Nanopatterning reveals an ECM area threshold for focal adhesion assembly and force transmission that is regulated by integrin activation and cytoskeleton tension

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Summary
Integrin-based focal adhesions (FA) transmit anchorage and traction forces between the cell and the extracellular matrix (ECM). To gain further insight into the physical parameters of the ECM that control FA assembly and force transduction in non-migrating cells, we used fibronectin (FN) nanopatterning within a cell adhesion-resistant background to establish the threshold area of ECM ligand required for stable FA assembly and force transduction. Integrin–FN clustering and adhesive force were strongly modulated by the geometry of the nanoscale adhesive area. Individual nanoisland area, not the number of nanoislands or total adhesive area, controlled integrin–FN clustering and adhesion strength. Importantly, below an area threshold (0.11 μm²), very few integrin–FN clusters and negligible adhesive forces were generated. We then asked whether this adhesive area threshold could be modulated by intracellular pathways known to influence either adhesive force, cytoskeletal tension, or the structural link between the two. Expression of talin- or vinculin-head domains that increase integrin activation or clustering overcame this nanolimit for stable integrin–FN clustering and increased adhesive force. Inhibition of myosin contractility in cells expressing a vinculin mutant that enhances cytoskeleton–integrin coupling also restored integrin–FN clustering below the nanolimit. We conclude that the minimum area of integrin–FN clusters required for stable assembly of nanoscale FA and adhesive force transduction is not a constant; rather it has a dynamic threshold that results from an equilibrium between pathways controlling adhesive force, cytoskeletal tension, and the structural linkage that transmits these forces, allowing the balance to be tipped by factors that regulate these mechanical parameters.

Key words: Cell adhesion, Fibronectin, Vinculin, Talin, Focal adhesion

Introduction
Integrin-mediated adhesion to extracellular matrix (ECM) components such as fibronectin (FN) transmits mechanical forces and ‘outside-in’ biochemical signals regulating tissue formation, maintenance, and repair (Hynes, 2002; Danen and Sonnenberg, 2003; Wozniak and Chen, 2009). Integrin receptors regulate their affinity for ligands by undergoing conformational activation through ‘inside-out’ signaling via binding of the cytoskeletal protein talin to the β-integrin tail (Hynes, 2002; Calderwood and Ginsberg, 2003; Shattil et al., 2010). Following activation and ligand binding, integrins cluster together into nanoscale adhesive structures that function as foci for the generation of strong anchorage and traction forces in stationary and migrating cells (Balaban et al., 2001; Beningo et al., 2001; Galbraith et al., 2002; Tan et al., 2003; Gallant et al., 2005). These focal adhesion (FA) complexes consist of integrins and actins vertically separated by a ∼40 nm core that includes cytoskeletal elements, such as vinculin, talin and α-actinin, and signaling molecules, including FAK, src, and paxillin (Geiger et al., 2001; Zamir and Geiger, 2001; Kanchanawong et al., 2010). Integrin clustering is a crucial step in the adhesive process promoting recruitment of cytoskeletal components, activation of signaling molecules, and enhancing adhesive force (Miyamoto et al., 1995a; Miyamoto et al., 1995b; Maheshwari et al., 2000; Roca-Cusachs et al., 2009; Bunch, 2010; Petrie et al., 2010). These integrin-based FAs serve as mechanosensors converting environmental mechanical cues into biological signals (Geiger et al., 2009).

Two central questions in mechanobiology are: (i) what information is encoded by the physical properties of ECM molecules?, and (ii) how are these physical properties sensed and interpreted by cells as instructions to assemble a force-transmitting adhesion complex (Bershadsky et al., 2006; Vogel and Sheetz, 2006; Gardel et al., 2010; Parsons et al., 2010)?
Previous work has shown that both ECM rigidity and ligand spacing influence FA and stress fiber assembly, cell spreading, migration speed, and adhesive forces (Massia and Hubbell, 1991; García et al., 1998a; Maheshwari et al., 2000; Wang et al., 2001; Coussen et al., 2002; Koo et al., 2002; Jiang et al., 2003; Arnold et al., 2004; Griffin et al., 2004; Engler et al., 2006; Cavalcanti-Adam et al., 2007; Petrie et al., 2010; Selhuber-Unkel et al., 2010). For instance, different average minimal RGD ligand spacings are required for spreading (440 nm) and FA assembly (140 nm) (Massia and Hubbell, 1991). More recent studies with precisely spaced RGD ligands confirmed that FA assembly, spreading, and migration require close integrin ligand spacing (<90 nm) and showed that cells cannot integrate signals from integrin–ligand complexes spaced more than 58 nm from each other (Arnold et al., 2004; Cavalcanti-Adam et al., 2006). However, the minimal area of integrin–ligand complexes required to assemble an adhesion complex and transmit force remains unknown.

The influence of the size of adhesive complexes on force transmission has also been investigated, but a generalized relationship between FA area and force has not been established. Nascent punctate nanoscale (<0.2 μm²) FAs assemble at the tips of filopodia and the leading edges of lamellipodia (Fig. 1A) in an actin polymerization-dependent but myosin-II-independent process to transmit traction forces (Beningo et al., 2001; Cai et al., 2006; Choi et al., 2008; Stricker et al., 2011). These nascent adhesions either undergo rapid turnover within the lamellipodium or mature into larger, elongated FAs under the influence of myosin-dependent cytoskeletal tension (Chrzanowska-Wodnicka and Burridge, 1996; Riveline et al., 2001; Cai et al., 2006; Choi et al., 2008; Gardel et al., 2008). Mature, large (1.0–10 μm²) FAs (Fig. 1A)

Fig. 1. Cells adhere to nanopatterned FN and form adhesive structures. (A) Nanoscale nascent adhesions (vinculin) formed at the lamellipodium and leading edge of a fibroblast (mature adhesions: red arrowhead; nascent adhesions: white arrowhead). (B) FN patterns on PDMS stamps are transferred onto the substrates with NHS esters resulting in the transfer and tethering of FN molecules onto the substrate. (C) Adhesive zone of nanoislands (500 nm x 4 shown); each zone consists of a center square (2 x 2 μm) surrounded by eight radially distributed adhesion pads. Each adhesion pad consists of 1, 2, 4 or 9 square nanoislands. Adhesive pads are presented in two orientations (45°, 90°) relative to the center pad. (D) FN nanopatterns retain high spatial fidelity in culture as visualized by direct imaging of Alexa-Fluor-555-labeled FN (FN 555) or indirect immunostaining using a polyclonal antibody against FN (poly FN). (E) Cells expressing GFP–vinculin assemble vinculin-containing FAs on nanopatterns.
anchor cells to the substrate to maintain cell morphology and
tensional homeostasis (Balaban et al., 2001; Beningo et al., 2001;
Tan et al., 2003). Using deformable substrates and traction force
microscopy, a linear relationship between FA area and traction
force was reported for large (>1 μm) FAs, indicating a constant
traction stress at these adhesive structures (Balaban et al., 2001;
Tan et al., 2003). However, other studies have measured widely
variable adhesive forces versus FA area for FAs (Beningo et al.,
2001; Goffin et al., 2006; Stricker et al., 2011). Recently, Gardel
and colleagues elegantly demonstrated a strong correlation
between FA size and traction force only during the initial
stages of FA maturation and growth, whereas mature adhesions
did not exhibit this relationship (Stricker et al., 2011). Moreover,
this group found a spatial dependence for traction forces across
an entire cell with higher traction forces transmitted by mature
FAs near the cell periphery. Together, these studies show that FA
function (i.e. force transmission) is not reliably predicted by FA
size.

To define additional ECM physical parameters that determine
adhesive complex function, we examined whether there is a
minimal area of integrin–ligand complexes required to assemble
an adhesive complex and transmit adhesive force. In the present
study, we used novel nanopatterned adhesive arrays of FN
nanoislands within a non-adhesive background to address this
question. We found that stable assembly of integrin–FN clusters
and generation of adhesive force depend on the area of individual
adhesive nanoislands and not the number of adhesive contacts
(number of nanoislands) or total adhesive area. Importantly, the
minimal area of integrin–FN clusters required for FA assembly
and force transmission exhibits a threshold that is not constant,
but is instead regulated by recruitment of talin and vinculin and
the cytoskeleton tension applied to these adhesive clusters. We
propose that this dynamic area threshold for stable FA assembly
and force transmission results from an equilibrium between
pathways controlling adhesive force, cytoskeletal tension, and
the structural linkage that transmits these forces. We further suggest
that perturbation of this force equilibrium is a local regulatory
mechanism for the assembly/disassembly of adhesive structures
and the transmission of adhesive forces.

**Results**

**FN nanoislands direct assembly of nanoscale adhesive
clusters**

To study mechanobiology responses at the scale of individual
adhesive structures, we engineered cell adhesion arrays with 250–
1000 nm square FN islands using modified subtractive contact
printing to covalently immobilize FN into defined nanopatterns on
a cell adhesion-resistant background (Coyer et al., 2011) (Fig. 1B;
supplementary material Fig. S1A). Several FN nanopattern
configurations were prepared to vary the nanoscale geometry in
terms of nanoisland size, number, and spacing (supplementary
material Fig. S2). We generated 10 μm diameter adhesive zones
with a center square (2×2 μm) surrounded by 8 radially distributed
adhesion pads containing nanoislands (Fig. 1C). This configuration
allowed for manipulation of the adhesive nanoisland geometry
independently from cell shape/spreading which was maintained
constant for all nanopattern configurations. This is a critical
consideration because both anchorage and traction forces are cell
shape and position dependent (Gallant et al., 2005; Stricker et al.,
2011). Two adhesion pad orientations (90°, 45°) relative to the
center square are present in the adhesive zone. Each adhesion pad

consists of 1, 2, 4 or 9 square nanoislands. These nanoislands
present adhesive areas (0.06–1.0 μm²) corresponding to the size of
small, nascent FAs (Beningo et al., 2001; Choi et al., 2008) and are
below the size of mature FAs (1–10 μm²) (Goffin et al., 2006;
Sniadecki et al., 2006). The edge-to-edge distance between
nanoislands is equal to the nanoisland side dimension. Adhesive
pattern geometry is designated by the length of the side of the
nanoislands and number of nanoislands; for example, 500 nxn4
refers to adhesive pads with 4 nanoislands with sides of 500 nm
(Fig. 1C). Printed nanoisland dimensions were confirmed by
atomic force microscopy imaging (Coyer et al., 2007; Coyer
et al., 2011). Adhesive zones were spaced 100 μm apart from each
other in order to support adhesion of a single cell (supplementary
material Fig. S1B).

NIH3T3 murine fibroblasts cultured overnight (>16 h) adhered
to FN adhesive zones as single cells and remained nearly spherical
(supplementary material Fig. S1B). To examine the stability and
fidelity of FN nanopatterns in the presence of cells, fibroblasts were
cultured overnight in serum-containing medium on patterns printed
with human FN labeled with Alexa Fluor 555. Examination by
fluorescence microscopy demonstrated that the printed pattern of
Alexa-Flour-555-labeled FN was retained with high-fidelity and
uniform intensity (Fig. 1D). Furthermore, immunostaining with a
polyclonal antibody that reacts with human, bovine and murine FN
showed no changes in nanopattern integrity or intensity (Fig. 1D).

No staining for FN outside the nanopatterned islands, including
between nanoislands, was detected. Vinculin-null mouse embryonic
fibroblasts expressing GFP–vinculin showed patterns of vinculin
recruitment to FN nanoislands that corresponded to the size and
location of the FN nanopatterns (Fig. 1E). These results demonstrate
that the FN nanopatterns retain high fidelity in terms of spatial
distribution and density in overnight culture, indicating that cells
cannot lay down secreted or serum-derived FN or reorganize the
printed FN on the surface.

**Assembly of stable integrin–FN clusters exhibits an ECM
area threshold**

We first examined whether assembly of stable, steady-state
integrin–FN clusters on the nanoislands is modulated by the
nanoscale geometry of the adhesion interface. Clustering of
ligand-bound integrins is an early step in the formation of dot-
like nascent adhesions (Wiseman et al., 2004; Gardel et al.,
2010). We performed integrin immunostaining using a cross-
linking and detergent extraction method that selectively retains
integrins bound to FN and removes non-ligated integrins (Garcia
et al., 1999; Keselowsky and Garcia, 2005). Cells were cultured
on FN nanopatterns overnight (>16 h) to allow the formation of
stable integrin–FN clusters. We have previously shown that both
integrin–FN complexes and adhesion strength reach stable,
steady-state values after 8 hours (Gallant et al., 2005; Michael
et al., 2009). Adherent cells were incubated in the cell-
impermeable reagent sulfo-DTSSP to cross-link integrins to
FN. Following SDS detergent extraction of cellular components,
including uncross-linked integrins, immunostaining for α5
integrin was performed. Previous analyses with function-
perturbing antibodies demonstrated that adhesion in the
NIH3T3 cell model is primarily mediated by α5β1-integrin–FN
without significant contributions from other receptors or
extracellular ligands (Gallant et al., 2005).

For the present analysis, Alexa-Flour-555-labeled FN and
Alexa-Flour-488-labeled secondary antibodies were used to
simultaneously visualize FN nanoislands (red) and bound integrins (green). After screening different nanopattern configurations, we selected four adhesive zone configurations for detailed analyses of integrin recruitment and localization. Three patterns presented the same adhesive pad area (1.0 μm²) but the pad area was distributed over 1 (1000 nm×1), 4 (500 nm×4) or 9 nanoislands (333 nm×9). Because less is known about the structure/size of nascent adhesions, a pattern (250 nm×4) with smaller adhesive pad area (0.25 μm²) distributed over 4 smaller nanoislands was also examined. Immunostaining revealed that integrin localization was restricted to the nanoislands within the adhesive pads and the center square only, and areas between the adhesive regions were mostly devoid of integrin staining (Fig. 2A). In some instances,
integron staining was present over non-adhesive areas spanning adhesive nanoislands corresponding to ‘cell bridging’ (Lehnert et al., 2004; Zimmermann et al., 2004; Rossier et al., 2010). We do not expect that integrins over the non-adhesive areas contribute significantly to adhesive force since there is no FN underneath them (Fig. 1D) to support anchorage to the substrate. Notably, the distribution and intensity of integrin staining on nanoislands differed significantly among the different geometrical patterns, with higher signal intensity to the peripheral adhesive pads for the larger nanoislands (500 and 1000 nm) and higher integrin localization to the center square for the smaller nanoislands (250 and 333 nm).

In order to perform quantitative analyses of integrin recruitment and clustering to FN nanopatterns, individual images were stacked and color coded to generate frequency maps of integrin–FN clustering (Fig. 2B,C). This analysis exploits the controlled spatial arrangement of the FN islands and allows us to extract the dominant spatial localization of integrins across multiple cells. As such, these frequency map images represent a better descriptor of integrin localization for the cell population compared to images of individual cells. This type of analysis has been used to identify spatial patterns of proliferation and cell density in multicellular assemblies and filters out low frequency occurrences, such as noise (Nelson et al., 2005; Nelson et al., 2006). Fig. 2B presents frequency maps for cells adhering to the four adhesive zone configurations, and higher magnification images for the adhesive pads in the 45° and 90° orientations are shown in Fig. 2C. In addition, the frequency maps were overlaid onto images of the FN nanoislands (Fig. 2D) to identify colocalization of integrin and FN within the adhesive pads (Fig. 2E). On 1000 nm×1 patterns, integrins were recruited to the adhesive pads and center square, and integrins uniformly localized to the available single FN nanoisland in the adhesive pads. On 500 nm×4 patterns, integrins were recruited to the center square as well as the nanoislands in the adhesive pads. Although integrins localized to the 4 nanoislands in the adhesive pad, there was higher frequency of localization to the nanoislands that were closer to the center square. This enrichment in integrin recruitment is most evident on the adhesive pad with the 45° orientation. For 333 nm×9 patterns, integrin recruitment was enriched to the center square compared to the adhesive pads, where the frequency maps revealed more diffuse integrin localization. In addition, integrins recruited to adhesive pads colocalized with the FN nanoislands, but only to those that were closer to the center square. On the 250 nm×4 patterns, integrins clustered exclusively on the center square and very little integrin staining was evident on the nanoislands. These results demonstrate that stable integrin–FN clusters exhibit a nanoscale area threshold (333 nm islands corresponding to 0.11 μm²) below which no stable complexes are formed.

It was clear from examining multiple images that the number of adhesive pads occupied by integrins for a given adhesive zone (each zone presents 8 adhesive pads to one cell) was dependent on the adhesive pad geometric configuration. We therefore scored the number of adhesive pads (out of 8) staining positive for integrin–FN clustering and generated histograms for adhesive pad occupancy by integrins (Fig. 2F). For 1000 nm×1 patterns, integrin–FN clusters predominantly occupied three or more adhesive pads with over 50% of cells showing occupancy on all 8 adhesive pads. In contrast, for 250 nm×4 patterns integrins showed low pad occupancy with over 90% of adhesive zones having two or less adhesive pads occupied by integrins. The 500 nm×4 and 333 nm×9 patterns resulted in integrin pad occupancies that were equally distributed from partial to full occupancy. These findings also support our conclusion that stable integrin–FN clusters exhibit a nanoscale area threshold (0.11 μm²), below which no stable complexes are formed.

To further characterize these integrin–FN clusters, we examined the contributions of Rho-kinase activity to steady-state integrin–FN cluster formation on nanopatterned substrates. Contractility inhibitors have divergent effects on nascent adhesions compared to mature FAs. Inhibitors of Rho-kinase and actomyosin contractility dissolve mature FAs and reduce corresponding adhesive forces (Burridge and Chrzanowska-Wodnicka, 1996; Amano et al., 1997; Balaban et al., 2001; Tan et al., 2003; Dumbauld et al., 2010). In contrast, inhibition of Rho-kinase upregulates the number of nascent adhesions in the lamellipodium (Alexandrova et al., 2008) whereas the formation rate of nascent adhesions is myosin-II-independent (Choi et al., 2008). We examined whether inhibition of contractility using Y-27632, a specific inhibitor of Rho-kinase that reduces myosin light chain phosphorylation, cell contractility, FA assembly and FA-dependent forces (Dumbauld et al., 2010; Kuo et al., 2011), influences assembly of integrin–FN clusters on nanoislands. Cells were cultured on FN nanopatterns overnight and treated with Y-27632 (10 μM) 30 min prior to analysis. For 500 nm×4 patterns, treatment with Y-27632 reduced the frequency of integrin localization within the nanoislands (Fig. 3A,B). However, treatment with Y-27632 did not eliminate integrin localization to FN nanoislands (Fig. 3D) or alter pad occupancy (Fig. 3E), especially when compared to the unoccupied adhesive pads for the 250 nm×4 islands (Fig. 2E). These results demonstrate that Rho kinase activity reduces, but does not eliminate, steady-state integrin–FN clustering, and does not affect pad occupancy. The partial myosin dependence suggests that these engineered nanoscale, steady state adhesions reflect the properties of initial adhesions that are constrained from subsequent increase in area by the geometric arrangement of the ECM.

Nanoscale adhesive geometry modulates adhesive force
To examine the effects of nanoscale adhesive geometry on adhesive forces, we quantified the force required to detach cells from the adhesive zones after a 16-hour culture time point using a spinning disk device (Garica et al., 1998b; Gallant et al., 2005). A detachment profile (adhesion fraction / versus shear stress t) was fit to a sigmoid curve to obtain the shear stress for 50% detachment (t_50), defined here as the cell adhesion strength. Fig. 4A presents typical detachment profiles showing sigmoidal decreases in the fraction of adherent cells as a function of shear stress for two nanopattern configurations. The rightward shift in the detachment profile for the 1000 nm×1 pattern compared to the center square-only pattern (no adhesive pads) reflects a 2.2-fold increase in adhesive force.

Cell adhesion strength was quantified for adhesive zone configurations with different adhesive pad areas, nanoislands sizes, and number of nanoislands. Fig. 4B summarizes results for adhesive force as a function of adhesive pad area and number of nanoislands per adhesive pad. The upper bound (top dashed line) represents the adhesion strength for a 10 μm diameter micropatterned area (adhesive area 78.5 μm²), whereas the lower bound (bottom dashed line) corresponds to the adhesion strength for a pattern with 2×2 μm center square but no adhesive
pads or nanoislands (adhesive area 4.0 μm²). For most nanopattern configurations, adhesion strength values were higher than the lower bound, indicating that FN nanoislands significantly contribute to adhesive force. A 650% reduction in total available adhesive area (10 μm diameter circle versus 1000 nm x 1 pattern) resulted in only a 25% reduction in adhesive force. This result is consistent with our previous work demonstrating that adhesive strength is controlled by small adhesive areas at the periphery of the cell (corresponding to FAs) and that the majority of the available adhesive interface does not contribute significantly to adhesive force (Gallant et al., 2005).

The adhesion strength value for all patterns with nanoisland dimensions below 333 nm was equivalent to the lower bound (no adhesive pads), indicating no appreciable contributions to adhesive force for these nanoislands (Fig. 4B). For example, there are no differences in adhesion strength for 250 nm islands regardless of whether each pad contained 2, 4 or 9 islands, and the adhesion strength for these nanoislands is equivalent to center-only patterns that have no nanoislands. This result is consistent with the integrin recruitment results and shows the functional consequences of the area threshold of integrin–FN clustering to adhesive force. Furthermore, we noticed that the 500 nm x 1 and 500 nm x 4 patterns, which have same nanoisland dimensions but different number of nanoislands (1 versus 4), and therefore, different adhesive pad areas (0.25 versus 1.0 μm²), produced equivalent adhesion strength values (245±15 versus 252±11 dynes/cm²). This result suggests that individual nanoisland area, independently from the number of nanoislands and pad adhesive area, controls adhesion strength. Indeed, as shown in Fig. 4C, when adhesion strength is plotted as a function of the individual nanoisland area, the data points from different nanopattern configurations collapse into a single curve. Non-linear regression with a logarithmic curve indicated that this functional dependence accounts for over 92% of the variance in the data (P<0.0007). Additionally, no differences in adhesion strength were observed between configurations with the same adhesive pad area (0.25 μm²) and number of islands (4) but with different inter-island spacings (0.75 versus 1.25 μm; Fig. 4D), indicating that the spacing between nanoislands does not contribute appreciably to adhesive force. This analysis demonstrates that, above an area threshold of 0.11 μm², individual nanoisland area, and not the geometric arrangement of the adhesive area (number of islands, island spacings, total pad area), regulates the adhesive force generated by stable integrin–FN clusters. Below this area threshold, no appreciable adhesive forces are generated, correlating with the lack of stable integrin–FN clusters at steady state.

**Fig. 3. Effects of Rho-kinase inhibition on integrin–FN clustering on nanoscale patterns.** (A) Frequency maps for integrin clustering to 500 nm x 4 patterns for control and Y-27632 (10 μM)-treated cells. (B) High magnification frequency maps for integrin recruitment to adhesive pads for the 45° and 90° orientations. Scale bars: 1 μm. (C) FN nanoislands in adhesive pads. (D) Binary images of area corresponding to colocalization of integrin recruitment and FN nanoislands for the 45° and 90° pad orientation; images are oriented such that the edge closer to the center pad is at the top. (E) Histograms of pad occupancy.

**Talin controls the stable assembly of integrin–FN clusters at nanoscale dimensions**

What determines the area threshold for assembly of stable integrin–FN clusters? Extrinsic, cell-independent factors related to ligand presentation/density could dictate integrin clustering. However, the observed area threshold corresponds to a nanoisland area that is considerably (at least 50-fold) larger than the minimal ligand spacing (~100 nm) required for focal adhesion assembly and spreading (Massia and Hubbell, 1991; Arnold et al., 2004; Cavalcanti-Adam et al., 2007). Alternatively, cell-dependent, intrinsic mechanisms could regulate this ECM area ‘switch’ in the assembly of integrin–FN clusters. We first postulated that the nanoscale area threshold in integrin–FN clustering results from an insufficient number of activated and bound integrins required to establish a stable nascent integrin cluster. Therefore, we hypothesized that increased integrin activation would overcome this ‘nanolimit’ for stable integrin recruitment.

Talin is an elongated (~60 nm) flexible anti-parallel dimer that interacts with several proteins in FAs (Critchley, 2009). Notably, the FERM domain in the globular N-terminus of talin [talin1(1–405)] binds to an NP(IL)Y motif in the cytoplasmic tail of integrin-β subunits to activate the integrin (Calderwood et al., 1999; Tadokoro et al., 2003; Bouaouina et al., 2008; Goult et al., 2010). To determine whether integrin activation can overcome the ‘nanolimit’ of integrin–FN clustering on 250 nm islands, cells were transfected with plasmids encoding GFP–
talin1(1–405) or GFP–talin1(1–405)A/E with an integrin binding-defective mutation (Bouaouina et al., 2008). Expression of talin1(1–405) significantly enhanced integrin clustering to the 250 nm islands compared to untransfected control cells, whereas the integrin binding-defective talin mutant had no effects on integrin recruitment (Fig. 5A,B). There were no significant differences in integrin recruitment on the larger 500 nm islands between talin head domain and controls (Fig. 5A,B). Expression of talin head domain also significantly altered the frequency of pad occupancy for the 250 nm \( \times \) 4 patterns. Talin1(1–405)-expressing cells on 250 nm \( \times \) 4 patterns mostly occupied three to six adhesive pads (Fig. 5C). Cells expressing the A/E mutant or control cells showed low pad occupancy on 250 nm \( \times \) 4 islands with over 95% of cells having two or less adhesive pads occupied by integrin clusters. The 500 nm \( \times \) 4 patterns exhibited integrin pad occupancies that were equally distributed from partial to full occupancy, but talin1(1–405) expression increased the full pad occupancy by 45% compared to A/E mutant and control cells (Fig. 5D). Importantly, adhesion strength values for talin1(1–405)-expressing cells on 250 nm \( \times \) 4 patterns were 1.8-fold higher than control talin1(1–405)A/E-expressing cells (Fig. 5E; \( P < 0.05 \)). These results demonstrate that talin-head domain triggers assembly of integrin–FN nanoclusters below the ECM area threshold and significantly increases adhesive force.

Vinculin regulates nanoscale assembly of integrin–FN clusters by balancing adhesive force and cytoskeletal tension

We next examined the role of the FA protein vinculin in the nanoscale organization of integrin–FN clusters. Vinculin contains a talin binding site in its globular head and actin binding sites in the tail domain, and interactions with these partners are regulated by an auto-inhibited conformation arising from strong head–tail binding (Cohen et al., 2006). Importantly, vinculin is a force-carrying component between adhesive sites and the cytoskeleton (Grashoff et al., 2010). The vinculin head domain promotes integrin clustering and increases residence times in mature FAs in spread cells (Cohen et al., 2005; Cohen et al., 2006; Humphries et al., 2007). We examined the effects of the vinculin head domain (V\( _H \), 1–851) on the area threshold of integrin–FN clustering using vinculin-null cells expressing V\( _H \). In contrast to cells expressing wild-type vinculin, cells expressing V\( _H \) assembled integrin–FN clusters on the 250 nm nanoislands (Fig. 6A,B). These results show that the vinculin head domain

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Fig. 4. Nanoscale adhesive geometry regulates cell adhesion strength. Cell adhesive force to FN nanopatterns was measured using a spinning disk assay. (A) Detachment profiles (adhesive fraction versus shear stress) for cells adhering to 1000 nm \( \times \) 1 and center-only patterns. Experimental points were fitted to a sigmoid curve to calculate the shear stress for 50% detachment, which represents the mean adhesion strength. Vertical dashed lines show the shear stress for 50% detachment for each profile. (B) Adhesion strength as a function of adhesive pad area for different nanoisland configurations. Values are means ± s.e.m. The top and bottom dashed lines correspond to the adhesion strengths for a 10 \( \mu \)m diameter circular area and the center-only pattern, respectively. (C) Adhesion strength as a function of individual nanos island area (log scale). Values are means ± s.e.m., and logarithmic (natural base) fit is shown (solid line). The dashed line corresponds to the adhesion strength for the center-only pattern. Results for 0.0625 \( \mu \)m\(^2\) and 0.250 \( \mu \)m\(^2\) comprise 3 (250 nm \( \times \) 2, 250 nm \( \times \) 4, 250 nm \( \times \) 9) and 2 (500 nm \( \times \) 1, 500 nm \( \times \) 4) nanoisland patterns, respectively. (D) Adhesion strength values for 250 nm \( \times \) 4 patterns with different inter-island spacings (0.75 versus 1.25 \( \mu \)m), showing no differences in adhesive force.
can overcome the nanoscale limit of integrin–FN clustering, presumably via interactions with talin. The finding that cells expressing wild-type vinculin did not assemble stable integrin–FN clusters on the 250 nm nanoislands could be explained by the auto-inhibitory interaction between vinculin head and tail domains. To examine this possibility, we cultured vinculin-null cells expressing the vinculin T12 mutant (VinT12) on the nanopatterned substrates. VinT12 has a mutated head-tail interface that reduces the head-tail affinity 100-fold and thus exposes binding sites for talin and actin (Cohen et al., 2005; Cohen et al., 2006). This mutant also drives integrin clustering and FA assembly (Cohen et al., 2005; Cohen et al., 2006; Humphries et al., 2007). Surprisingly, expression of VinT12 failed to promote assembly of integrin–FN clusters on the 250 nm nanoislands even though robust clusters were assembled on the 500 nm patterns (Fig. 6A,B). Expression of V_H also increased pad occupancy (Fig. 6C) on 250 nm islands, whereas VinT12 expression did not enhance pad occupancy. Pad occupancy was similar for V_H- and VinT12-expressing cells on 500 nm islands (Fig. 6D). This result supports an alternative explanation in which the nanoscale area threshold for integrin-FN clustering is regulated by cytoskeletal tension.

Fig. 5. Talin head expression drives integrin–FN clustering at the nanoscale. Integrin binding analysis for talin-expressing cells. (A) Fluorescence microscopy images for integrin binding (green) to FN (red) adhesive zones. Scale bars: 1 μm. (B) Frequency maps for integrin recruitment on 250 nm x 4 and 500 nm x 4 patterns generated by stacking individual images. For 250 nm patterns, expression of talin1(1–405) induced recruitment and clustering of integrins compared to control cells. (C,D) Frequency histograms for pad occupancy on (C) 250 nm x 4 and (D) 500 nm x 4 patterns. (E) Cell adhesive force response to FN nanopatterns with talin head expression. Bar graphs represent fold change in adhesion strength over talin1(1-405)A/E-transfected cells adhering to 250 nm x 4 patterns (means ± s.d.; *P<0.05).
cytoskeletal tensile force and the integrin clusters are disassembled or detached from the adhesive interface because of limited adhesive size of the nanoislands. To test this hypothesis, vinculin-null cells expressing VinT12 cultured overnight on the 250 nm islands were treated with blebbistatin (20 μM) 60 min prior to analysis. Blebbistatin is an inhibitor of non-muscle myosin IIA that blocks actin–myosin contractility (Allingham et al., 2005). Cells expressing VinT12 and treated with blebbistatin displayed integrin–FN clusters on 250 nm islands similar to those in VH-expressing cells (Fig. 7A), whereas VinT12 did not induce any recruitment. Blebbistatin treatment also increased adhesive pad occupancy compared to control VinT12-expressing cells (Fig. 7B). These results demonstrate that a reduction in the cytoskeletal tension applied to integrin–FN nanoclusters in the presence of a vinculin mutant that drives integrin clustering allows for stable assembly of adhesive structures below the ECM area nanolimit. Taken together, these findings support a model where stable FA assembly and ECM–cell adhesive forces are regulated by the force equilibrium between cytoskeletal tension and adhesive forces.

**Discussion**

A standing question in mechanobiology is how cells sense geometrical ECM cues and transmit local forces at FAs (Bershadsky et al., 2006; Vogel and Sheetz, 2006; Gardel et al., 2010; Parsons et al., 2010). Several studies have shown that ECM ligand spacing regulates FA and stress fiber assembly, cell spreading and migration, and adhesive forces (Massia and Hubbell, 1991; García et al., 1998a; Maheshwari et al., 2000;
that increase integrin activation or clustering overcame this nano-limit. Inhibition of myosin contractility in cells expressing a vinculin molecule that enhances the coupling between the force-generating actin cytoskeleton and integrins also restored integrin–FN clustering below the area threshold. We conclude that the size of the integrin–FN clusters required for stable FA assembly and force generation has an ECM area threshold that is not constant, but is instead regulated by intracellular proteins (talin, vinculin) that influence the force equilibrium between the adhesive force generated by the integrin–FN clusters, which is related to the nanoscale area and number of FN-bound integrins, and the cytoskeletal tension applied to the clusters.

A critical advantage of our patterning strategy is that the overall cell shape is constrained within the 10 µm diameter adhesive zone for all nanopatterm configurations. This approach allows decoupling of integrin–FN cluster formation from cell spreading and cells cannot spread (or retract). Importantly, the distance between the adhesive pads containing the nanosilands and the center square is fixed across all nanopattern configurations, ensuring equivalent force loading in all experiments. Although we observed a higher frequency of integrin–FN clusters on nanosilands that were closer to the center square, we do not expect that this reduced distance alters force loading because this distance is significantly smaller than the overall distance between the adhesive pad and the center square. Indeed, our calculations estimate that enrichment of integrin–FN clusters towards the center square altered the resultant adhesive force by less than 1.3%. Finally, while the focus of the present study was to analyze the assembly of stable, steady-state integrin–FN clusters and adhesion strength, in the future, it will be of interest to perform time course analyses of integrin–FN complex assembly and adhesive forces. Unfortunately, this experiment is not presently possible due to technical limitations associated with live cell imaging of nanometer-scale adhesive clusters on the gold-coated substrates.

We propose a force equilibrium model for the ECM-area-regulated assembly of stable integrin–FN clusters (Fig. 8). Activated integrins bind to ECM ligands in the adhesive interface and assemble into small adhesive clusters containing talin and vinculin. The actin cytoskeleton applies tensile force to these nascent adhesive clusters via actin-myosin contractility. This cytoskeletal force is balanced by the adhesive force generated by the integrin–FN clusters. Below an area threshold (0.11 µm²), the adhesive force cannot support the cytoskeletal tension and the integrin cluster is unstable and is disassembled or detached from FN. Above this area threshold, the integrin–FN cluster is large enough to generate sufficient force to support the applied tension (Fig. 8A). The ECM area threshold for assembly of stable integrin–FN clusters is therefore regulated by (i) the adhesive force generated by the integrin–FN clusters, which is related to the nanoscale ECM area and number of bound integrins, and (ii) the cytoskeletal tension applied to the clusters. Increases in the adhesive force generated by the integrin–FN clusters via integrin activation or clustering through binding to talin head or vinculin head result in a stable adhesive cluster that can support cytoskeletal tension with smaller adhesive areas (Fig. 8A). Conversely, decreases in the applied cytoskeletal tension via inhibition of myosin contractility result in a reduction in the force driving disassembly of the integrin clusters (Fig. 8A).

Fig. 8B provides quantitative relationships that fully support this model. Because the size and position of the adhesive clusters and

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Fig. 7. Actomyosin contractility controls stabilization of integrin–FN clusters at nanoscale dimensions. (A) Fluorescence microscopy images for integrin binding (green) to FN (red) adhesive zones of VinT12 cells on 250 nm×4 patterns in the presence or absence of blebbistatin (20 µM). Scale bars: 1 µm. (B) Frequency maps for integrin recruitment on 250 nm×4 for VinT12 cells in the presence or absence of blebbistatin (20 µM), generated by stacking individual images. (C) Frequency histograms for pad occupancy of VinT12 cells on 250 nm×4 patterns in the presence or absence of blebbistatin (20 µM).

Cousen et al., 2002; Koo et al., 2002; Jiang et al., 2003; Arnold et al., 2004; Cavalcanti-Adam et al., 2007; Petrie et al., 2010; Selhuber-Unkel et al., 2010). However, whether the geometric organization of the ECM ligand regulates FA assembly and force transmission has not been addressed. Here, we used nanopatterned substrates with defined geometrical arrangements of FN to restrict the formation of adhesive structures to the nanoscale dimensions characteristic of FAs to study how nanogeometry regulates assembly of integrin–FN clusters and the generation of ECM–cell anchorage forces. The smallest nascent adhesion size reported is 0.19 µm² (Choi et al., 2008), although these authors speculated that the actual size is smaller. Our nanopatterning approach offers the ability to identify even smaller adhesive structures and we showed formation of tiny 0.06 µm² adhesions (Figs 6, 7). We demonstrated that integrin clustering on FN islands and adhesive force were modulated by nanoscale ECM area; below a threshold of 0.11 µm², no stable integrin–FN clusters were assembled or adhesive forces generated. Expression of talin head or vinculin head domains

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cell morphology are defined in our experimental system, we can convert the measured values for adhesion strength (Fig. 4) to the cell–ECM adhesive force generated on the nanoislands ($F_{Adh}$) as a function of nanoisland dimension (solid black line). For the cytoskeletal tension ($F_{CSK}$), we considered two estimates based on traction forces measured on deformable substrates. Gardel and colleagues recently reported the traction stresses during the initial stages of FA assembly (Stricker et al., 2011); the range for maximum traction forces for these nascent complexes is indicated by the gray box in Fig. 8B. Plotted also is the traction force for larger, mature FAs derived by Geiger and colleagues (Balaban et al., 2001) that increases with FA size (dashed gray line) (Balaban et al., 2001). Several noteworthy observations are evident from Fig. 8B. First, $F_{CSK}$ is larger than $F_{Adh}$ for small nanoislands but there is a cross-over point for which $F_{Adh}$ becomes larger than $F_{CSK}$. This cross-over point corresponds to the area threshold. For either $F_{CSK}$ model, the area threshold occurs below areas with dimensions below 333 nm ($L < L_{critical}$). For nanoislands with dimensions smaller than the threshold area ($L < L_{critical}$), the applied cytoskeletal tension exceeds the integrin–FN adhesive force and no stable integrin–FN complexes are assembled. For nanoislands with dimensions larger than the threshold area ($L > L_{critical}$), the integrin–FN adhesive force can support the applied cytoskeletal tension and stable integrin–FN clusters assemble on nanoislands. For either $F_{CSK}$ model, the area threshold occurs below areas with dimensions below 333 nm ($L < L_{critical}$) in excellent agreement with our experimental data for integrin–FN cluster assembly (Fig. 2). Remarkably, when the value for adhesion strength for the talin head domain on 250 nm nanoislands is included (open symbol), the resulting $F_{Adh}$ exceeds the $F_{CSK}$, also in agreement with the result that expression of talin head promotes assembly of stable integrin–
FN clusters on the 250 nm nanoislands (Fig. 5). This finding shows that talin-based integrin activation stabilizes integrin–FN clusters and enables these nanoscale structures to transmit large adhesive forces.

This force equilibrium model for ECM-area-controlled assembly of integrin–FN clusters provides a simple, local regulatory mechanism for the assembly/disassembly of adhesive structures. During leading edge protrusion in cell migration, small (\(~0.19 \mu m^2\)) nascent adhesions (Choi et al., 2008) assemble and either disassemble or become stable and grow into mature adhesions (Parsons et al., 2010). The mechanism(s) regulating these adhesion dynamics is not clear but two models have been proposed (Parsons et al., 2010). In the first model, nucleation of adhesive clusters is initiated by integrin binding, clustering and recruitment of cytoskeletal proteins (e.g. vinculin, talin), and these nascent structures grow and mature in response to contractile forces. In the second model, FA assembly is coupled to actin polymerization wherein vinculin and FAK bind to Arp2/3 and these complexes then bind ECM-bound integrins to stabilize the nascent adhesion in a myosin-independent fashion. The force equilibrium model is consistent with elements of first model and provides a simple biophysical nanoscale switch to control the nucleation and growth of adhesive structures. The finding that integrin activation via binding of talin head or driving clustering via vinculin head domain overcomes the nanoscale limit for stable integrin–FN cluster assembly supports the explanation that integrin activation and binding drive assembly of nascent adhesive clusters (Cohen et al., 2005; Cohen et al., 2006; Humphries et al., 2007; Zhang et al., 2008). The assembly of integrin clusters on 250 nm nanoislands (Fig. 5) in the presence of blebbistatin is also consistent with previous reports showing that myosin activity is not required for formation of nascent adhesions (Alexandrova et al., 2008; Choi et al., 2008). Our results with the vinculin head domain driving assembly of integrin–FN nanoclusters suggest that Arp2/3 is not directly involved in this process because this vinculin domain lacks that the proline-rich stalk (amino acids 851–880) that contains the Arp2/3 binding site (DeMali et al., 2002). Finally, the force-balance model also provides an attractive explanation for mechanosensitive changes in FA assembly. For instance, application of local external forces to adhesive clusters would perturb the local force balance and drive FA growth to increase the net adhesive force. This prediction is consistent with the observation that application of external forces to adhesive plaques results in Rho-dependent directional focal adhesion growth (Riveline et al., 2001). Similarly, changes in substrate stiffness could alter the local force balance to regulate the size of FA structures without significant changes in local cytoskeletal forces, in good agreement with published results (Yeung et al., 2005; Aratyn-Schaus et al., 2011).

In conclusion, we demonstrate that integrin–FN clustering and adhesive force are strongly modulated by the geometry of the nanoscale adhesive area. Stable assembly of integrin–FN clusters and adhesive force depend on the area of individual adhesive nanoislands and not the number of adhesive contacts or total adhesive area. Importantly, the minimal size of integrin–FN clusters required for FA assembly and force transmission exhibits an area threshold that is not constant, but is instead regulated by recruitment of talin and vinculin and the cytoskeleton tension applied to these adhesive clusters. We propose that this dynamic area threshold results from an equilibrium between pathways controlling adhesive force, cytoskeletal tension, and the structural linkage that connects these forces. This force equilibrium acts as a simple, local regulatory mechanism for the assembly/disassembly of nascent adhesive structures and the transmission of adhesive forces.

**Materials and Methods**

**Cells and reagents**

NIH3T3 fibroblasts (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% newborn calf serum (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin (Invitrogen). Cell culture reagents, including human plasma FN and Dulbecco’s phosphate-buffered saline (PBS), were purchased from Invitrogen. BSA was purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies against α5 integrin (ab921), Millipore, Billerica, MA, USA), and human FN (anti-FN polyclonal antibody, Sigma-Aldrich) were used for immunostaining. Alexa Fluor-488-conjugated secondary antibodies and Alexa Fluor 555 succinimidyl ester were purchased from Invitrogen. Cross-linker 3,3-dithiobis(sulfosuccinimidylpropionate) (DTSSP) was purchased from Pierce Chemical (Rockford, IL, USA). Polys(methacrylic acid) (PMADMA) elastomer and curing agent (Sygard 184) were produced by Dow Corning (Midland, MI, USA). ZEP520A was purchased from Zeon Chemicals (Tokyo, Japan). Amyl acetate was produced by Mallinckrodt Baker (Phillipsburg, NJ, USA), and n-methyl pyrrolidone (NMP, 1165 Remover) was obtained from MicroChem (Newton, MA, USA). Oligonucleotides (GATCCTGCAGTAGC-ACCGGTGCTACTGCA-3’ and 9AGCTTGCAGTAGC-ACCGGTGCTACTGCA-3’ sequences underlined) were annealed together, creating HindIII-compatible overhangs at each end. This product was then ligated into a linearized pXFO vector which had been digested with HindIII. Finally, the pXFO– vinculin constructs were digested from the pEGFP-C1 with AgeI and SacI and ligated into the SacI and AgeI-digested pXFO vector. The pXFO-400-gfp-vinculin WT, V9 and T12 vectors transcribe the eGFP-vinculin gene from the tetracycline-inducible promoter. All vectors were verified by sequencing the ligation points.

**Retroviral stocks**

Retroviral plasmids pTJ66-tTA and pXFO were previously described (Gersbach et al., 2006), pGFP-C1 WT vinculin and eGFP-T12 vinculin plasmids have been described (Chen et al., 2005). One 4gel restriction site was inserted into the multiple cloning site of pXFO, the retroviral expression vector. The oligonucleotides 5’-AGCTTGCAGTAGC-ACCGGTGCTACTGCA-3’ and 5’-AGCTTGCAGTAGC-ACCGGTGCTACTGCA-3’ (AgeI sequences underlined) were annealed together, creating HindIII-compatible overhangs at each end. This product was then ligated into a linearized pXFO vector which had been digested with HindIII. Finally, the pXFO– vinculin constructs were digested from the pEGFP-C1 with AgeI and SacI and ligated into the SacI and AgeI-digested pXFO vector. The pXFO-400-gfp-vinculin WT, V9 and T12 vectors transcribe the eGFP-vinculin gene from the tetracycline-inducible promoter. All vectors were verified by sequencing the ligation points.

**Retroviral stocks**

Retroviral stocks were produced by transient transfection of helper virus-free nX amphotropic producer cells with plasmid DNA as previously described (Byers et al., 2002). Vinculin-null mouse embryonic fibroblasts (Xu et al., 1998), a kind gift from Eileen Adamson (Sanford-Burnham Medical Research Institute), were plated on tissue culture polystyrene at 2×10^5 cells/cm^2 24 h prior to retroviral transduction. Cells were transfected with 0.2 μg/cm^2 each of complexes pTJ66-tTA and pXFO-400-gfp-vinculin retroviral supernatant supplemented with 4 μg/ml histidine-methionine and 10% fetal bovine serum, and centrifuged at 1200 g for 30 min in a Beckman GS-6R centrifuge with a swinging bucket rotor. Retroviral supernatant was replaced with growth medium (DMEM, 10% fetal bovine serum, 100 μU/ml penicillin G sodium, 10 μg/ml streptomycin sul fate, 1% non-essential amino acids, 1% sodium pyruvate). Five days after transduction, eGFP-expressing cells were FACS sorted, expanded, and either used for experimentation or cryopreserved in liquid nitrogen for later use. Expression of vinculin constructs was verified by western blot and immunofluorescence microscopy (data not shown).

**Nanopatterned surfaces**

Mixed self-assembled monolayers (SAMs) of tri(ethylene glycol)-terminated (EG3) and carboxylic acid-terminated (EG6-COOH) alkanethiols on gold were used to present anchoring groups for covalent immobilization of FN within a non-fouling background as reported earlier and showed in supplementary material Fig. S1 (Coyer et al., 2007; Coyer et al., 2011).
Cell seeding and integrin cross-linking
Alexa-Fluor-488-conjugated secondary antibodies (anti-rabbit IgG, 10 μg/ml) diluted in blocking buffer for 1 h at 37°C. Images were captured using a Nikon Eclipse E400 fluorescence microscope, Nikon CFI Apo TIRF 60× oil/1.49 NA objective and Image-Pro image acquisition software (Media Cybernetics, Bethesda, MD, USA) at fixed exposures and illumination conditions. Frequency map images were produced by stacking and averaging individual images for a given condition using Image Pro software. In order to evaluate pod occupancy, individual images with positive integrin staining for the center area (indicating a cell) were scored for the number of adhesive pads within an adhesive cluster with positive integrin localization and normalized by the total number of patterns counted to generate a cumulative distribution.

Adhesive force measurements
Cell adhesion strength was measured using a spinning disk system (Gallant et al., 1998b; Gallant et al., 2005). Patterned coverslips with adherent cells cultured overnight were spun in PBS + 2 mM dextrose for 5 min at a constant speed in a Spot-RT camera, and Image Pro analysis system. Sixty-one fields (80–100 cells/field) were obtained by multiplying the nanopattern area by the stress constant (5.5 nN/m) and averages individual images for a given condition using Image Pro software. In order to evaluate pod occupancy, individual images with positive integrin staining for the center area (indicating a cell) were scored for the number of adhesive pads within an adhesive cluster with positive integrin localization and normalized by the total number of patterns counted to generate a cumulative distribution.

Calculation of adhesive forces for force equilibrium model
Adhesion strength data (Fig. 4) was converted to adhesive forces using a simple mechanical equilibrium model for a nearly spherical adherent cell (Gallant et al., 2005; Gallant and Garcia, 2007). Traction forces based on those derived by Geiger et al. (1997) for 50% detachment and 50% retraction and 50% detachment and 50% retraction

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References


Fig. S1. Cell adhesive substrates with nanopatterned geometries. (A) Anchoring groups (COOH) on mixed self-assembled monolayers on gold are activated with EDC/NHS to produce amine-reactive NHS-esters. A flat elastomer stamp uniformly coated with FN is brought into contact with a high-precision nanotemplate to selectively remove FN from the stamp and create the desired FN patterns on the stamp. Pressing the stamp onto the substrates with NHS-esters results in the transfer and tethering of FN molecules onto the substrate. (B) NIH3T3 fibroblasts cultured overnight on nanopatterned substrates attach as single cells and remain rounded (top: fluorescence microscopy image of FN nanopatterns, bottom: phase contrast image of cells).
Fig. S2. Characteristics of adhesion cluster designs analyzed.

<table>
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<th>Number of Islands per pad</th>
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10 µm Diameter

Fig. S3. Blebbistatin treatment (20 µM, 60 min prior to analysis) has no effect on the lack of assembly of integrin-FN clusters on 250 nm islands for cells expressing wild-type vinculin. Fluorescence microscopy images for integrin binding (green) to FN (red) adhesive zones on 250 nm×4 patterns; scale bar: 1 µm.