Vinculin transmits high-level integrin tensions which are dispensable for focal adhesion formation

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Running title: Vinculin elevates integrin tensions

Abstract

Focal adhesions (FAs) transmit force and mediate mechanotransduction between cells and the matrix. Previous studies revealed that integrin-transmitted force is critical to regulate FA formation. As vinculin is a prominent FA protein implicated in integrin tension transmission, this work studies the relation among integrin tensions (force), vinculin (protein) and FA formation (structure) by integrin tension manipulation, force visualization and vinculin knockout (KO). Two DNA-based integrin tension tools are adopted: tension gauge tether (TGT) and integrative tension sensor (ITS), with TGT restricting integrin tensions under a designed T_{tol} (tension tolerance) value and ITS visualizing integrin tensions above the T_{tol} value by fluorescence. Results show that large FAs (area >1 μm^2) were formed on the TGT surface with $T_{\text{tol}} = 54$ pN but not on those with lower T_{tol} values. Time-series analysis of FA formation shows that focal complexes (area <0.5 μm^2) appeared on all TGT surfaces 20 min after cell plating, but only matured to large FAs on TGT with $T_{\text{tol}} = 54$ pN. Next, we tested FA formation in vinculin knockout (KO) cells on TGT surfaces. Surprisingly, T_{tol} value of TGT required for large FA formation is drastically decreased to 23 pN. To explore the cause, we visualized integrin tensions in both wildtype and vinculin KO cells using ITS. The results showed that integrin tensions in FAs of wildtype cells frequently activate ITS with $T_{\text{tol}} = 54$ pN. With vinculin KO, however, integrin tensions in FAs became lower and unable to activate 54 pN ITS. Force signal intensities of integrin tensions reported by 33 pN ITS and 43 pN ITS were also significantly reduced with vinculin KO, suggesting that vinculin is essential to transmit high-level integrin tensions and involved in transmitting intermediate-level integrin tensions in FAs. However, the high-level integrin tensions transmitted by vinculin are not required by FA formation.

Keywords: vinculin, focal complex, focal adhesion, integrin tension, tension gauge tether

SIGNIFICANCE

Cells form focal adhesions (FAs) to stabilize cell adhesion and mediate mechano-sensing which are crucial for many cell functions including cell survival. Integrins are the core proteins in FAs transmitting tensions which reciprocally facilitate FA formation and maturation. Vinculin, another FA protein, is known to participate in integrin tension generation. However, the relation among integrin tensions, vinculin and FA formation has not been quantitatively investigated. This work adopts molecular tension tools to control and visualize integrin tensions in wildtype cells and vinculin knockout cells. We discovered that vinculin is essential in producing high-level integrin tensions (shearing 18-bp dsDNA) in FAs. However, vinculin-transmitted integrin tensions are not required for FA formation.

Introduction

Integrin family are the major transmembrane proteins mediating cell adhesion (1). Integrins link cell interior cytoskeleton to the extracellular matrix (ECM) (2) and transmit tensions in and out of the cells. Integrin tensions not only play a physical role that directly regulates cell adhesion (3) and promote cell migration (4), but also activate multiple biochemical signaling cascades (5,6) and regulate a myriad of cell physiological functions such as proliferation (7), differentiation (8), cancer cell metastasis (9), and so on. During integrin-mediated cell adhesion, one interesting feature is that integrins tend to cluster into micron-sized plaques, named focal adhesions (FA), at the cell-matrix interfaces. FAs are known to stabilize cell adhesion (10) and promote cell mechanotransduction (11). Because the prominent role of FAs in cellular functions, the formation and function of FAs have been extensively studied from structural, biochemical and biomechanical perspectives. One converging conclusion is that while FAs are central for transmitting and transducing cell adhesive force, FA formation is reciprocally signaled and regulated by the integrin-transmitted adhesive force (here termed integrin tensions). It has been shown that integrin tensions are critical for FA formation and maturation (12). Consequently, cell traction force reduction by inhibiting myosin II suppresses the maturation of focal complexes to FAs (13), and stretching force externally applied on cells enhances FA formation and maturation (14). Clearly, force and structure are two inter-dependent aspects of FAs which interplay with each other.

As supramolecular assemblies, in addition to integrins, FAs recruit hundreds of different types of proteins including scaffolding molecules (adhesion receptors, adaptors and cytoskeletal proteins) and signaling molecules (kinases, proteases, etc.) (15,16). Some well-studied proteins include talin which links integrins to the actin cytoskeleton and assists in force transmission (17,18), focal adhesion kinase (FAK) which is one of the major integrin signaling mediators (19), and zyxin which marks matured FA sites (20). In particular, vinculin stands out as an important protein consistently recruited to FAs (21) and regulating force transmission between integrins and cytoskeleton (22,23). In the past, cell traction force microscopy (TFM) (24,25) has revealed tremendous insights to force-structure interplay in FAs. It has been shown that vinculin is important for the efficient generation of cell traction forces, as the cell bulk forces were significantly reduced with vinculin impaired or knocked out in cells (26,27). However, few studies have manipulated integrin molecular tension level and vinculin expression in parallel during the study of FA formation. The relation among integrin tensions, vinculin and FA formation awaits further examination.

In recent years, DNA-based (28-32) or peptide-based (33) integrin tension tools have provided powerful new avenues for the study of cell mechanobiology. Among these molecular tension tools, tension gauge tether (TGT) has a unique application in terms of global integrin tension knock-down. TGT is a dsDNA-based linker that self-destructs (with two strands separated apart) under a force higher than its tension tolerance (T_{tol}, tunable in the range of 12-54 pN) (28). With TGT, it has been demonstrated that the T_{tol} value required for FAs to assemble is 54 pN in wildtype cells, suggesting that FA formation requires integrin ligand strength that can sustain ~54 pN force. However, one potential bias in the research (28) is that integrin tension knockdown by TGT also affects cell adhesion status which may indirectly impact FA formation and skew the measurement of integrin tensions during FA formation. To eliminate this bias, a platform is needed to ensure cell stable adhesion while retaining the ability of integrin tension control.

In this study, we developed a platform to specifically manipulate integrin tensions without disrupting cell adhesion. On this platform, we tested FA formation in both wildtype and vinculin knockout HeLa cells. The result confirms that the formation of large FAs (area>1 μm^2) in wild type HeLa cells indeed requires TGT with T_{tol} = 54 pN. More interestingly, we found that T_{tol} value required for large FA formation is drastically reduced to 23 pN with vinculin knocked out. We explored the underlying biomechanical mechanism for the reduction of integrin tension requirement for FA formation. A fluorescent tension sensor ITS (integrative tension sensor) has been applied to visualize integrin tensions in both wildtype and vinculin KO cells. The results show that vinculin KO significantly reduces integrin tension level in cells, consequently lowering the tension level required for FA formation. Overall, this study revealed that vinculin is dispensable for FA formation and elevates integrin tension level required for FA formation.

Methods and Materials

TGT and ITS Synthesis

DNA materials:

(5' → 3')	**TGT**	**ITS**
Upper strand	/5ThioMC6-D/ CCC ACC AGC GAC GCC CGG	/5ThioMC6-D/ CCC ACC AGC GAC GCC CGG /3BHQ_2/
Lower strand (T_{tol} = 12 pN)	/5Biosg/CCG GGC GTC GCT GGT GGG	/5Biosg/T/iCy3/ CCG GGC GTC GCT GGT GGG
Lower strand (T_{tol} = 23 pN)	CCG GGC G/iAmMC6T/C GCT GGT GGG	/5Cy3/CCG GGC G/iAmMC6T/C GCT GGT GGG
Lower strand (T_{tol} = 33 pN)	CCG GGC GTC GC/iAmMC6T/ GGT GGG	/5Cy3/CCG GGC GTC GC/iAmMC6T/ GGT GGG
Lower strand (T_{tol} = 43 pN)	CCG GGC GTC GCT GG/iAmMC6T/ GGG	/5Cy3/CCG GGC GTC GCT GG/iAmMC6T/ GGG
Lower strand (T_{tol} = 54 pN)	CCG GGC GTC GCT GGT GGG/3BioTEG/	/5Cy3/CCG GGC GTC GCT GGT GGG/3BioTEG/

Table. 1. Single-stranded DNAs ordered for TGT and ITS synthesis.

Above DNA sequences use the exact format for ordering DNA products in Integrated DNA Technologies, Inc. /5ThioMC6-D/ is a thiol modification at the 5' end of DNA. /3BHQ_2/ is the Black Hole Quencher 2. /5BiosG/ is a biotin tag. /iAmMC6T/ refers to an internal thymine (dT) modified with an amine with C6 as a spacer. The amine is used for biotin labeling by reacting with NHS-PEG12-Biotin (A35389, ThermoFisher Scientific). Although Integrated DNA Technologies already offers /iBiodT/ modification that modifies an internal thymine with a biotin tag, /iBiodT/ has been proven to be too short to efficiently immobilize TGT and ITS on the surface. In the application of TGT or ITS, the linker between the biotin and DNA construct needs to be sufficiently long to avoid steric hindrance during DNA surface immobilization. Here, we resorted to using NHS-PEG12-Biotin which is 5.6 nm long, similar to TGT/ITS's length and sufficiently long for the immobilization of TGT and ITS.

The DNA sequences are slightly different from the ones used in previous publications of TGT, as they have been optimized to reduce DNA secondary structures and increase DNA solubility and thermal stability.

Conjugating RGD to upper-strand DNA: TGT and ITS are conjugated with integrin peptide ligand RGD (Arginine-Glycine-Aspartate). RGD is conjugated to the upper DNA strands shown in table 1, producing the upper DNA strands shown in table 2. In the past, we have optimized the conjugation procedures substantially to increase the yield and obviate the need of purification processes such as gel electrophoresis or HPLC, which is labor demanding and causes material loss. The up-to-date procedure is detailed as follows:

A mixture solution of 500 mM TCEP (Tris (2-Carboxyethyl) phosphine) (77720, bond-breaker, ThermoFisher Scientific) and 500 mM EDTA (AM9260G, Invitrogen) in PBS (phosphate buffered saline) is prepared. This solution is to break the disulfide bond in /5ThioMC6-D/ on the DNA strand to a thiol group and makes it accessible for thiol-maleimide reaction. 10 μL of the above solution is added to 100 μL 2 mM thiol-DNA-BHQ2 in PBS. The solution is incubated for 30 min at room temperature (Solution 1).

In parallel, 10 mg RGD-NH_2 (PCI-3696-PI, Peptides International) is dissolved in 200 μL ultrapure water. The RGD-NH_2 solution is directly added to 2 mg solid sulfo-SMCC (A39268, Thermo Scientific) in a vial dipped in an ultra-sonicator to quickly dissolve the solid sulfo-SMCC. 22 μL 10× PBS is added to the solution afterwards, making the solution in final 1× PBS. It is advised to avoid directly using 1× PBS to dissolve RGD-NH_2 and sulfo-SMCC, as sulfo-SMCC dissolves poorly in 1× PBS. This mixture is incubated for 10 min at room temperature (Solution 2).

Solution 1 and 2 are then mixed and incubated for 1 h at room temperature and overnight at 4 ºC. RGD is now conjugated to the DNA through sulfo-SMCC which is a heterolinker with a maleimide (reacting with the thiol of the DNA) at one end and an NHS ester (reacting with the amine of RGD-NH_2) at the other end. The unreacted SMCC, RGD and TCEP are removed via ethanol precipitation (The protocol is common to find online). In our tests, this synthesis protocol consistently had high yield with >90% DNA conjugated with RGD. This purity is often sufficient for cell experiment, as the unconjugated DNA is passive for integrin tension control or force imaging. The RGD-DNA is re-dissolved in PBS. 100 μM is the typical stocking concentration.

Conjugating PEG12-biotin to lower-strand DNAs (23, 33 and 43 pN strands):

For TGT or ITS constructs with intermediate T_{tol} values, the biotin is conjugated to a thymine modified with an amine in the middle of a lower-strand. The modified thymine is indicated by/iAmMC6T/ shown in Table 1. NHS-PEG12-biotin is conjugated to the thymine through amine-NHS ester reaction. PEG12 is a spacer with 12 repeats of poly-ethylene glycol (PEG) that provides a long handle for biotin tag and also increases the solubility of the conjugate product.

The conjugation procedure is straightforward. Here the 23 pN lower-strand DNA (CCG GGC G/iAmMC6T/C GCT GGT GGG) is used as an example. 100 μM×200 μL DNA in PBS is prepared. The DNA solution is added to one vial of NHS-PEG12-Biotin (A35389, no-Weight format, ThermoFisher Scientific) which contains 1 mg NHS-PEG12-Biotin in the dry state (wax-like material). Molar concentration of NHS-PEG12-Biotin (molecular weight: 826 Da) is 6 mM in the mixture. Let the mixture react for 2 h at the room temperature. Ethanol precipitation is conducted to purify the DNA conjugated with PEG12-Biotin. We use /iBioPEG12T/ to indicate this modification. The DNA-PEG12-Biotin is re-dissolved in PBS. 100 μM is the typical stocking concentration.

TGT and ITS:

Both TGT and ITS are assembled by hybridizing the corresponding upper strand and lower strand (Table 2). Solutions of lower strand and upper strand were mixed at a molar ratio of 1:1.2 at a desired concentration, typically at a concentration of 10-50 μM, and annealed by heating the mixture to 90ºC and cooling it back to room temperature. These duplexes after hybridization can be stored in a -20ºC freezer for more than a year without noticeable quality degradation. 10 μM is the typical stocking concentration.

(5' → 3')	**TGT**	**ITS**
Upper strand	/RGD/ CCC ACC AGC GAC GCC CGG	/RGD/ CCC ACC AGC GAC GCC CGG /3BHQ_2/
Lower strand ($T_{\mathrm{tol}} = 12$ pN)	/5Biosg/CCG GGC GTC GCT GGT GGG	/5Biosg/T/iCy3/ CCG GGC GTC GCT GGT GGG
Lower strand ($T_{\mathrm{tol}} = 23$ pN)	CCG GGC G/iBioPEG12T/C GCT GGT GGG	/5Cy3/CCG GGC G/iBioPEG12T/C GCT GGT GGG
Lower strand ($T_{\mathrm{tol}} = 33$ pN)	CCG GGC GTC GC/iBioPEG12T/ GGT GGG	/5Cy3/CCG GGC GTC G/iBioPEG12T/C GGT GGG
Lower strand ($T_{\mathrm{tol}} = 43$ pN)	CCG GGC GTC GCT GG/iBioPEG12T/ GGG	/5Cy3/CCG GGC GTC GCT G/iBioPEG12T/C GGG
Lower strand ($T_{\mathrm{tol}} = 54$ pN)	CCG GGC GTC GCT GGT GGG/3BioTEG/	/5Cy3/CCG GGC GTC GCT GGT GGG/3BioTEG/

Table 2. Single-stranded DNAs ready for TGT and ITS assembly.

Preparation of PLL-PEG-TGT Surfaces

All TGTs were immobilized on surfaces through neutravidin-biotin bonds. A glass bottom petri dish (D35-14-1.5-N, In Vitro Scientific, Mountain View, CA) was incubated with 1 mg/ml PLL-PEG-Biotin in ultra-pure H_2O for 30 min at 4°C, and then the surface was washed by ultra-pure H_2O three times. PLL supports cell adhesion by electrostatic adsorption and PEG suppresses nonspecific integrin-substrate interaction. PLL-PEG-Biotin provides biotin tags for neutravidin coating. The surface was then incubated with 200 μg/ml neutravidin in PBS for 30 min at 4°C, and then washed with PBS and incubated with 0.1 μM biotin-TGT in PBS for 30 min at 4°C. Finally, the surface was washed by PBS three times without drying. Cell solution was loaded onto the TGT surface immediately after the washing procedure.

Preparation of ITS Surfaces

ITS was also immobilized on a glass surface through neutravidin-biotin bonds. During ITS application, the surface is co-coated with fibronectin to provide unrupturable integrin ligands for cell normal adhesion. A glass bottom petri dish was incubated with a mixture solution with 200 µg/ml BSA-biotin (biotin-conjugated Bovine serum albumin, A8549, Sigma-Aldrich, USA) and 5 µg/ml fibronectin (1918-FN, R&D System) in PBS for 30 min at 4 °C. Both BSA and fibronectin are physically adsorbed on the glass surface. The surface was then incubated with 200 µg/ml neutravidin for 30 min at 4°C, and then washed with PBS and incubated with 0.1 µM biotin-ITS for 30 min at 4°C. Finally, the surface was washed by PBS three times without drying. Cell solution was loaded onto the ITS surface immediately after the washing procedure.

Cell culture and transfection

Wildtype and vinculin KO HeLa cells (Abcam, ab265580) were cultured in Dulbecco's modified eagle's medium (DMEM, ATCC 30-2002) supplemented with 10% fetal bovine serum (FBS, ATCC 30-2020), 100 U/mL of penicillin and 100 µg/mL of streptomycin (Gibco 15140122). Cells were passaged every three days. HeLa cells were transfected with mEmerald-Integrin-Beta 3 (Addgene plasmid #54130) or GFP-Talin1 (Addgene plasmid #26724). Plasmid transfection was performed in HeLa cells with Lipofectamine LTX (15338-030, Invitogen) in opti-MEM media (11058021, Gibco) according to manufacturer's instructions. Briefly, 2.5 µg plasmid was mixed with 5 µL Lipofectamine LTX in 500 µL opti-MEM culture media and incubated at room temperature for 15 min. The mixture was then added to HeLa cells at 70% confluency in a 2-inch petridish with 2 mL culture medium. The transfection rate was checked after 24 h.

Preparation of cell solution

Wildtype HeLa cells and vinculin KO HeLa cells were detached by mild detaching solution [100 mL 10 × HBSS + 10 mL 1 M HEPES (PH7.6) + 10 mL 7.5% sodium bicarbonate + 2.4 mL 500 mM EDTA (Ethylenediaminetetraacetic acid) + 878 mL H_2O] for 7 minutes, the harvested cells were centrifuged at 300 g for 3 min and resuspended in DMEM medium with 1% penicillin-streptomycin (catalog No. 15140122, Gibco) at a seeding concentration of 10^6 cells/ml. After plating cells on PLL-PEG-TGT surfaces, they were incubated in a cell culture incubator for 90 min or other appropriate times based on experimental design.

FA imaging by vinculin or zyxin immunostaining

Wildtype HeLa cells and vinculin KO HeLa cells were plated on TGT surfaces. The cells were incubated for 90 min or other stated times at 37 °C. For immunostaining, cells were fixed with a 4% paraformaldehyde (catalog No. 15710, Electron Microscopy Sciences) solution for 15 min at room temperature. Cell samples were rinsed thrice with PBS. Next, the cells were permeabilized with 0.5% Triton X detergent at room temperature for 10 min and washed thrice with PBS. After cell fixation and permeabilization, 5% BSA was added to the cell samples and the samples were incubated at room temperature for 1 h (or overnight at 4 °C). The cells were rinsed thrice using PBS. This step was followed by the preparation of two antibody solutions. For vinculin immunostaining, dilutions of 2.5 µg/ml of primary antibody (FAK100, Millipore, mouse anti-vinculin) and 2.5 µg/ml of secondary antibody (A31553, Invitrogen, goat anti-mouse Alexa 405) were prepared using PBS. For zyxin immunostaining, dilutions of 2.5 µg/ml of primary antibody (NBP1-90349, Novus Biologicals, rabbit anti-zyxin) and 2.5 µg/ml of secondary antibody (A11008, Invitrogren, goat anti-rabbit Alexa 488) or (ab175652, Abcam, goat anti-rabbit Alexa 405) were prepared using PBS. First, the cells were incubated at room temperature in a primary antibody solution for 1-2 h (or overnight at 4 °C). This step was followed by rinsing of the cell samples thrice using PBS and 5 min incubation time was maintained throughout three wash cycles. The cell samples were incubated in the secondary antibody solution (previously prepared) at room temperature for 1-2 h (or overnight at 4 °C). Cell samples were rinsed thrice using PBS, and imaging was performed immediately. Cells were imaged by a total internal reflection fluorescence (TIRF) microscope.

FA imaging by integrin β_3, talin transfection

For transfection, corresponding cells were cultured in a 35-mm Petri dish up to 70–80% of confluency. Plasmid DNA (2–3 µg) was diluted in 500 µl Opti-MEM (11058021; Thermo Fisher Scientific) spiked with 2–3 µl Plus reagent (15338100; Thermo Fisher Scientific). The plasmid solution was mixed and incubated for 10 min at room temperature. After that, 5 µl Lipofectamine-LTX (15338100; Thermo Fisher Scientific) was added to the plasmid solution, which was then mixed and incubated at room temperature for 30 min. The medium in a Petri-dish of cells was exchanged with 2 ml fresh complete medium added with the plasmid mixture. Cells were incubated in an incubator for 18–24 h. Before experiments, cells were detached with EDTA solution and plated on the imaging platform.

Microscopy and image processing

All static and time-lapse imaging was performed using TIRF microscopy setup (Nikon Ti-2) with a 100× oil immersion objective (TIRF 100×, NA = 1.49). An Andor camera DU-897 X-11940 (model IXON-L-897) was used as the imaging device. FA sizes were analyzed by a python code developed in our laboratory. Imaging of fixed samples was done at room temperature and live-cell imaging was done at 37°C with a lens heater. The wavelengths of laser as the excitation light were selected as 405, 488, 561, and 640 nm for corresponding fluorophores. The image acquisition software provided with microscope (NIS-Element AR 5.11.00) was used to acquire all images.

FA size analysis coding

Focal adhesion size analysis was performed in python. The code utilizes the OpenCV package, which is an open source computer vision and machine learning software library. The code automatically outlines and calculates the area of a focal adhesion, based on an intensity threshold defined by user input. The code is freely distributed at https://github.com/jaustin22/Focal_Adhesion_Analysis.git.

Results and Discussions

Developing a platform enabling integrin tension control without disrupting cell adhesion

TGT is a molecular tension modulator globally and quantitatively knocking down integrin tensions in cells (28). Immobilized on a surface at a density of ~1000/µm^2, TGT binds to integrins and sustains the tensions transmitted by integrins upon cell adhesion. TGT ruptures with a high probability if the integrin tension is higher than the designed tension tolerance (T_{tol}), hence abolishing integrin tensions above the T_{tol} value. TGT has been applied to test the integrin tension level required for cell adhesion and FA formation in a previous study (28). The result shows that cells adhered only on surfaces with $T_{\mathrm{tol}} \geq 43$ pN and FAs were formed only on the surface with $T_{\mathrm{tol}} \geq 54$ pN. Because both cell adhesion and FA formation are impacted by TGT, and cell adhesion is one prerequisite condition for FA formation, this experiment design may cause a potential bias in testing integrin tension required for FA formation, as TGT may indirectly affect FA formation by altering cell adhesion status.

To study FA formation by integrin tension control without disrupting cell adhesion, we developed a platform that supports cell adhesion by an integrin-independent approach. We functionalized glass surfaces with a composite polymer consisting of poly-L-lysine (PLL) as the backbone and poly-ethylene glycol (PEG) as the branches (Fig. 1A and 1B). The PLL backbone is a long peptide consisting of ~100 lysines, with 20% of lysines conjugated with biotinylated PEG polymers. Physically adsorbed on a glass surface, the PLL supports cell adhesion by electrostatic adsorption (34) and remains passive to integrin signaling and FA formation (35), while the PEG branches form a non-fouling coating that minimizes non-specific integrin-substrate interaction (36). On top of the PEG coating, TGT is immobilized by biotin-neutravidin-biotin interaction (Fig. 1A). T_{tol} values are selectable in the range of 12-54 pN (Fig. 1C). On this platform, cells adhere and spread normally regardless of T_{tol} values of TGT, or even without TGT, thus decoupling cell adhesion from testing integrin tension-dependent processes including FA formation.

FA formation in wildtype HeLa cells on PLL-PEG-TGT surfaces

On PLL-PEG-TGT surfaces, we tested integrin tensions required for FA formation. HeLa cells were adopted as the cell model and plated on a series of PLL-PEG-TGT surfaces with various T_{tol} values in the range of 12-54 pN, including a PLL-PEG surface without TGT as the control. All surfaces supported cell adhesion regardless of the T_{tol} values as shown in Fig. 1D. Cells spread out normally and the spreading area is comparable to each other among all surfaces (Fig. 1E), further confirming the cell adhesion and spreading on PLL-PEG-TGT surfaces.

We co-imaged talin and vinculin as the protein markers to visualize FAs. In Fig. 1D, HeLa cells have been cultured for 90 min on the TGT surfaces, and the clustering of talin and vinculin was examined. We found that talin and vinculin formed clusters on all TGT surfaces. Talin clusters are consistently co-localized with vinculin clusters. The cluster sizes are positively correlated with T_{tol} values. On 12-33 pN surfaces, the cluster size is typically smaller than 0.5 μm^2. These small clusters are likely focal complexes which are abundant under cell bodies but have a low segregation level (the clusters are not well separated from each other and continuously distributed on the surface). Signal intensity of these clusters is comparable to the fluorescence background under cell bodies, suggesting that the majority of talin and vinculin are not clustered. On the 43 pN TGT surface, talin and vinculin clusters appear in an intermediate state exhibiting a transition from focal complexes to FAs. Only on the 54 pN TGT surface, cells formed distinct FAs which have fluorescence intensity standing out from the background. We quantified the cluster sizes using python-based programming code (see methods section). Shown in Fig. 1F, the code automatically outlines and calculates the cluster sizes. Cluster sizes of both talin and vinculin increase along the T_{tol} values as shown in Fig. 1G. The largest size increase occurs at the transition from T_{tol}=43 pN to T_{tol}=54 pN. This sharp cluster size transition, paired with the spatial segregation level of clusters (qualitatively observable in Fig. 1D), demonstrates that the T_{tol} of 54 pN is indeed needed for the formation of large FAs. The average cluster size is 1.12 ± 0.24 μm^2 on the 54 pN TGT surface. Areas greater than 1 μm^2 have been previously adopted as the cluster size threshold in identifying FAs from focal complexes (37).

Temporal dynamics of FA formation on PLL-PEG-TGT surfaces

To test temporal dynamics of FA formation with integrin tension control, we compared cells plated on two TGT surfaces (T_{tol} = 12 pN and 54 pN, respectively) and fixed at four different time points, shown in Fig. 2A. To mark the FA formation with high fidelity, we adopted integrin β_3 and zyxin as FA markers, with integrin recruited in the early stage of FA formation and zyxin supposedly recruited at the late stage (38). HeLa cells were transfected with integrin β_3, then fixed and immunostained with zyxin to visualize FAs. During the initial adhesion stage (10 min) of cell plating on surfaces, small clusters of both integrin and zyxin are visible on both TGT surfaces. The clusters grew larger with a size of 0.5 μm^2 on both TGT surfaces with 20 min incubation. After 20 min, the fates of these clusters diverged, with clusters shrinking on the 12 pN TGT surface and clusters continuing growth on the 54 pN TGT surface. After 1 h incubation, the clusters on the 54 pN surface matured into stable FA, which is marked by the large cluster area and clear cluster segregation. The cluster size analysis for integrin β_3 and zyxin is displayed in Fig. 2B and 2C. This experiment revealed that TGT surface with T_{tol} = 12 pN can support focal complexes formation during cell initial adhesion stage. However, these focal complexes shrunk and were unable to mature to FAs in longer incubation time. In contrast, the TGT surface with T_{tol} = 54 pN supports the maturation from nascent adhesions to matured adhesions.

FA formation in Vinculin KO cells on PLL-PEG-TGT surfaces

As integrin tensions are required for FA formation, vinculin has been shown to be responsible for transmitting myosin-powered traction force to integrins (39). A recent study also demonstrated that vinculin is required for the subpopulation of integrins tensions >7 pN in mouse embryonic fibroblasts (40). However, it remains unknown whether vinculin-transmitted integrin tension plays a major role in FA formation. Here we ask the question whether vinculin-transmitted tension is related to the integrin tension required for FA formation. To answer this question, we plated vinculin KO HeLa cells and wildtype HeLa cells on PLL-PEG-TGT surfaces for 90 min. FA formation in these two cell lines was analyzed on TGT surfaces (Fig. 3A) The Vinculin KO cells were purchased from Abcam which validated the vinculin-null status in cells. Both cells were transfected with talin-GFP for the examination of FA formation. Vinculin KO is also confirmed by vinculin immunostaining as shown in Fig. 3B.

With vinculin knocked out, remarkably, HeLa cells formed distinct FAs on most of TGT surfaces as long as $T_{\mathrm{tol}} \geq 23$ pN (Fig. 3C). As the control, wildtype HeLa cells still requires TGT with $T_{\mathrm{tol}} = 54$ pN for FA formation, consistent with Fig. 1. This result shows that vinculin is dispensable for FA formation, being consistent with previous report (41). Moreover, our result reveals that vinculin adversely affects FA formation and abolishes FA formation on the TGT surfaces with T_{tol} values lower than 43 pN. Considering that vinculin is involved in integrin tension transmission, we speculate that integrin tensions transmitted by vinculin could be the culprit hampering FA formation on ligand weak surfaces with low T_{tol} values.

Vinculin is required for transmitting high-level integrin tensions

To find out the biomechanical mechanism why vinculin KO cells have reduced integrin tension requirement for FA formation, we visualized and calibrated the integrin tensions in vinculin KO cells using integrative tension sensor (ITS, Fig. 4A) (31,42). While ITS and TGT share similar DNA constructs and both have designed T_{tol} values, their applications are distinctly different. While TGT is used to modulate integrin tensions by globally restricting integrin tensions below the T_{tol} value, ITS is used to visualize integrin tensions above the T_{tol} value by fluorescence. On a TGT surface, integrins can only bind to the RGD ligand present on the TGT constructs to achieve the global integrin tension control in cells. However, on an ITS surface, abundant fibronectin is always provided as stable integrin ligand for integrin binding which ensures normal cellular functions. Only a portion of integrins binds to ITS constructs and rupture them to activate ITS to fluoresce. This design is to minimize the influence of ITS rupture to normal cellular functions so that ITS functions only as a passive reporter of local integrin tension events.

First, we compared actin structure associated with FAs in vinculin KO cells and wildtype cells on fibronectin-coated surfaces. Fig. 4B shows that stress fibers were formed normally in wildtype HeLa cells, but prohibited in vinculin KO cells. F-actin was still recruited to FAs in vinculin KO cells but the F-actin filaments (stress fibers) connecting FAs disappeared. This suggests that vinculin is required for the normal assembly of stress fibers which are known as a force source of integrin tensions (43). Next, we compared integrin tensions in vinculin KO cells and wildtype cells on the ITS surface with $T_{\mathrm{tol}} = 54$ pN. As shown in Fig. 4C, wildtype HeLa cells produced clear fluorescent signal, indicating that a subpopulation of integrin tensions are above 54 pN. The force signal is co-localized with talin clusters marking the FAs. In contrast, vinculin KO cells had nearly zero force signal on the 54 pN ITS surface, suggesting that the generation or transmission of high-level integrin tensions (the tensions capable of shearing 18-bp dsDNA) requires the presence of vinculin in the cells. Relative intensity analysis was performed to compare force signals between the two cell types, shown in Fig. 4D. Clearly, vinculin is required to transmit high-level integrin tensions in cells.

Vinculin KO significantly reduces the population size of intermediate-level integrin tensions

Since we have demonstrated that vinculin KO HeLa cells were unable to produce integrin tensions >54 pN, we sought to determine how vinculin KO affects integrin tensions in other force ranges. We tested wildtype and vinculin KO cells on five ITS surfaces with T_{tol} values in the range of 12-54 pN. Representative force images are shown in Fig. 5A and Fig. 5B. Force signal intensity analysis was carried out on the five ITS surfaces for both cell types, shown in Fig. 5C (vinculin KO cells) and Fig. 5D (wildtype cells). Force signal intensities are similar between the two cell types on the 12 pN surface and the 23 pN surface. However, the signal intensity of integrin tensions in vinculin KO cells on the 33 pN surface and on the 43 pN surface are reduced by 22% and 68% (defined by $100\% \times (I_{WT} - I_{KO})/I_{WT}$), respectively, in comparison to wildtype cells. The result clearly shows that vinculin is not only required for the transmission of high-level integrin tensions, but also involved in transmitting the intermediate-level integrin tensions (the tensions capable of activating ITS with $T_{\mathrm{tol}} = 33$ pN or 43 pN). Clearly, vinculin substantially elevates the force level of integrin tensions in cells, resulting in higher requirement for integrin ligand strength or integrin-ligand bond strength during FA formation.

Conclusions

This paper investigated the role of vinculin in integrin tension transmission and FA formation. To study integrin tension level required for FA formation, we developed a platform that provides global integrin tension restriction without disrupting cell adhesion. The new platform provides an unbiased measure and reported that the integrin ligand strength (the TGT) required for FA formation in wildtype HeLa cells is comparable to the shearing force of an 18-bp dsDNA (TGT with $T_{\mathrm{tol}} = 54$ pN). Next, this work revealed that vinculin KO cells form FAs on all 23-54 pN TGT surfaces, suggesting that the integrin ligand strength required for FA formation in vinculin KO cells is drastically reduced. Our work revealed that vinculin is dispensable for FA formation, in consistency with previous reports (41), and also demonstrated that integrin tensions required for FA formation are likely in the range of 10-20 pN, as FAs were formed normally on the TGT surface with $T_{\mathrm{tol}} = 23$ pN (but not on the TGT surface with $T_{\mathrm{tol}} = 12$ pN) with vinculin knocked out.

To investigate why vinculin adversely impacts FA formation by raising the required integrin ligand strength, we adopted ITS to visualize integrin tensions and calibrate the profile of tension distribution in vinculin KO cells. ITS revealed that integrin tensions above 54 pN is completely abolished in vinculin KO cells. The signal intensities of integrin tensions reported by 43 pN ITS and 33 pN ITS are also reduced by 68% and 22%, respectively, in comparison to wildtype cells. The result clearly shows that vinculin is essential for the transmission of high-level integrin tensions (reported by 54 pN ITS), and important for the efficient transmission of intermediate-level integrin tensions (reported by 43 pN and 33 pN ITS). We proposed that the high-level integrin tensions transmitted by vinculin may be applied to integrins to test integrin-ligand bond strength. If the integrin-ligand bond cannot withstand the pulling of vinculin force, the focal complexes would be disassembled without developing to FAs. With vinculin knocked out, the population of high-level integrin tensions are substantially reduced, and focal complexes have the chance to develop to FAs on TGT surfaces with low T_{tol} values. Collectively, our results showed that FA formation requires a modest force level of integrin tensions in the range of 10-20 pN. During FA formation, vinculin would significantly elevate the force level of integrin tensions. While the biological significance of vinculin-elevated integrin tensions is not fully understood, such high-level integrin tensions are actually not required for FA formation.

We speculated how vinculin may elevate integrin tensions in FAs. By F-actin imaging, we have shown that stress fibers in vinculin KO cells are severely prohibited. Stress fibers are actomyosin structures which are well known to produce contractile force on FAs. Vinculin is clearly required for the efficient assembly of stress fibers in cells. Therefore, vinculin may elevate integrin tensions by facilitating the formation of stress fibers. Another possibility is that vinculin may stabilize the molecular structure transmitting force from actin network to integrins. A recent study shows that vinculin-talin and vinculin-catenin complexes have a lifetime of >1000 s under force (44). This high mechanical stability of vinculin complexes may provide the structural basis to transmit high-level integrin tensions or to extend the force dwell time, resulting in the rupture of TGT with high T_{tol} values.

We'd also like to discuss the accuracy of T_{tol} values of TGT and ITS used in the study. Overall, $T_{\mathrm{tol}} = 12$ pN based on DNA unzipping conformation and $T_{\mathrm{tol}} = 54$ pN based on DNA shear conformation are values that have been experimentally calibrated with a force dwell time of 2 seconds (45,46) at room temperature. 23 pN, 33 pN and 43 pN values are theoretically interpolated values based on the P. G. deGennes model of DNA rupture (47). A latest study calibrated the critical forces dissociating dsDNA at 37^{o}C, the cell culture temperature, showing that 15-bp DNA and 11 bp DNA (43 pN TGT) actually has critical dissociation forces at the level of 33 pN and 21 pN, respectively, with a force dwell time of seconds and at 37^{o}C (48). These results suggest that current T_{tol} values may not be accurate, either due to the insufficiency of the P. G. deGennes model or due to the temperature difference (dsDNA calibration vs cell culture) or both. In this consideration, we suggest that the T_{tol} values of 12-54 pN used in this paper should be considered as nominal values which indicate the relative TGT strengths from low to high.

Another factor affecting the accuracy of T_{tol} values is force dwell time. The critical force rupturing dsDNA is also dependent on force dwell time (49). The dwell time of integrin tensions in cells has not been not fully calibrated, and is likely not a constant, making it more challenging to determine the absolute T_{tol} values in a certain cellular force assay. However, it is worth noting that the mechanical rupture of dsDNA is much more sensitive to the force level than to the force dwell time. As an example, it has been shown that the dsDNA lifetime under a ~10 pN unzipping force is 1 sec, but the lifetime becomes ~10,000 sec under a 5 pN unzipping force (49). There is also evidence suggesting that the dwell time of integrin tensions in FAs are in the range of seconds to minutes (50,51), matching the time range for the calibration of T_{tol} values. Overall, we consider TGT as a suitable tool to evaluate the relative force levels of molecular tensions transmitted by integrins.

The monotonicity of T_{tol} values should also remain unchanged for the same ensemble of forces regardless of the force dwell time. This claim is evident as the integrin tension signals reported by the ITS clearly exhibit monotonic decrease along the T_{tol} values (Fig. 5). In this regard, TGT and ITS are capable of modulating and reporting integrin tensions at different force levels. The claim is also exhibited by vinculin KO cells on ITS surfaces with $T_{\mathrm{tol}} = 12$pN and 54 pN, respectively. Force signal is vibrant on the former one and nearly zero on the latter one, suggesting that TGT and ITS reacted differentially to integrin tensions at different force levels. Note that the DNA constructs of all TGTs (or ITSs) share the same length and sequence, therefore requiring the same amount of mechanical energy to rupture them apart. The different force signal intensities shown on the series of ITS surfaces prove that DNA rupture by integrins is not dictated by mechanical energy produced by the force, but by the force level.

Besides the study of FA formation, the PLL-PEG-TGT platform developed here decouples cell adhesion from other integrin-dependent cellular activities. As cell adhesion is a basic cellular function, prohibition of cell adhesion would certainly disable other subsequent cellular activities. Without PLL-PEG, TGT alone may impact cell adhesion status and indirectly affect the cellular activities of interest, making the interpretation of TGT data difficult. Ensuring cell adhesion by an integrin-independent manner, PLL-PEG-TGT platform provides a less biased surface for the study of many integrin-dependent cell mechanobiological processes (e.g. neuron growth cone development, filopodium formation, gene expression dictated by integrin signaling, etc.). We expect to see more applications of this platform in the future.

Acknowledgements

This work was supported by National Science Foundation (1825724) and National Institute of General Medical Sciences (1R35GM128747).

Author Contributions

X.W. and J.A. conceived the project and designed the experiments. J.A. performed the experiments and collected the data. Y.T. prepared PLL-PEG surfaces. K.P. assisted in ITS preparation. J.A. and X.W. analyzed the data. J.A. wrote the first version of the manuscript. X.W. revised and finalized the manuscript.

Declaration of Interests

The authors declare no competing interests.

Figure Legends

FIGURE 1 Calibrate integrin ligand strength required for FA formation with PLL-PEG-TGT surfaces. (A) A PLL-PEG-TGT platform is developed to knock down integrin tensions without disrupting cell adhesion. TGT is immobilized on glass surfaces coated with composite polymer PLL-PEG which supports cell adhesion by electrostatic adsorption and suppresses non-specific integrin-substrate interaction. (B) Molecular structure of PLL-PEG. PLL is a long peptide consisting of ~100 lysines, and 20% of lysines are conjugated with biotinylated PEG polymers. (C) T_{tol} values are selectable in the range of 12-54 pN by changing the biotin location on the TGT. (D) HeLa cells were incubated on PLL-PEG-TGT surfaces for 90 min, with vinculin and talin imaged to mark adhesion clusters. Talin and vinculin are highly co-localized in the clusters. (E) Cells adhered on all surfaces including the TGT-null PLL-PEG surface, with comparable cell spreading areas. 10-20 cells were analyzed for each condition. (F) Vinculin cluster area were analyzed by Python-based code. (G) Vinculin cluster area monotonically increases along with T_{tol} values of TGT. The cluster area increases most significantly from T_{tol} = 43 pN to T_{tol} = 54 pN. 50-70 clusters were analyzed for each T_{tol} condition. The error bar defines the data range (excluding outliers) and the inner box defines the standard deviation and the median value. This applies to all other error bars in this paper.

FIGURE 2 Time series of integrin and zyxin clusters formed on PLL-PEG-TGT surfaces. (A) HeLa cells were plated on TGT surfaces with T_{tol} = 12 pN and 54 pN, respectively, and then fixed at various timepoints to observe the development of integrin and zyxin clusters. Integrin β_3 marks initial cluster formation, and zyxin supposedly marks mature focal adhesion. (B) Integrin β_3 cluster area versus cell incubation time. The left side shows cluster areas on the 12 pN TGT surface from 10 to 60 min, and the right side shows cluster areas on the 54 pN TGT surface from 10 to 60 min. (C) Zyxin cluster area versus cell incubation time. The results demonstrate that both integrin β_3 and zyxin clusters were able to grow larger by time on the 54 pN TGT surfaces but not on the 12 pN TGT surfaces.

FIGURE 3 HeLa cells with vinculin knocked out and wildtype HeLa cells on PLL-PEG-TGT surfaces. (A) Both Vinculin KO cells and wildtype cells express talin-GFP which reports FA formation. Cells were incubated on the surfaces for 90 min. (B) Vinculin immunostaining confirms that vinculin is indeed knocked out in the Vinculin KO cells. (C) Talin cluster areas versus T_{tol} values of TGT. The cluster area increases significantly with T_{tol} value changing from 12 pN to 23 pN. (D) Talin cluster areas versus T_{tol} values of TGT. The cluster area increases significantly with T_{tol} value changing from 43 pN to 54 pN.

FIGURE 4 Visualize integrin tensions in vinculin KO cells using integrative tension sensor (ITS). (A) Schematics of ITS application. ITS shares similar DNA constructs to TGT, but serves to image integrin tensions, not to modulate integrin tensions. ITS is conjugated with a quencher-dye pair to visualize integrin tensions. The surface is co-coated with fibronectin (FN) which provides stable integrin ligands for cell adhesion. (B) vinculin KO HeLa and Wildtype HeLa cells incubated on fibronectin-coated surfaces for 90 min. FAs were marked by talin-GFP and F-actin was stained with phalloidin. Stress fibers connecting FAs are visible in the wildtype cells but few in vin KO cells. (C) vinculin KO HeLa and Wildtype HeLa cells incubated on ITS surfaces with T_{tol} = 54 pN for 90 min. FAs were marked by talin-GFP and integrin tension signal were reported by 54 pN ITS. The third row is an overlay of talin (green) and ITS (magenta). (D) Relative fluorescence intensities of ITS signal (T_{tol} = 54 pN) between wildtype and vinculin KO HeLa cells. Integrin tension signal reported by 54 pN ITS in vinculin KO cells is nearly zero.

FIGURE 5 Integrin tension signals in vinculin KO and wildtype HeLa cells on a series of ITS surfaces. (A) Vinculin KO cells incubated on 12 pN-54 pN ITS surfaces for 90 min, with talin marking FAs. (B) Wildtype cells incubated on 12 pN-54 pN ITS surfaces for 90 min. (C) Relative fluorescence intensities of ITS signals in vinculin KO cells. The 54 pN ITS showed nearly zero signals, but ITS surfaces with other T_{tol} values showed observable force signals. (D) Relative fluorescence intensities of ITS signals in wildtype cells.

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