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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

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AUTHOR CONTRIBUTIONS

F.C., N.W., T.H. conceived the project and designed the experiments. F.C., S.D., B.L., R.S., K.A., B.T., S.P. performed experiments and/or analyzed data. R.S. developed image analysis suite for focal adhesion tracking. F.C., N.W., and T.H. wrote the manuscript with feedback from all authors.

SUPPLEMENTARY MATERIALS

Supplementary information includes experimental procedures, tables, figures, and references.

TRCs that may enable us to distinguish TRC population from a pool of heterogeneous tumor cell population.

Keywords

Cell adhesion and spreading; tension gauge tethers; tumor repopulating cells; focal adhesions; smFISH

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% CO₂. 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/µm² (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 ratio between control cells and TRCs (Figs. 3b,c and Supplementary Fig. 5). For control cells, the number of FAs per cell with an area $> 1.5 \mu\text{m}^2$ increased by ~two fold between 1 hour and 4 hour of cell plating (Fig. 3b inset). In contrast, the number of FAs per cell with an area $> 1.5 \mu\text{m}^2$ remained low and unchanged for TRCs (Fig. 3b inset). The average FA aspect ratio for control cells increased by 40% between 1 and 4 h of cell plating (Supplementary Fig. 5, left inset) but remained almost constant for TRCs (Supplementary Fig. 5, right inset).

3.4 Cdc42 restores cell spreading in TRCs

We next explored if cell spreading can be restored by transient overexpression of *Rac1* or *Cdc42* in TRCs. Transient overexpression of Cdc42-GFP restored cell spreading capacity in TRCs (Fig. 3d, left panel). More than 80% of cells, exhibiting GFP fluorescence, readily spread with typical filopodia-like extensions (Supplementary Fig. 6) and cells with low Cdc42-GFP fluorescence did not spread (Supplementary Fig. 7). In contrast, overexpression of Rac1-GFP did not restore normal cell spreading (Fig. 3d, right panel; Supplementary Figs. 6) and ~20% of cells spread partially with small lamellipodia-like protrusions (Supplementary Fig. 8). No cell spreading was observed in TRCs upon overexpression of RhoA-GFP or GFP-only (Supplementary Fig. 9).

We performed smFISH after transient transfection of *Cdc42* in TRCs to test if there exists a threshold of *Cdc42* to initiate TRC spreading (Fig. 3e, left). Spherical TRCs uniformly showed fewer than 25 transcripts per 100 μm^2 of projected cell area. This density indeed appears to be a threshold for cell spreading, as all transfected cells exceeded this density, and CSI decreased with increasing *Cdc42* density beyond this density (Fig. 3e, right). Conversely, knocking down *Cdc42* in parental B16-F1 control cells with 2 different siRNA constructs reversed their spread morphology, resulting in more than 80% Cdc42-knock down cells exhibiting round morphology similar to TRCs (Supplementary Fig. 10).

To determine the effect of Cdc42 on FA formation, we transiently transfected TRCs with a Cdc42-GFP plasmid and plated them on a >100 pN surface. After 4 h of cell plating, we fixed and immunostained TRCs with primary anti-vinculin antibody to visualize FAs. Remarkably, TRCs transfected with Cdc42-GFP plasmid, in addition to spreading, were able to form focal adhesions (Supplementary Fig. 11). FA area quantification shows the number of adhesions similar to control cells (Supplementary Fig. 11b, Fig. 3b). Conversely, when *Cdc42* was knocked down in control cells with siRNA #1, they exhibited FA formation trend similar to TRCs (Supplementary Fig. 11b and Fig. 3c). Therefore, Cdc42 also has a role in FA assembly in TRCs in addition to TRC spreading.

3.5 Overexpression of Cdc42 rescues sensitivity to molecular tension cues

Finally, we tested whether the overexpression of Cdc42 can restore TRCs' sensitivity to mechanical cues. After transiently transfecting freshly isolated TRCs with Cdc42-GFP plasmid, GFP positive cells were collected using fluorescence activated cell sorting and applied to molecular tension surfaces with T_{tol} values of 43 pN, 56 pN, and >100 pN (Fig. 4a). Cdc42- overexpressing TRCs increased cell spreading with increasing T_{tol} , similar to control cells (Fig. 4b, blue bars). In addition, we computed CSI of TRCs on different T_{tol} surfaces and found that CSI values decreased with increasing T_{tol} suggesting the increase in protrusions leading to a more irregular cell spreading pattern with higher tension surfaces, similar to control cells (Fig. 4b, green bars).

4. DISCUSSION

Our TGT platform allows us to control the cell's mechanical environment at the single bond force level. This not only increased our understanding of integrin signaling [11,15,21] but

also revealed force relevance of various mechanosensitive ligand-receptor interactions including notch [17,22], selectins [23,24], T-cell receptors [25], and B cell receptor activation [26]. Because TRCs have been shown to depend on $\alpha_v\beta_3$ integrins to adhere and grow in a soft-3D-fibrin-gel system [9], we engineered TGT constructs to offer the same ligands (cyclic-RGDfK peptide) specific to $\alpha_v\beta_3$ integrins [14] to measure molecular forces exerted during cell adhesion and capture their cell spreading response. It remains to be explored if there is any difference in TRC adhesion and spreading through non-RGD integrins (e.g., LVD-binding integrins, α_A domain integrins, and non- α_A domain integrins) [27].

Previous studies have shown that cell spreading and gene regulation are modulated by the bulk stiffness of the underlying substrate [28,29]. However, the mechanism connecting the substrate stiffness to cell spreading and consequently fate-determination remain unresolved. Because cell spreading is known to be strongly coupled with cellular traction force generation [21,30], we recently investigated if capping the molecular forces at a predetermined value at the single integrin level has any impact on cell spreading and found B16-F1 control cells, together with many other cell lines, progressively increase spreading with the increase of single-molecular tension cues [12]. This evidence strongly suggests that ultimately the tension tolerance of single bonds can dictate the differential spreading response. Therefore, at the most fundamental level, the rigidity sensing and resultant changes in cell spreading can occur by sensing the single-molecular forces across ligand-receptor bonds. In this work, we showed that highly tumorigenic TRCs, a subpopulation of B16-F1 control cells, exhibit an unusual behavior of suppressed spreading. When we plated TRCs on different molecular tension tolerance surfaces, they did not change their spreading characteristics. The inability to spread and sense the tension cues are due to the reduced expression of *Cdc42*. Once *Cdc42* expression was restored, TRCs were able to spread through FA formation and exhibited molecular tension-dependent spreading just like B16-F1 control cells. It remains to be tested if *Cdc42* upregulation has any negative effect on the tumorigenicity of TRCs in animal models.

As TRCs do not spread, one can imagine that TRCs may exert a high cortical tension to limit the extent of cell spreading. However, cell stiffening response measurement as a function of the bulk stiffness of polyacrylamide hydrogels [31] showed that B16-F1 TRCs are much softer than the parent cell line suggesting cortical tension is much lower [31]. We also showed that TRCs stiffen with increasing substrate rigidity and such stiffening response is mediated by *Cdc42* [31]. In integrin-mediated mechanotransduction, downstream focal adhesion kinase (FAK) is one of the molecules that controls *Cdc42* activity [32]. Recently, downregulation of FAK has been reported to regulate histone 3 lysine residue 9 (H3K9) demethylation via *Cdc42* [33], providing a potential link between *Cdc42* and TRC self-renewal. However, the role of *Cdc42* in translating outside physical cues into meaningful intracellular signals and its contribution to regulating cell spreading and self-renewal was not well understood. In this work, we showed that *Cdc42* can control focal adhesion maturation and cell spreading in response to molecular tension cues received through single integrins, and that both processes are severely perturbed in TRCs. Future studies will reveal whether this unique feature is shared by all TRCs irrespective of tumor origin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. TRCs do not spread on any TGT surfaces.

a, A schematic of a cell on a TGT surface. TGTs with varying T_{tol} ranging from 12 to 56 pN were immobilized on biotinylated BSA passivated glass surfaces via biotin-neutravidin interactions. Biotinylated cyclic-RGDfK peptide, immobilized directly on the surface, was represented as >100 pN. **b**, TRCs adhere to surfaces with T_{tol} 43 pN. Interestingly, TRCs do not spread on any TGT surfaces. **c**, Projected cell area of TRCs ($n=33, 33, 38, 35$ for 43 pN, 50 pN, 56 pN, and >100 pN respectively) are presented in a box-and-whisker plot showing no significant changes across any TGT surfaces (p values are >0.09, 0.07 and 0.99 for 43 pN and 50 pN, 50 pN and 56 pN, and 56 pN and >100 pN, respectively). **d**, A box-and-whisker plot shows a dimensionless parameter-CSI of cells on varying T_{tol} surfaces. No significant changes in CSI values were observed across any TGT surfaces (p values are >0.78, 0.47, 0.29 for 43 pN and 50 pN, 50 pN and 56 pN, and 56 pN and >100 pN, respectively).

Figure 2. Single-mRNA-transcript statistics revealed a dissimilarity in RhoA and Cdc42 expression in TRCs leading to suppression in cell spreading.

a, Representative images showing mRNA-transcript statistics of RhoA, Rac1, and Cdc42 in single control cells and TRCs. **b**, Correlation analysis between RhoA and Cdc42 transcripts (top) and RhoA and Rac1 transcripts (bottom) is shown here. RhoA and Cdc42 expression in control cells are tightly correlated while TRCs tend to exhibit a heterogeneous expression pattern. Each dot represents a single cell (ρ , Pearson correlation coefficient). **c**, RhoA: Cdc42 and RhoA: Rac1 in control cells and TRCs are significantly different ($p < 1.35 \times 10^{-58}$ and 5.86×10^{-13} for RhoA: Cdc42 and RhoA: Rac1 respectively).

Figure 3. TRCs' inability to spread corresponds to very few focal adhesions but can be restored by *Cdc42* overexpression.

a, Quantification of TIRF microscopy images show very dissimilar FA formation of control cells and TRCs expressing mCherry-vinculin. Color bar represents FA intensity relative to maximum intensity. **b-c**, Histogram plot of FA area in control cells and TRCs is shown here after 1 hour ($n=10$ for both control cells and TRCs) and 4 hour ($n=10$ and 15 for control cells and TRCs respectively) of cell plating. The number of FAs with area $>1.5 \mu\text{m}^2$ per cell in control cells was significantly different between 1 hour and 4 hour ($p<0.02$), but not in TRCs ($p>0.44$) (insets). **d**, Representative images of TRCs with overexpression of Rac1 and Cdc42 show that Cdc42 alone, but not Rac1, can facilitate cell spreading in TRCs with filopodia-like extensions (inset). A TRC not transfected with Cdc42 did not spread

(arrowhead, DIC image; cell boundary, fluorescence image). e, Single mRNA-transcripts were quantified after transient overexpression of Cdc42 in TRCs. A representative image of Cdc42-overexpressed-TRC is shown (left). A plot of CSI vs. Cdc42 density is shown in blue representing overexpressed Cdc42 (n=46, Pearson's correlation coefficient $\rho = -0.62$) and red representing endogenous Cdc42 transcripts in TRCs (n=168, $\rho = 0.07$). A clear threshold of ~ 25 Cdc42 transcripts per projected cell area is observed for TRC spreading initiation.

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Figure 4. Overexpression of Cdc42 in TRCs causes increasing cell spreading capability with increasing T_{tot} .

a, TRCs transfected with Cdc42-GFP can spread more with increasing T_{tot} . **b**, Summarized data of Cdc42 overexpressed TRC spreading on 43 pN, 56 pN, and >100 pN surfaces ($n=22, 29, 22$ for 43 pN, 56 pN, and >100 pN surfaces respectively). Significant differences in projected cell area were observed between 43pN and 56 pN, and 56 pN and >100 pN ($p < 2.89 \times 10^{-8}$ and 5.43×10^{-6} respectively). Similarly, significant differences in CSI between 43pN and 56 pN, and 56 pN and >100 pN were observed ($p < 0.04$ and 9.33×10^{-4} respectively).