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Cdc42-dependent modulation of rigidity sensing and cell spreading in tumor repopulating cells

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ABSTRACT

Recently, a robust mechanical method has been established to isolate a small subpopulation of highly tumorigenic tumor repopulating cells (TRCs) from parental melanoma cells. In order to characterize the molecular and mechanical properties of TRCs, we utilized the tension gauge tether (TGT) single-molecule platform and investigated force requirements during early cell spreading events. TRCs required the peak single molecular tension of around 40 pN through integrins for initial adhesion like the parental control cells, but unlike the control cells, they did not spread and formed very few mature focal adhesions (FAs). Single molecule resolution RNA quantification of three Rho GTPases showed that downregulation of *Cdc42*, but not *Rac1*, is responsible for the unusual biophysical features of TRCs and that a threshold level of *Cdc42* transcripts per unit cell area is required to initiate cell spreading. Cdc42 overexpression rescued TRC spreading through FA formation and restored the sensitivity to tension cues such that TRCs, like parental control cells, increase cell spreading with increasing single-molecular tension cues. Our single molecule studies identified an unusual biophysical feature of suppressed spreading of TRCs that may enable us to distinguish TRC population from a pool of heterogeneous tumor cell population.

1. Introduction

Cancer cells within a tumor ecosystem possess a remarkable capacity for self-renewal. Cancer stem cells, a small subpopulation of stem-cell-like-cells driving growth and progression of tumors, were first described in leukemia [1]. Following this work, several reports indicate the existence of cancer stem cells in solid tumors such as the breast [2], brain [3], skin [4], prostate [5], and the lungs [6]. However, evidence for the existence of cancer stem cells in solid tumors has been rather controversial primarily due to unreliably expressed antigen based selection techniques [7,8]. This prompted the need for the development of a robust technique to isolate a highly tumorigenic subpopulation of cancer cells. Recently, we successfully isolated a small fraction of highly tumorigenic cells, which we termed tumor repopulating cells (TRCs), from the B16-F1 melanoma cell line by culturing them in soft-3D-fibrin gels [9]. However, very little is known about the biophysical characteristics of such tumorigenic cells. In this study, we utilize a number of single-molecule techniques including single molecule fluorescence in situ hybridization (smFISH) [10] and our recently developed tension gauge tether (TGT) technique [11] to reveal the molecular and mechanical features of TRCs.

2. Materials and methods

2.1. Cell culture

Melanoma cell lines B16-F1 and B16-F10 cells were maintained in rigid culture dishes with high-glucose DMEM (Invitrogen) cell culture medium containing 10% FBS (Hyclone) at 37 °C with 5% CO_2.

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The medium was supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μg/ml penicillin-streptomycin. Soft 3D fibrin gels (90 Pa) were prepared as described previously [9]. TRCs from these cell lines were grown in soft 3D fibrin gels using the same growth medium.

2.2. Surface functionalization

Tension gauge tethers of different tension tolerance were immobilized on passivated glass bottom dishes at a cyclic-RGDfK ligand density of ~600 ligands/μm^2 (one ligand in every 40 nm) as described elsewhere [11,12]. An earlier study showed that cell spreading and FA formations are not supported when the spacing between integrin binding sites is more than 58 nm [13]. Briefly, glass surfaces were incubated with biotinylated BSA (Sigma) for 20 min at room temperature, then washed with PBS and further incubated with NeutrAvidin (Thermo Fisher) for 10 min at room temperature. These surfaces were washed again with PBS and incubated with TGTs of different tension tolerance or biotinylated RGDfK peptides (Peptides International). Following surface functionalization, cells were seeded on the surface.

2.3. Cell area, volume, fluorescence intensity measurements

ImageJ (NIH) was used to measure projected cell spreading area, perimeter, and volume. 3D volume was estimated from the Z-stack images. Cell shape index (CSI), a geometric measure of circularity, is a non-dimensional parameter calculated based on the projected cell area and perimeter using the following relation, $CSI = \frac{4*\pi*Area}{Perimeter^2}$. CSI values range from 0 to 1, 1 being a perfect circle while values less than 1 indicate complex spread pattern.

Statistical testing. All statistical analysis was carried out using a two-tailed Student's t-test unless noted otherwise.

3. Results

3.1. TRCs do not spread on any TGT engineered surfaces

TGTs are rupturable DNA tethers with tunable tension tolerances, T_{tol} [11]. Here, one strand of the DNA is immobilized to a glass surface via biotin-neutravidin interactions and other strand is conjugated to cyclic-RGDfK peptide, specific to $\alpha_v\beta_3$ integrins [14]. T_{tol} is highest (56 pN) when the biotin is positioned on the opposite end of the rupturable duplex DNA tether (force is applied in a *shearing mode*) (Fig. 1a). T_{tol} progressively decreases to lowest value (12 pN) as the biotin is moved toward the same end of the duplex DNA tether (force is applied in an *unzipping mode*) (Fig. 1a). T_{tol} values can thus be tuned monotonically as a function of the distance between the biotin and the integrin ligand (Fig. 1a) [11,12,15]. Glass surfaces were passivated with biotinylated bovine serum albumin (BSA) prior to coating with neutravidin [12,16,17]. TGTs of varying T_{tol} or cyclic-RGDfK peptide (ruptures at significantly higher tension forces, >100 pN [12,18]) were then immobilized to the passivated surface through neutravidin-biotin linker (Fig. 1a).

Freshly isolated TRCs from soft 3D fibrin gel were plated on surface presenting TGTs of nominal T_{tol} values of 12, 23, 33, 43, 50, 56 or >100 pN (23 and 33 pN not shown). TRCs did not attach to surfaces with T_{tol} < 40 pN suggesting that they require about 40 pN peak force through integrins during initial cell adhesion. Interestingly, TRCs exhibited round morphology and projected cell area did not increase with increasing T_{tol} on any surface supporting cell adhesion, indicating their inability to spread in response to increasing mechanical stimuli across single molecular bonds (43, 50, 56, >100 pN) (Fig. 1 b, c).

Parental B16-F1 control cells also required 40 pN for initial cell adhesion, but unlike TRCs, they spread well on TGT surfaces, and cell spreading increased with increasing T_{tol}, suggesting that single molecular forces are sensed to promote cell spreading. (Supplementary Fig. 1, [12]). These data show that there is a fundamental difference in cell spreading between parental B16-F1 control cells and 3D fibrin selected TRCs.

To further quantify the differences in cell morphology, we computed cell shape index (CSI), a dimensionless parameter for geometric circularity measurement. TRCs exhibit CSI values close to 1 across all TGT surfaces (Fig. 1d). In contrast, parental B16-F1 control cells exhibited progressively lower CSI values as T_{tol} increased, due to complex cell spreading patterns [12]. Suppression of cell spreading in TRCs is not specific to B16-F1 cells because a similar difference was also observed between more aggressive B16-F10 melanoma cell line and their TRCs (Supplementary Fig. 2). TRCs also failed to spread on surfaces coated with natural ligands, fibronectin or type-I collagen, showing that suppression of cell spreading is not due to the use of synthetic ligands (Supplementary Fig. 3).

3.2. Single-mRNA quantification shows altered expression of RhoA and Cdc42 in TRCs

Because Rho-family small GTPases Rac1 and Cdc42 are known to regulate cell spreading, integrin clustering, and focal adhesion (FA) formation [19], we examined mRNA levels of Rac1 and Cdc42 in TRCs using qPCR. Transcription levels of both Rac1 and Cdc42 were significantly lower in TRCs compared to control cells (Supplementary Fig. 4). To understand and correlate phenotypic changes like cell spreading and FA formation with changes in gene expression at the single cell level, we utilized smFISH to visualize and quantify individual transcripts in fixed cells [10]. We imaged Rho-family small GTPases RhoA, Rac1, and Cdc42 mRNA molecules simultaneously and quantified the mRNA transcripts from single cells (Fig. 2a). We observed positive correlations between *RhoA* and *Cdc42* (Fig. 2b, top panel) and between *RhoA* and *Rac1* (Fig. 2b, bottom panel) transcripts, with differences in absolute numbers of transcripts likely attributable to differences in cell volume. Since *RhoA* has an antagonistic relationship with *Rac1* and *Cdc42* [20], we quantified *RhoA* to *Cdc42* and *RhoA* to *Rac1* ratios in each cell (Fig. 2c). Average *RhoA* to *Rac1* ratios in the B16-F1 control cells and TRCs were similar, 2 and 3, respectively (Fig. 2b, bottom panel). However, the average ratio of *RhoA* to *Cdc42* in TRCs was ~2.7 fold higher than in control cells, potentially contributing to cell spreading suppression in TRCs. We also observed a large cell-cell variation of *RhoA* to *Cdc42* ratio in TRCs but not in control cells (Fig. 2c).

3.3. Many focal adhesions are formed by control melanoma cells but not TRCs

Since *Cdc42* is involved in integrin clustering and FA formation [19], we hypothesized that downregulation of *Cdc42* expression in TRCs may translate into fewer mature FAs. To test this, we utilized TIRF microscopy to monitor FAs in live cells expressing mCherry-vinculin. Control cells and TRCs were plated for 1 and 4 h on >100 pN passivated surfaces. In contrast to control cells, the number of mature FAs per individual TRC remained very low even after 4 h of cell plating, and failed to exhibit increases in area and polarization characteristic of mature FAs (Fig. 3a). To quantify mature FA characteristics, we compared differences in FA area and aspect