Force-Signaling Coupling at Single Focal Adhesions

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Abstract:

Cell adhesion to extracellular matrices (ECM) is regulated by integrin receptors. After binding to ECM proteins, integrin clustering occurs to form focal adhesion (FA) complexes. These complexes contain proteins that link the cell cytoskeleton to the ECM, providing cell anchorage by mechanical transmission of adhesive forces that drive signaling, proliferation, and tissue morphogenesis. These complexes also act as signaling effectors that regulate cell division, migration, and differentiation. However, not much is known about the relationship between force generation and FA signaling, as there are many signaling proteins that interact within FAs. One of these proteins, focal adhesion kinase (FAK), is known to be a key player in the entirety of this signaling mechanism. As such, it has been postulated that FAK is also involved in force modulation at FAs. Therefore, in this thesis I analyze the relationship between FAK concentration (intensity of phosphor-FAK Y397 stained fluorescence) and force exerted at single adhesions measured with the use of microfabricated post-array-detectors (mPADs). With the information garnered from studies like these, we can build upon and continue to detail the model of cellular adhesion which we have very little information about. This research was conducted in a regenerative medicine setting, and as such, human mesenchymal stem cells (hMSCs) were used for this study. In a preliminary trial, this analysis revealed that FAK concentration is independent of forces exerted at focal adhesions after comparison between control and blebbistatin treated groups. However, in a secondary trial, a significant relationship was shown between total FAK concentration and force, as well as phosphor-FAK Y397 and force ($P < 0.05$). This secondary analysis suggests that FAK may be involved with force modulation.

Introduction:

Cells are highly dependent on environmental factors for survival, as these can affect trends in cell motility, cell proliferation, and differentiation [3]. One key environmental factor is cellular adhesion to an extracellular matrix (ECM) as many internal cellular processes rely on force modulation between the cell and its environment. Specialized protein structures called focal adhesions (FAs) allow cells to physically adhere to their external environment [5]. FAs also act as a point of force transmission between the ECM and the cytoskeleton of a cell. Specifically, FAs are able to relay changes in forces produced by the actin cytoskeleton to internal signaling

molecules and to external ECM proteins [1]. FAs themselves also serve as a major hub for signaling molecules [5]. Therefore, in understanding FAs, we can also begin to understand a variety of fundamental cell behaviors from a new point of view.

Research into cell force transduction has moved towards understanding the variety of proteins functioning within and around the FA complex. While this encompasses proteins like talin and vinculin, focal adhesion kinase (FAK) is a protein identified to be a key regulator of FAs [5]. Primarily, FAK has been shown to regulate FA assembly and disassembly through interactions with structural proteins that make up the FA and interactions with signaling proteins, such as paxillin and Src [3]. FAK interacts with these proteins though phosphorylation, utilizing its main kinase domain. However, FAK has also been discovered to have a variety of functions that pertain to entities other than FAs. FAK has been shown to aid in the formation and degradation of cadherins (for cell-cell adhesion), aid in p53 degradation to enhance cell survival, and aid in cell motility through phosphorylation of rho-family GTPase activators and inhibitors [5]. Although we know a great deal about the basics of FAK, we have yet to understand the underlying mechanisms in a multitude of cases.

In other contexts, still more is unknown about FAK. Human mesenchymal stem cells (hMSCs) are human derived stromal cells that are able to differentiate into a variety of specialized cells types [6]. A large amount of research has been conducted between FAK and hMSCs on the topic of differentiation. As FAK is involved with survival, cell motility, and environmental modulation. FAK has been proposed to be a differentiation factor, especially for osteoblasts [4]. Yet, little research has been conducted on understanding the FA mediation characteristic of FAK in hMSCs. With hMSCs being a key player in regenerative medicine, understanding how they adhere to their environment is important in aiding treatments and therapies that require hMSCs. For example, hMSCs are used in seeding 3D matrices to create tissues. Understanding how they adhere to that matrix is important in comprehending specific cell phenotypic expression occurences under varied conditions.

With a multitude of interactions made possible by distinct activation states of FAK, the variety of functions FAK is involved with, and the sizable number of FAK's mediators, an in depth analysis of FAK is still being developed [3]. Yet, there are avenues that are progressing. By understanding how adhesion force, a unifying commonality between FAK and its

downstream phosphorylation targets is affected by FAK, we were able to gain insight into specific interactions FAK is having with FAs to modulate force. This approach views the cellular adhesion mechanism as a 'function' with various forces as inputs and FAK concentration as an output. By observing how FAK reacts to various forces, we are able to develop a solid relationship between these two factors. Although our first trial in this study was not successful, we expect to test other variables and develop the inner workings of this cellular adhesion 'function'.

The study detailed in this thesis explored the idea proposed above, and strives to help solidify a link between force transduction mechanisms and FAK. A few studies of this nature have been already conducted, involving established cell lines [7]. In response, this study was conducted with low passage hMSCs. By using hMSCs, we were also able to gain understanding of the role FAK plays in this specific cell type, as FAK has only been studied as a differentiation factor in this context. As we have begun to understand FAK and its many forms, we are also finally beginning to understand how FAK acts as a force transduction mediator. Therefore the research presented here on the role of FAK in force mediation within hMSCs is the next logical step in unraveling the ambiguity behind the proteins involved with focal adhesions.

Materials and Methods:

In order to find the relationship between FAK and adhesion force, we first quantified each. By exposing FAK proteins in fixed cells to primary and secondary antibodies, we stained for FAK with a fluorescent tag. In quantifying the intensity of the fluorescence, we can generalize the relative concentration of FAK. As for quantifying force, we can use microfabricated post-array-detectors (mPADS) to calculate the force a cell generates at a focal adhesion. mPADs are a series of pillars in a hexagonal pattern that can be made to have varying 'stiffness' by changing pillar height. Shorter pillars will be harder to deflect, while taller ones will deflect easily. Cells are seeded onto mPADs, and the resulting post deflections are measured to determine the force at single focal adhesions in the cell.

Cell Culture: Cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 1% sodium pyruvate, and 1% penicillin-streptomycin. mPAD device silicon masters were prepared as described previously [2]. In brief, elastomeric micropost arrays were

fabricated using PDMS replica molding. To make microfabricated post array templates, 1:10 PDMS prepolymer was cast on top of silanized mPAD device silicon masters, cured at 110 °C for 30 min, peeled off gently, oxidized with oxygen plasma (Plasma-Preen; Terra Universal), and silanized overnight with (tridecafluoro-1,1,2,2,- tetrahydrooctyl)-1-trichlorosilane (Sigma–Aldrich) vapor under vacuum.

Traction Force Measurements: To make the final PDMS mPAD device, 1:10 PDMS pre-polymer was cast on the template, degassed under vacuum for 20 min, and cured at 110 ℃ for 20 h and gently peeled off the template on a 25 mm diameter #1 circular coverslip (Electron Microscopy Services). Peeling induced collapse of the mPADs was rectified by sonication in 100% ethanol, followed by supercritical drying in liquid CO_2 using a critical point dryer (Samdri-PVT-3D; Tousimis). Flat PDMS stamps were generated by casting 1:20 PDMS pre-polymer on flat and silanized silicon wafers. Stamps were coated in a saturating concentration of fibronectin (FN, Thermo Fisher D307) (50 μg/ml in PBS) for 1 h. These stamps were washed in sterile distilled water and dried under a stream of nitrogen gas. Subsequently, fibronectin-coated stamps were placed in contact with surface-oxidized mPAD substrates (UVO-Model 342; Jelight). mPAD substrates were labeled with 5 μg/ml DiD' (Invitrogen) in distilled water for 10 min. mPAD substrates were subsequently transferred to a solution of 0.2% Pluronics F127 (Sigma–Aldrich) for 30 min, to prevent nonspecific protein absorption.

mPADS were then seeded with hMSCs (Passage Number = 4). Once hMSCs had been cultured and expanded, hMSCs were seeded at 50,000 cells per device in growth medium and then allowed to spread overnight. Before staining, 100 μM of blebbistatin, a compound that reduces actin-myosin contractility, was administered to three samples out of 6. This treatment allows for comparison of FAK localization and concentration between cells that exert normal amounts of force, and cells that are inhibited in generating forces. For staining of FAs, cells cultured overnight on FN-coated surfaces were rinsed and permeabilized in cytoskeleton-stabilizing buffer (0.5% Triton X-100, 10 mM PIPES buffer, 50 mM NaCl, 150 mM sucrose, 3 mM $MgCl_2$, 1 μg/mL leupeptin, 1 μg/mL aprotinin) for 10 min, fixed in 3.7% formaldehyde for 5 min, blocked in 33% goat serum in PBS, and incubated with primary antibodies against FA components overnight (Abcam 39967 against pFAK Y397) followed by AlexaFluor 405-labeled secondary antibody for one hour (Jackson Labs).

Traction Force Microscopy: Following successful staining, confocal images of FAs were taken with a Nikon C2 module connected to a Nikon Eclipse Ti inverted microscope with a high magnification objective (60X Plan Apo VC Water Immersion objective, NA 1.2). Cell spreading and FA size were quantified and calculated using Nikon NIS-Elements Analysis Software and ImageJ. For final force measurements, the top and bottom of the posts were sequentially imaged and the deflection measured. The resulting force, *F*, was calculated using Euler-Bernoulli beam theory shown below:

$$F = \delta \frac{3\pi E D^4}{64 L^3}$$

E, *D*, *L* and δ are the Young's modulus, post diameter, post height, and post deflection respectively

Statistical Analysis: After forces were calculated, Welch's t-tests, Mann-Whitney tests, linear regressions and post hoc tests were performed in GraphPad Prism. A p-value < 0.05 was considered significant.

Results:

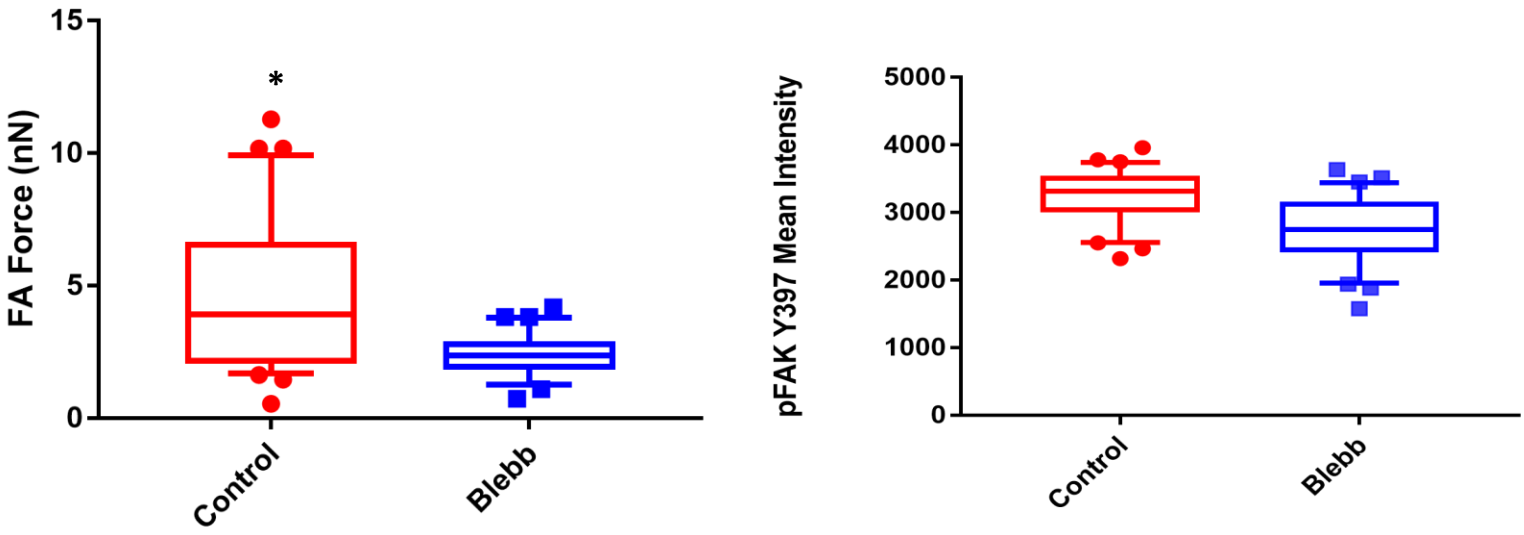

Figure 1. Focal Adhesion Force and pFAK Y397 Mean Intensity per group

Box-Whisker Plots (median, 10th, 25th, 75th, and 90th percentile) for focal adhesion force and pFAK mean intensity per group. Samples treated with blebbistatin were treated with a concentration of 100 μM for one hour. Welch's t-test was conducted on the mean intensity data between each group and the Mann-Whitney test was conducted on force data between each group. * indicates $P < 0.05$ between groups. For each group (control and blebbistatin treated) 35-40 focal adhesions were analyzed.

From the data gathered from images of the mPADs, we can clearly see that untreated hMSCs exerted significantly higher forces compared to blebbistatin treated cells. Such a result is expected, as blebbistatin is a contractility inhibitor and should decrease the amount of force exerted by cells as it inhibits actin contractility. We also see that although there was a slight increase in pFAK means intensity, there was no significant difference in terms of pFAK concentration between the two groups. These two factors were then combined and viewed as a scatter plot, to assess how local adhesive force relates to pFAK Y397 levels at single FAs.

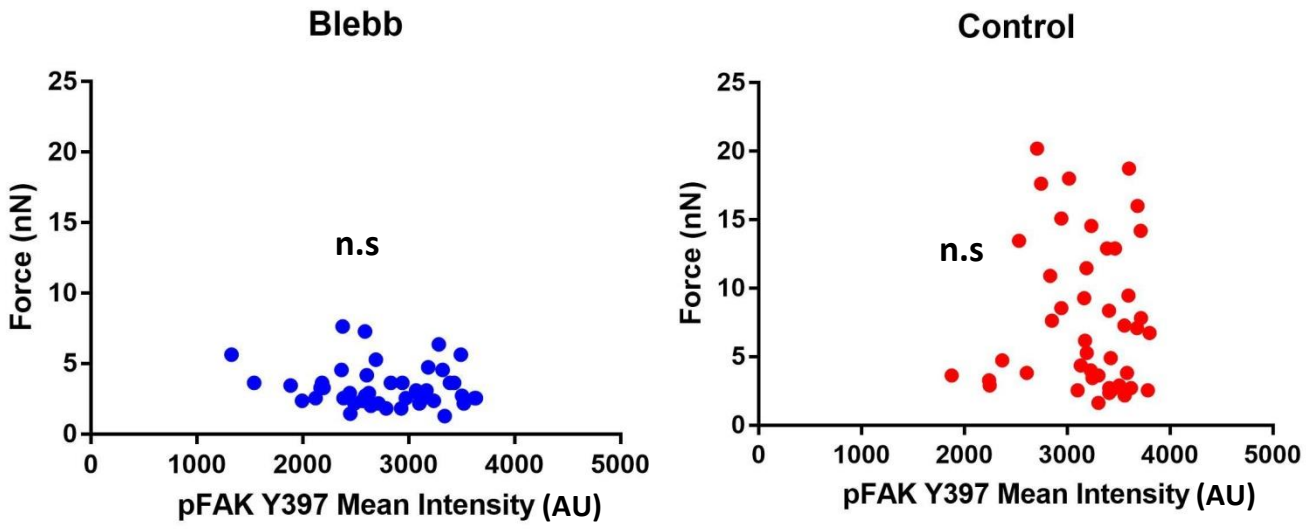

Figure 2. Focal Force vs. pFAK Y397 Mean Intensity

Scatter Plots for focal adhesion force vs. pFAK mean intensity. For each group (control and blebbistatin treated) 35-40 focal adhesions were analyzed. Linear regressions were found to be not significant ($P > 0.05$).

By comparing total force and mean intensity against each other, we can see that neither group exhibited a correlation between pFAK Y397 mean intensity and force at a single focal adhesion. Linear regressions showed non-significant deviation from a slope of zero. However, the decrease in force between is still retained and apparent in the blebbistatin treated group.

Discussion:

FAK is able to interact with FAs and regulate their formation, but FAK has also been suspected to play a role in unexpected places. FAK is able to interact with cadherins, maintain FA structure during endosomal transport, and conduct signaling within the nucleus [3]. As such, FAK is part of modulating cell survival and motility. In addition to these, from the data collected, we are able to say that FAK is marginally involved with force transduction in hMSCs. pFAK Y397 staining revealed that the untreated hMSCs had higher levels of pFAK Y397 at focal adhesions, which suggests that higher forces promote FAK Y397 phosphorylation. We also see that contractility is important for FAK Y397 phosphorylation. It is notable to consider how force and intensity scatter plots showed no significance between these two factors. From this, we find that local FA force is independent of FAK Y397 phosphorylation levels at single FAs. The relationship between force and signaling remains unclear, and our preliminary study suggests that signaling is independent of local force at FAs. However, on a macroscopic level, FAK

signaling still depends on cell contractility. All in all, this study shows us that it is force that can drive concentrations of signaling proteins and begins to reveal insights about how FAK is involved in the larger scheme of focal adhesions.

Error Sources and Future Work: With only one experiment, our results require further replication before we can draw any conclusions. Originally, this experiment also stained for total FAK within the cell, but the resulting staining was too diffuse to quantify. This could partly be attributed to the FAK antibody used. We may need to identify an improved FAK antibody for hMSCs. In addition, during analysis and quantification of forces generated by the hMSCs, it was found that cells were generating lower forces compared to how much these cells are able to spread. This decrease on overall force generated by hMSCs is most likely due to the fixation process, which reduces the forces generated by 20 to 30%. We may need to develop a way to account for this decrease in force in our force-deflection calculations.

To further extend this study, it might be useful to observe how other contractility inhibitors can influence FAK concentrations. Or perhaps observing the effects of a FAK inhibitor may be more useful in order to observe if the relationship between FAK and force is truly driven by force, or if FAK is able to directly drive it. While much is still unknown about FAK, focal adhesions, and cellular adhesion, studies like these are able to reveal small interesting facets that when taken together form a larger detailed picture on how complex systems function. In understanding FAK, we can understand more about cellular adhesion, allowing us to begin to finally consider how focal adhesion can influence key cellular processes from a mechanistic view.

Research Update: The trial described above was repeated and the control data was analyzed. The figure below depicts the data obtained from this second trial.

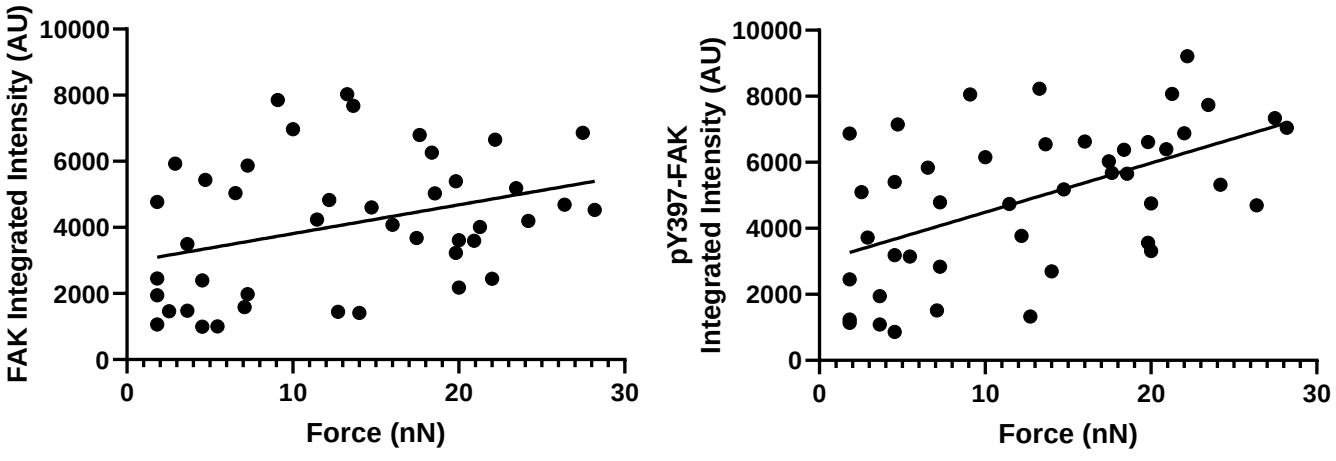

Figure 3. Focal Force vs. pY397-FAK Intensity and FAK Intensity

Scatter Plots for focal adhesion force vs. pFAK intensity and total FAK intensity. For each group 40+ focal adhesions were analyzed out of 5+ cells. Linear regressions were found to be significant ($P < 0.05$) with FAK having a linear regression with an R2 value of 0.12. pFAK had an R2 value of 0.28.

The preliminary trial described was repeated in an effort to decrease the cell density of the samples, preserve more force within the focal adhesions and validate the results of the first trial. Upon this secondary analysis, forces were indeed preserved much better comparatively to the preliminary trial, and although cell density was still high, it was less than the preliminary trial. The data shows statistically significant positive linear trends between FAK concentration and force. This suggests that FAK may indeed play a larger role in force modulation. However it is important to note that the R2 values for linear regression are fairly low, showing only a significant weak relationship. In order to further cement our findings, the trial will be repeated one more time in order to reach an acceptable cell density, with an analysis stretching across more cells, so as to include more focal adhesions in the analysis. Perhaps with more isolated cells, a stronger relationship will be shown.

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