

TGF β_1 -induced EMT in PCa cells and its confirmation. a) Phenotype changes of PCa cells before and after TGF β_1 treatment. (–): TGF β_1 -untreated and (+): TGF β_1 -treated cells. The changes (%) in expression level of E-cadherin and vimentin proteins were marked under the band of each protein in western blotting. b) Enhanced cell mobility after EMT induction. Wound healing rate over 24 hrs monitoring was increased after 10 ng/mL TGF β_1 treatment. (n = 3, mean $\pm$ standard deviation) c) Anchorage-independent cell growth of cancer cells was enhanced after TGF β_1 treatment. The surviving cell colonies were stained with crystal violet, and the number of colonies per field was increased. (n = 3, mean $\pm$ standard deviation) d) Changes in recovery yields of PCa cells on aEpCAM, aHER-2, and aEGFR-immobilized surfaces after TGF β_1 treatment. (n = 5, mean $\pm$ standard error).

Figure 3.

Enhanced sensitivity of EMT-induced PCa and BCa cells on G7 PAMAM dendrimer-immobilized platform with novel triple antibody cocktails. (a and b) To distinguish between two different populations, TGFβ₁-untreated cells were labeled with green fluorescence. Antibody cocktails were able to bind with PCa (a) and BCa (b) cells in immunopanning assays regardless of TGFβ₁-induced EMT, which were further enhanced by using G7 dendrimers as a multivalent binding mediator. (c and d) Under flow, capture efficiencies of PCa (c) and BCa (d) cells on antibody cocktail-dendrimer conjugates were further increased by E-selectin surface treatment. (n = 5–10, mean ± standard error) The measured recovery yields and capture efficiencies were compared by statistical analysis using two-way ANOVA, followed by Tukey's multiple comparisons tests with 95% simultaneous confidence intervals (Prism software). ns: not significant; **p < 0.01; and ***p < 0.001.

Figure 4. Enhanced capture efficiency of EMT-induced PCa and BCa cells-spiked blood samples on the G7 PAMAM dendrimer-immobilized platform with novel triple antibody cocktails and E-selectin. (a and b) To distinguish between two different populations in hematological cells, TGF β_1 -untreated (pre-EMT) and treated (post-EMT) cells were labeled with green and red fluorescence, respectively. The capture efficiency enhanced by hiring G7 dendrimers as a multivalent binding mediator and antibody cocktails was further enhanced by E-selectin. (c and d) Under flow, capture efficiencies of PCa (c) and BCa (d) cells on antibody cocktail-dendrimer conjugates were further increased by E-selectin surface treatment. (n = 5–10, mean $\pm$ standard error).

Figure 5.

Enhanced capture efficiency and purity of CTCs from PCa patient blood samples on our capture surfaces (ABmix + dendrimers + E-selectin). (a) CTCs and leukocytes were distinguished using immunostaining against red-labeled epithelial marker, cytokeratin (CK), green-labeled leukocyte marker, CD45, and blue-labeled nuclei marker, DAPI. CK-positive, CD45-negative, and DAPI-positive cells are defined as CTCs, while CK-negative, CD45-positive, DAPI-positive cells are as leukocytes. (b) Compared to the control, aEpCAM-immobilized PEGylated platform, our multifunctional surfaces showed significantly higher number of CTCs in clinical pilot study of PCa patients. Although the numbers of CTCs per patients varied, the enhancement of CTC capture on our surfaces was significant, compared to the control ($p < 0.01$, two-way anova). (c) Capture purity, the percentage of CTCs among all captured cells on the platforms, was also increased via our approach by 2.6–6.8 fold.

Table 1.

A summary of Combination Indexes (*CI*s) to evaluate the antibody cocktail effect to capture pre- and post-EMT PCa and BCa cells.

	Bliss independent model		Highest Single Agent approach (the highest single antibody)	
	<i>CI</i> _{untreated}	<i>CI</i> _{treated}	<i>CI</i> _{untreated}	<i>CI</i> _{treated}
LNCaP	1.0	0.3	1.0 (aEpCAM)	0.2 (aEGFR)
PCa-2b	1.2	1.1	1.0 (aEGFR)	1.1 (aEGFR)
PC-3	3.6	2.3	2.8 (aEGFR)	1.9 (aEGFR)
PC-3*	1.7	1.4	1.4 (aEGFR)	1.3 (aEGFR)
MB361	0.8	1.2	1.3 (aEpCAM)	1.3 (aEpCAM)
MCF-7	1.1	1.5	1.4 (aEpCAM)	1.9 (aEGFR)
MB231	0.9	0.8	0.7 (aEGFR)	0.7 (aEGFR)

Note. The *CI* values of PC-3* were obtained from the recovery yields of the G7-platforms functionalized with aEpCAM, aHER-2, and aEGFR.