The Physical Interaction of Myoblasts with the Microenvironment during Remodeling of the Cytoarchitecture

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Abstract

Integrins, focal adhesions, the cytoskeleton and the extracellular matrix, form a structural continuum between the external and internal environment of the cell and mediate the pathways associated with cellular mechanosensitivity and mechanotransduction. This continuum is important for the onset of muscle tissue generation, as muscle precursor cells (myoblasts) require a mechanical stimulus to initiate myogenesis. The ability to sense a mechanical cue requires an intact cytoskeleton and strong physical contact and adhesion to the microenvironment. Importantly, myoblasts also undergo reorientation, alignment and large scale remodeling of the cytoskeleton when they experience mechanical stretch and compression in muscle tissue. It remains unclear if such dramatic changes in cell architecture also inhibit physical contact and adhesion with the tissue microenvironment that are clearly important to myoblast physiology. In this study, we employed interference reflection microscopy to examine changes in the close physical contact of myoblasts with a substrate during induced remodeling of the cytoarchitecture (de-stabilization of the actin and microtubule cytoskeleton and inhibition of acto-myosin contractility). Our results demonstrate that while each remodeling pathway caused distinct effects on myoblast morphology and sub-cellular structure, we only observed a ~13% decrease in close physical contact with the substrate, regardless of the pathway inhibited. However, this decrease did not correlate well with changes in cell adhesion strength. On the other hand, there was a close correlation between cell adhesion and β1-integrin expression and the presence of cell-secreted fibronectin, but not with the presence of intact focal adhesions. In this study, we have shown that myoblasts are able to maintain a large degree of physical contact and adhesion to the microenvironment, even during shot periods (<60 min) of large scale remodeling and physiological stress, which is essential to their in-vivo functionality.

Introduction

Many living cells proliferate and survive while being strongly associated with the extracellular matrix (ECM), which can have significant effects on their functions [1,2]. The interaction of cells with the ECM and microenvironment is largely mediated through a complex set of interactions between trans-membrane and internal protein complexes and the cytoskeleton (CSK) [1,2]. Trans-membrane protein complexes such as integrins are composed of α and β subunits that can assemble into twenty-four different heterodimers [3,4]. These dimers bind to ECM proteins such as collagen and fibronectin and possess internal cytoplasmic domains that interact with focal adhesion (FA) proteins [1,2]. FAs and integrins mediate the adhesion and interaction of cells with the underlying substrate and allow cells to sense mechanical cues (mechanosensitivity) and respond to local mechanical forces (mechanotransduction) arising in the microenvironment [2,8–11]. Indeed, stretching of the FA and integrin protein complexes through acto-myosin contractility is believed to increase the interaction of FA proteins with actin filaments and lead to integrin clustering [12]. Myosin-II (myoII) is also required for the recruitment of focal adhesion kinase (FAK), zyxin and vinculin; it is not the case for the recruitment of paxillin, talin, and α2-integrin [13]. Conversely, the loss of acto-myosin contractility results in the disassembly of well-defined FA sites and integrin clustering which leads to a loss of cell adhesion [14–16].
Flow chamber assays have been extensively employed to study the fundamental nature of cell adhesion to the microenvironment to better understand the role that integrins and all FA proteins play in cell-substrate interaction. Previous studies have shown that adhesion strength is directly proportional to the number of active integrin bonds that link the cell with its substrate [16–19]. Although it has been acknowledged that integrins contribute the most to the strength of the adhesion, focal adhesion kinase (FAK) also contributes to adhesion strengthening by modulating the binding of integrin available on the cell membrane [18] and inducing a rapid increase in adhesion strength upon integrin activation [15,18]. Several studies used microprinting techniques to create functionalized islands of defined size on which cells were firmly attached and then exposed to a shear stress in order to demonstrate a direct relationship between cell contact area and adhesion strength [12,15,16]. Traditionally, the area bounded by the cell contour has been taken as a measure of cell contact area (or cell size). However, as revealed by interference reflection microscopy (IRM) (sometimes referred to as or reflection interference contrast microscopy), many cells do not display homogenous contact with the substrate throughout their total spreading area [20–23]. These heterogeneously distributed regions of close cell-substrate association indicate that only a fraction of their total spreading area is in close physical contact with the surface. Although IRM was developed for the study of thin films, it has many applications has become useful for the study of cell-substrate interactions [14–16,20–31]. Indeed, it is possible to characterize the nature of close cellular contact with an underlying substrate by examining dark regions in the IRM image [20–23]. By quantifying the total area of low intensity pixels one can define a close contact area (CCA), which corresponds to the total area of the cell membrane that is within ~100 nm of the substrate [20–23]. This subtle, yet important, distinction between total cell spreading area (or size) and CCA has not been explored previously and it is unclear how CCA relates to the dynamics of cellular adhesion.

In this study, we employed IRM to study the nature of the interaction of C2C12 mouse myoblasts with a substrate. Myoblasts are muscle pre-cursor cells that fuse and form myotubes (myogenensis). This process is highly mechano-sensitive, requiring an intact CSK and strong contact and adhesion to the microenvironment [32–38]. It has been demonstrated that myoblasts cultured on hydrogels of increasing stiffness leads to optimal myogenesis on substrates having an elasticity similar to resting muscle tissue [37]. Conversely, the in-vivo microenvironment of a myoblast is highly dynamic, constantly undergoing mechanical stretch and compression. Importantly, it has been shown that when cells experience stretch and compression, dramatic CSK remodeling, depolymerization and fluidization takes place [39,40]. Therefore, in the case of myoblasts, it is unclear if significant remodeling of the CSK and loss of acto-myosin contractility results in a significant loss in CCA and/or adhesion to a substrate.

The objectives of this study were to characterize how the induction of CSK remodeling and inhibition of acto-myosin contractility alters CCA, cell spreading area and adhesion. We demonstrate that myoblasts are capable of maintaining strong adhesion and CCA during CSK depolymerization, the loss of acto-myosin contractility and FA remodeling over short timescales (up to 1 hour). Evidence suggests that during these remodeling processes, integrins and cell-secreted fibronectin remain intact which act to maintain cell adhesion. As well, we also observed a complex relationship between CCA, cell spreading area and adhesion. It is important for myoblasts to maintain contact and adhesion with the microenvironment even as it undergoes mechanical or physiological stress, in order to maintain and rebuild muscle tissue. It is clear from this study that myoblasts are able to maintain adhesion and CCA during dramatic remodeling of the cytoarchitecture over short time scales. We speculate, that this may be part of a coping mechanism to remain in contact with the microenvironment during short periods of activity and during the early myogenic processes [9,37,41].

Results

Effect of Inhibitors on the Morphology of the Cytoskeleton

C2C12 mouse myoblast cells were treated for one hour with one of the following inhibitors: the actin depolymerizing inhibitor cytochalasin-D (CytD), the microtubule (MT) depolymerizing inhibitor nocodazole, the rho kinase (ROCK) inhibitor Y27632, the myosin light chain kinase (MLCK) inhibitor (ML7) and an inhibitor which directly inhibits myoII, blebbistatin. After treatment, cells were fixed and stained to visualize the actin CSK, the MT CSK and the nucleus with laser scanning confocal microscopy (LSCM) (Fig. 1). As can be seen in the figure, untreated cells display classic adherent cell actin stress fibres, MT and nuclear morphologies (Fig. 1A). Treatment with CytD results in complete loss of filamentous actin while the MT remains intact (Fig. 1B). A similar effect is observed in response to Y27632 (Fig. 1D). Conversely, nocodazole causes the loss of filamentous MTs while leaving actin stress fibres intact (Fig. 1C). MyoII inhibition with ML7 and blebbistatin caused the loss of actin stress fibres, while MTs remain intact (Fig. 1E, F).

Time-lapse IRM Imaging and Analysis

In order to investigate the effects of inhibitor treatments on cellular contact with the substrate, we performed IRM time-lapse imaging on cells before and during a 60 min exposure to each inhibitor (Fig. 2). IRM images were acquired every 15 mins for a total of 60 mins without (control) or following the addition of an inhibitor (n = 3 cells for each condition). In every case the t = 0 min image is acquired immediately prior to the introduction of an inhibitor. Control cells imaged over 60 mins reveal almost no significant changes in the IRM images, aside from small intensity fluctuations. In the case of treatment with CytD, it can be

![Image](image-url)
observed that the low intensity regions of the cell at \( t = 0 \) min (corresponding to close contact between the cell and substrate) diminish over the course of 60 mins indicating a decrease in total CCA. Likewise, similar results were also observed for nocodazole and Y27632, ML7 and blebbistatin (data not shown).

In order to quantify the CCA we wrote a script in ImageJ to extract the total area of pixels corresponding to close contact (Fig. 3) in a manner similar to those described previously [20–23]. Low to median intensity pixels in intensity normalized IRM images typically correspond to regions of close physical contact between the cell and substrate when acquired with a high NA objective at visible wavelengths. To calculate CCA, raw IRM images (Fig. 3A) were FFT band-pass filtered resulting in a uniform-intensity background due to the removal of low frequency shadowing typically observed in the raw images (Fig. 3B). In order to further isolate the cell from the substrate, the background was smoothed and lightened using the background subtraction plugin (Fig. 3C). The resulting image was then thresholded to form a binary image (Fig. 3D) where the regions of close contact are distinguished from the background (any artifacts caused by debris on the glass substrate were easily identified and were subtracted manually). The zero intensity pixels in the binary image were then simply summed to provide a measure of CCA. As can be seen in Fig. 3A and D, the image processing accurately captures regions of median to low intensity pixels in the raw IRM images while ignoring high intensity pixels and the background. In all cases, the filters and filter settings were kept constant for all images in a single time-lapse sequence and for all sets of data under varying inhibitor and control conditions. However, as these settings are user defined it is possible to introduce bias into the measurement. In Fig. 3E–G, three user defined thresholding settings are shown which capture varying amounts of the low to median intensity pixels in the IRM image. The cell was then treated with 10 \( \mu \)M EDTA for 60 mins and the absolute CCA as a function of time was calculated (Fig. 3H) using the thresholding settings shown (Fig. 3E–G). By normalizing the data to a percentage change in CCA we show that the three plots are very similar. Therefore, as long as the user sets a reasonable threshold (i.e. capturing only regions within the cell), the change in CCA can be compared between individual cells and experiments. This methodology allowed us to measure the change in CCA over time for control cells and cells exposed to one of the five inhibitors (Fig. 4). Importantly, cells treated with EDTA consistently demonstrated a ~75% decrease in CCA (Fig. 3I, J). Indeed, cells were observed to come off the tissue culture plate (confirmed by focusing above the substrate after the EDTA treatment) and only leaving behind debris. This experiment confirms that dark areas in the IRM images were due to close association between the cell and substrate and that this interaction is dependent on the presence of calcium.

Cell Size Dynamics in Response to Inhibitor Treatments

The average change in cell CCA, under a given set of conditions and as a function of time, was quantified as above and is shown in Fig. 4. Control cells (Fig. 4A) reveal no significant changes in CCA over the 60 min imaging time. However treatment with CytD (Fig. 4B) reveals a rapid decrease in CCA within 15 mins of treatment that remains fairly constant over the remaining imaging time. Nocodazole treatment (Fig. 4C) results in a slow decrease in CCA over 60 mins. Cells exposed to Y27632 (Fig. 4D) only begin to decrease CCA after 30 mins of exposure to the inhibitor. Cells treated with ML7 (Fig. 4E) respond in a similar manner as the CytD treated cells, with rapid decrease in CCA within 15 mins followed by little change. Finally, blebbistatin treatment also results in a slow decrease in CCA, similar to nocodazole treatment (Fig. 4F). What can be observed is that treatments always result in a statistically significant decrease in contact area when compared to the control (\( p < 0.04 \) for each case). However, there is no clear statistical difference between any of the inhibitors after 60 mins (\( p > 0.1 \) for each case). Overall, after a 60 min treatment with any inhibitor the average decrease in CCA was ~13%. On the other hand, the absolute cell spreading area (determined by calculating the total area defined by the cell boundary) was shown to significantly decrease by ~50% in response to the various inhibitors compared to the control (\( p < 0.01 \) with no significance between them \( p > 0.1 \)), except in the case of blebbistatin which does not change with respect to the control (Table 1). In order to confirm that the change in CCA was not simply due to the cells contracting and lifting off from the substrate, we measured cell height (Table 1) by recording the distance between the apical and basal membrane above and below the nucleus in LSCM images \( n = 10 \) cells for each condition). We observed no appreciable change in cell height after exposure to each inhibitor \( p > 0.2 \). It is tempting to interpret this as due to a decrease in volume, however, volume measurements with traditional confocal microscopy are challenging due to the nature of the axial PSF. Therefore, we only conclude the apparent decrease in CCA is not due to the cells moving up and away from the surface.

Changes in Adhesion Strength during Inhibitor Treatments

In order to examine if changes in CCA are correlated to changes in cell adhesion strength we fabricated a parallel plate flow chamber (Fig. 5A–C). Cells were pretreated with each inhibitor for 60 min prior to the onset of a high flow producing a
shear stress of 65 Dyne/cm² [42]. The percentage of cells remaining after 30 min of continuous shear stress allowed us to qualitatively determine the change in adhesion strength relative to untreated (control) cells (Fig 5E, D). Consistent with previous work, we observed a ~9% decrease in the number of control cells exposed to shear stress [14]. What is immediately evident is that loss of the actin CSK in response to CytD treatment results in a complete loss of cell adhesion as no cells remained after 30 min of shear stress. We also observed a significant decrease in the number of cells remaining after treatment with nocodazole, ML7 and blebbistatin (p<0.05) when compared to control cells. Interestingly, ROCK inhibition with Y27632 did not cause a statistically significant decrease in remaining cells compared to the control (p>0.9).

Remodeling of Adhesion Structures in Response to Inhibitor Treatments

FA, integrins and extracellular matrix proteins are critical structures that link the CSK to the extracellular environment. In order to probe the influence of the various inhibitors on the molecular basis of cellular CCA and adhesion we performed IRM imaging on live cells transiently expressing vinculin-EGFP (Fig. 6). In untreated cells, vinculin-EGFP displays typical punctate morphologies at FA sites (known to be linked to actin stress fibres) and corresponds well to darker regions of the IRM image as expected (Fig. 6A). However, there are a number of lower intensity regions in the IRM image with no vinculin-EGFP content. Interestingly, cells treated with Y27632 continue to display point-like vinculin-rich structures, though smaller in size and number (Fig. 6D). Nocodazole (Fig. 6C) and ML7 (Fig. 6E) treatment reduced the vinculin to only a few point-like structures whereas CytD (Fig. 6B) and blebbistatin (Fig. 6F) resulted in a diffuse distribution of vinculin-EGFP throughout the cell. As shown above (Fig. 3), treatment with the calcium chelator, EDTA, resulted in the complete detachment of cells from the substrate (under no flow). Therefore, cell-substrate adhesion is clearly dependent on the presence of calcium, implicating integrin function [3,4]. We also fixed and methanol extracted cells to image the distribution of β1-integrin and cell-secreted fibronectin on the surface after 60 min of inhibitor treatment. In all cases LSCM images were collected at the planes containing the basal membrane and substrate in order to examine β1-integrin (Fig. 7) and fibronectin (Fig. 8) at the cell-substrate interface. After CytD treatment there is a complete loss of β1-integrin (Fig. 7B), but in all other cases it is found throughout the total cell contact area. β1-integrin was observed distributed diffusely throughout the cell spreading area, rather than localized in fibrillar or focal sites, as expected for C2C12 cells cultured on non-functionalized glass substrates for only 24 hours [43,44]. After 24 hours of culture, cell-secreted fibronectin was found deposited on the glass substrate and in higher amounts beneath the cells before and after treatment with inhibitors. No fibronectin staining was observed on glass substrates without cells, which excludes the possibility of non-specific binding of the primary and secondary antibodies (data not shown). Taken-together, the results presented in this study reveal a complex relationship between the inhibition of various structures and pathways in the cell and changes in CCA, absolute cell size, cell adhesion and the presence and distribution of FA sites, β1-integrin and cell-secreted fibronectin. The results are summarized in Table 2 and are discussed further below.

Discussion

Myoblasts are muscle precursor cells that can be mechanically stimulated to fuse and form myotubes and eventually new muscle tissue [32–36]. This process relies on the ability of these cells to sense mechanical properties and mechanical cues in their surrounding microenvironment [37]. This ability requires robust cell adhesion to the microenvironment via integrins and FA sites as well as an intact CSK for the transmission and conversion of mechanical information into biochemical signaling [2,8,9]. Importantly, when cells are exposed to physical stretch and compression this results in CSK remodeling, depolymerization and fluidization [32–36,39,40] which will change the nature of adhesion to the microenvironment. It remains unclear if myoblasts can maintain physical adhesion and contact to a substrate during CSK remodeling even though these processes are fundamentally linked to their function. Therefore, in this study we utilized IRM to examine the contribution of the CSK and acto-myosin contractility on the maintenance of cell-substrate contact and adhesion in C2C12 mouse myoblasts. We employed five inhibitors to examine the early response of myoblasts to actin and MT CSK remodeling as well as the inhibition of acto-myosin contractility regulators.

In previous studies, the relationship between total cell contact area and adhesion strength has been explored by confining cells to microprinted islands of adhesive molecules [14–16]. This allows the total contact area of the cell to be defined by the island size. In contrast, we employed IRM imaging to only determine the area occupied by the cell membrane close to the substrate, which we define as the close contact area (CCA). In this study, we examined the relationship of CCA with cell adhesion regardless of area defined by the cell membrane contour. This provides a more direct measure of what fraction of the cell remains in close physical contact with the substrate during dynamic remodeling of the cytoskeleton and if this contact corresponds to adhesion strength. Five specific inhibitors were employed in this study to remodel the actin CSK (CytD), the MT CSK (nocodazole) and inhibit regulators of acto-myosin contractility such as ROCK (Y27632), MLCK (ML7) and myoII (blebbistatin). Each caused distinct morphological changes in the cytoarchitecture. CytD causes a total loss of F-actin while leaving MTs intact. Nocodazole results in the collapse and depolymerization of MTs while leaving F-actin intact. Y27632 and ML7 both inhibit upstream regulators of myoII (ROCK and MLCK, respectively) and cause a loss of acto-myosin contractility. In addition we also inhibited myoII directly with blebbistatin. The loss of acto-myosin contractility results in the partial loss of a well-defined actin CSK while leaving MTs intact. Moreover, the loss of intact actin stress fibres in response to CytD

**Figure 3. Computational analysis of cell contact area in IRM images.** (A) Raw IRM image of a cell adhered to a glass substrate (scale bar = 25 μm, and applies to all). (B) The same image after applying a FFT band-pass filter to create a uniform intensity background. (C) The image after applying a background subtraction/lightening filter to isolate sites of close cellular contact from the background. (D) The image is then thresholded to form a binary image where regions of close contact (corresponding to grey values lower than the background intensity in (C)) are set to zero intensity. Artifacts arising from debris are manually removed and then the zero intensity pixels are summed to provide a measure of cell CCA. (E-G) The effect of different user specified thresholds (shown in red) yield different values of the (H) absolute CCA as a function of time. (I) Normalizing the data yields the change in CCA and all three curves fall on one another. (J) Time-lapse imaging of C2C12 cells during exposure to EDTA. All cells showed a consistent decrease in contact area of about 75% and that CCA is calcium dependent.

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and blebbistatin treatment results in the complete loss of intact FA sites. Other treatments only resulted in a decrease in the number and size of FA sites. Conversely, $\beta_1$-integrin was found distributed throughout the total cell area (defined by the cell contour) after treatment with each inhibitor with the exception of CytD, in which there was a complete loss of $\beta_1$-integrin.

However, although each inhibitor caused clear morphological changes in the cytoarchitecture and adhesion sites we only observed a $\sim13\%$ decrease in CCA with no statistically significant dependence on the inhibitor used. In each case, with the exception of blebbistatin, cells also decreased by $\sim50\%$ in total size relative to the control. Therefore, the ratio of CCA to absolute cell size increases during exposure to these inhibitors except for blebbistatin in which the ratio decreases. This implies that the proportion of the cell area in close contact with the surface actually increases in response to CSK, ROCK and MLCK inhibition but decreases in response to direct myoII inhibition. We also confirmed that the decrease in CCA was not due to cells contracting and moving away from the surface, as the average cell heights remain constant with each treatment. In order to examine if changes in CCA are reflected in adhesion strength, we subjected cells to a shear flow assay. Cells were first treated with each inhibitor for 60 mins prior to exposure to a shear stress. Although each inhibitor produced a similar decrease in cell CCA, cell adhesion had a clear dependence on the inhibitor used. Loss of the actin or microtubule CSK resulted in a 100% or $\sim50\%$ decrease in cell adhesion respectively. On the other hand inhibition of ROCK did not cause any significant change in cell adhesion. Whereas inhibition of MLCK or direct inhibition of myoII caused a $\sim25\%$ decrease in cell adhesion. This implies that though MLCK and ROCK both act to promote myoII contractility, it appears that MLCK may play a larger role in the maintenance of downstream cell adhesion. In each case, cell-secreted fibronectin was found distributed over the glass surface and in higher amounts directly underneath the cell body. Therefore, with the exception of CytD-treated cells, adhesion is likely maintained through integrin mediated links to underlying ECM proteins.

In brief, the CCA dynamics described above do not tend to correlate very well with adhesion strength, which has been previously shown to be dependent on the $\beta_1$-integrin expression and intact vinculin-rich FAs [16–19]. As expected, we found that the strength of adhesion is correlated with the interplay between $\beta_1$-integrin expression and the existence of intact vinculin-rich FAs. Indeed, depolymerization of actin CSK induced a loss of $\beta_1$-integrin expression along with point-like FAs. These findings suggest that the actin CSK is important for cell contact, cell-substrate adhesion strength and cell FA maturation. However, the MT CSK has also an equally important role in governing cell contact and adhesion, showing a significant reduction in cell-substrate adhesion strength in MT-depleted cells. Although the role of MTs in governing cell mechanics and cell adhesion are often ignored, an increasing number of studies have shown that they play a crucial role in governing cell traction forces and other

| Treatment | Change in CCA (%) | Cell Height ($\mu$m) | Cell Area ($\mu$m$^2$) |
|-----------|------------------|---------------------|-----------------------|
| Control   | $1\pm3\%$       | 11.0±2.0            | 2519±815              |
| CytD      | $-18\pm5\%$     | 10.2±1.2            | 1247±503              |
| Nocodazole| $-14\pm4\%$     | 11.0±1.2            | 1186±814              |
| Y27632A   | $-9\pm2\%$      | 10.1±1.3            | 1219±410              |
| ML7       | $-12\pm4\%$     | 10.2±2.7            | 1376±659              |
| Blebbistatin| $-13\pm3\%$    | 9.6±1.8             | 2687±826              |

Table 1. CCA, Cell Height and Cell Area after 60 min of exposure to various inhibitors.

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Figure 4. Dynamic changes in C2C12 close contact area over 60 mins. Cells were treated with various inhibitors and IRM images were acquired every 15 mins for 60 mins. (A) Control, (B) Cyt-D, (C) nocodazole, (D) Y-27632A, (E) ML-7 and (F) blebbistatin treated cells (n = 3 cells for each condition). In each case, except for the control, a significant decrease in contact area occurs after 60 mins of exposure to each inhibitor. However, there was no statistically significant dependence on the type of inhibitor used. On average, the $\Delta$CCA was $\sim13\%$ for each condition, except for the control.

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Figure 5. Flow chamber adhesion assay and changes in cell adhesion strength. (A) In face flow chamber dimensions: L = 2 cm and W = 1 mm. (B) SU-8 master used to fabricate flow chamber with a height of 160 μm. (C) Water with red dye shows the flow chamber working principle with bent stainless steel needle attached to the inlets and outlets allowing media to be pumped through the chamber. (D) Typical adhesion assay where cells were exposed to shear stress over a period of 30 min. Cells were counted before and after flow to quantify changes in cell-substrate adhesion strength (scale bar = 500 μm). (E) Bar graph showing the ratio of cells remaining on the glass substrate after exposition to a shear stress of 65 Dyne/cm² over a period of 30 min. There is no significant difference between the control and Y27632 treatment (p>0.1). All other treatments yield a significant decrease relative to the control (p<0.04).

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mechanical dynamics [45–47] as they form part of the highly cross-linked CSK network in the cell. Interestingly, inhibition of myoII by blebbistatin also induced a significant decrease in cell adhesion but maintained a significantly stronger adhesion than actin-deprived cells despite the loss of point-like FAs. It has been shown that the recruitment of vinculin to FAs enhances adhesion in fibroblast cells [16]. Our findings demonstrate that intact and point-like FAs are not necessary for maintaining cell adhesion but β1-integrin is essential. Though each inhibitor used in this study had a clear effect on cell adhesion, it is evident that they do not disrupt the majority of cell contacts [24]. Clearly, FAs can be maintained in the absence of an intact MT CSK and during the inhibition of ROCK and MLCK [9,48]. However, the dynamics of FAs during maturation, motility and actin contractility require the activity of myoII [1,9,48].

We have found that there is a complex relationship between cellular CCA, cell-adhesion and regulators of cytoskeletal remodeling and acto-myosin contractility. These structures are critical in myoblast cells that possess distinct mechanotransduction pathways, which are activated in response to local mechanical forces [37,49,50]. IRM imaging has revealed that the cellular CCA is decreased by ~13% after 60 mins of exposure to relatively high concentrations of inhibitors. Regardless of the dramatic remodeling of the cytoarchitecture, myoblasts are still able to maintain a large degree of contact and adhesion to the substrate [24]. Laminin specific dystroglycan complexes and hemidesmosomes also play an important role in the adhesion and physiology of myoblasts and myotubes [37,38]. The presence of specific integrin proteins in dystroglycan complexes and hemidesmosomes provides binding specificity to laminin, which is found in the ECM [51]. Importantly, myoblasts do not form dystroglycan complexes which are reserved for post-fusion myotubes [52]. In the case of hemidesmosomes, these protein complexes also form a link to laminin by mediating the interaction with intermediate filaments.

### Table 2. Changes in CCA, Cell Area, Adhesion, FAs and β1-integrin for control cells and cells exposed to various inhibitors for 60 min (↓ = decrease, NC = no change from the control, D = diffuse, PL = point-like, NP = not present and P = present).

| Treatment  | CCA  | Cell Area | Adhesion | FAs  | β1  |
|------------|------|-----------|----------|------|-----|
| Control    | NC   | NC        | ↓ 9%     | PL   | P   |
| CytD       | ↓    | ↓         | ↓ 100%   | D    | NP  |
| Nocodazole | ↓    | ↓         | ↓ 48%    | PL   | P   |
| Y27632     | ↓    | ↓         | ↓ 9%     | PL   | P   |
| ML7        | ↓    | NC        | ↓ 23%    | D    | P   |
| Blebbistatin| NC  | NC        |          |      |     |

Figure 6. Overlay of IRM imaging and vinculin expression after 60 mins. Simultaneous IRM and fluorescence imaging of live cells expressing Vinculin-EGFP after exposure to inhibitors for 60 min. (A) Control (scale bar = 20 μm and applies to all), (B) Cyt-D, (C) nocodazole, (D) Y-27632A, (E) ML-7 and (F) blebbistatin treated cells. Cells treated with nocodazole, Y27632 and ML7 posses smaller but point-like FA structures, similar to the control. Vinculin becomes diffuse after treatment with CytD and blebbistatin.

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Figure 7. Fixed and stained images of β1-integrin and the membrane of C2C12 cells. Cells were exposed to inhibitors for 60 min and then fixed and stained for the presence of β1-integrin (greyscale and red in the merge) and the plasma membrane (green in the merge). LSCM images were acquired at the cell-substrate interface. (A) Control (scale bar = 20 μm and applies to all), (B) Cyt-D, (C) nocodazole, (D) Y-27632A, (E) ML-7 and (F) blebbistatin treated cells. In all cases, integrin-β1 is well distributed over the cell contact area. However, after 60 min of CytD treatment a significant decrease in integrin-β1 was observed, correlating to a significant decrease in cell adhesion strength (Fig. 5).

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all cases, cell-secreted fibronectin was distributed over the substrate
nocodazole, (D) Y-27632A, (E) ML-7 and (F) blebbistatin treated cells. In all
cases, cell-secreted fibronectin was distributed over the substrate and found in higher concentrations underneath the cell body.

Figure 8. Fixed and stained images of fibronectin and the
membrane of C2C12 cells. Cells were exposed to inhibitors for
60 min and then fixed and stained for the presence of fibronectin (Fn,
greyscale and green in the merge) and the plasma membrane (red in the
merge). LSCM images were acquired at the cell-substrate interface.

(A) Control (scale bar = 20 μm and applies to all), (B) Cyt-D, (C)
nocodazole, (D) Y-27632A, (E) ML-7 and (F) blebbistatin treated cells. In all
cases, cell-secreted fibronectin was distributed over the substrate
and found in higher concentrations underneath the cell body.

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[53]. However, the myoblasts used in this study were cultured on
unfunctionalized glass, which eliminates the contribution these
complexes, in addition to the low expression of dystroglycan
complexes in non-fused single myoblast cells [52]. Regardless, in
vivo, these structures would only act to enhance the ability
of myoblasts to maintain adhesion during physiologically or me-
chanically mediated CSK remodeling. Although the inhibitors
used in this study represent extreme conditions, it is clear that
myoblasts can still maintain close contact with the substrate.

Extensive, future work can be undertaken to further elucidate a
detailed mechanistic picture of which proteins are involved in the
maintenance of CCA, FA sites and adhesion strength during
cytoskeletal remodeling. However, our results clearly implicate the
role of integrin-mediated links to the ECM. Emerging approaches
might include assessing changes in FA and ECM composition,
specifically at the cell-surface interface, by employing mass-
spectroscopy and proteomic strategies [54,55]. As recently shown,
FA sites are extremely complex and can consist of hundreds of
different proteins which may change in abundance in response to
inhibitors and stress [54,55]. This flexibility may permit the
maintenance of FAs, CCA and adhesion during periods of stress.

The ability to maintain cell-substrate contact and adhesion
under mechanical or biochemical stress may be part of a coping
mechanism which enables myoblasts to quickly recover after
removal of such stress [24]. In this study, we have shown that
although the physiology of myoblast cells requires the integration
of a mechanical and biochemical continuum between the ECM,
integrins, FAs and the CSK, it is not necessarily required to
maintain cell-substrate contact during short periods of stress. It
appears that myoblasts are able to maintain a high degree of
physical contact with the microenvironment during cytoarchitec-
tural remodeling. This is important to myoblast function as
mechanical stretch and compression causes large changes in CSK
morphology and acto-myosin contractility [12,56]. Therefore,
although cell-substrate contact, adhesion and mechanosensitivity/
mechanotransduction pathways are functionally integrated, they
clearly posses distinct and time-dependent cytoarchitectural
requirements.

Materials and Methods

Cell Culture and Transfections

C2C12 mouse myoblast cells were obtained from the American
Type Culture Collection (CRL-1772). Cells were maintained in a
37°C, 5% CO₂ incubator and cultured in DMEM with 10% fetal
bovine serum (FBS) and 50 μg/ml streptomycin and 50 U/ml
penicillin antibiotics (all from Hyclone Laboratories Inc.). For
microscopy experiments cells were plated in 35 mm glass bottom
dishes (Mat Tek) the day before experiments. In some cases
C2C12 cells were transfected vinculin-GFP (kindly provided by
Benny Geiger) using Lipofectamine 2000 (Invitrogen) according to
the manufacturer’s instructions.

Inhibitors

Cells were treated with either cytochalasin-D dissolved in dH₂O
(15 μM), nocodazole dissolved in DMSO (15 μM), Y27632
dissolved in dH₂O (15 μM), ML-7 dissolved in dH₂O (40 μM)
or blebbistatin dissolved in DMSO (15 μM). All inhibitors were
purchased from Sigma and the concentrations noted are the final
concentrations in the cell culture dish.

Immunofluorescence Staining

Briefly, cells were first fixed with 3.5% paraformaldehyde and
permeabilized with Triton X-100 at 37°C. Staining for actin was
accomplished with Phalloidin Alexa Fluor 346 (Invitrogen).
Following this MTs were stained on ice with a mouse monoclonal
anti-α-tubulin (Sigma) primary antibody and an Alexa Fluor 488
rabbit anti-mouse immunoglobulin (Invitrogen) secondary antibody.
DNA was labeled with DAPI (Invitrogen), also on ice. For staining
of β1-integrin or fibronectin cells were first fixed by paraformal-
dehyde and subsequently extracted by methanol. For integrin
staining, anti-β1-integrin antibody [P5D2] (Abcam) and Alexa
Fluor 546 rabbit anti-mouse immunoglobulin (Invitrogen) were used
as primary and secondary antibodies, respectively. Cells were also
stained with wheat germ agglutinin coupled to Oregon Green 488
(Invitrogen) to reveal the cell membrane. For fibronectin staining,
anti-human fibronectin antibody F3648 (Sigma) and Alexa Fluor
547 goat-anti-rabbit immunoglobulin (Invitrogen) were used,
respectively. Cells were also stained with wheat germ agglutinin
coupled to TRITC (Invitrogen) to reveal the cell boundaries.

Microscopy

All images of living and fixed cells were acquired on a TIE A1-R
laser scanning confocal microscope (Nikon) with a 60X/NA1.4 oil
immersion lens. Images of fixed cells were acquired with a
standard LSCM configuration with appropriate laser lines and
filter blocks. IRM imaging was performed with a 640 nm diode
laser in a standard LSCM IRM configuration [20], in which the
excitation and emission light is of the same wavelength. IRM
time-lapse imaging was performed with a perfect focus system (Nikon)
to prevent focus drift. Cells were maintained at 37°C in a
temperature-controlled stage and buffered in order to maintain
pH. Images were acquired every 15 mins for a total of 60 mins.

Image Analysis

All images were processed and analyzed with ImageJ (http://
rsweb.nih.gov/ij/). Brightness/contrast adjustments were the
only processing steps applied to raw IRM, immunofluorescence
or vinculin-EGFP images presented in this study. For quantifica-
tion of IRM images, low intensity regions of the image were
characterized to record changes in close cellular contact with the
surface (described in the main text). To isolate these close contact
regions and determine their total area we first created an image
stack containing a particular cell imaged at 0, 15, 30, 45 and
60 min. We then applied a FFT band-pass filter followed by a
background lightening in order to isolate the cell from the
background. Thresholding was applied to create a binary image
mask of the contact regions of the cell. Finally, any pixels
corresponding to regions of the cell in close contact with the surface in the resultant binary image were summed to calculate the change in close contact area as a function of time.

Parallel Plate Flow Assay

A polydimethylsiloxane (PDMS) microfluidic flow chamber, 1000 µm in width, 160 µm in height and 20 mm long, was produced using standard microfabrication techniques briefly described here. First a negative master was made with a SU-8 photosist. Liquid-state 1:10 ratio PDMS was poured on the master, solidified by baking at 80 °C for at least 2 hours and then carefully peeled off. Inlets and outlets, 0.75 mm in diameter, were punched at both ends of the chamber. The PDMS layer was bonded to a standard glass microscope slide after being plasma treatment (100 W for 30 sec), creating an enclosed channel. Finally, tubing was attached to the inlets and outlets allowing media to be flowed through the chamber. Cells were cultured inside the chamber overnight and the next day cells were first exposed to each inhibitor for 60 min under no flow. The cells were exposed to an average flow of 330 µl/s, for 30 min, which corresponded to a shear stress at the wall of the chamber of 65 dyn/cm² [42]. All experiments were preformed inside a cell culture incubator. Using a microscope, cells were counted from the same region of interest before and after exposure to shear stress.

Statistical Tests

All values quoted in this study are the average ± standard deviation. For comparisons between populations of data a student’s t-test and one-way ANOVA combined with a Tukey post-test were performed to determine statistical significance (α = 0.05).

Author Contributions

Conceived and designed the experiments: AEP. Performed the experiments: DM DT CG NVB. Analyzed the data: DM DT AEP. Contributed reagents/materials/analysis tools: DM DT AEP. Wrote the paper: DM DT CG NVB AEP.

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