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The Effects of Increased Extracellular Deformation, Pressure and Integrin Phosphorylation on Fibroblast Migration

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Abstract

Wound healing requires fibroblast migration. Increased pressure slows migration and ulcer healing. Pressure also induces $\beta 1$ integrin phosphorylation. We hypothesized that $\beta 1$ phosphorylation influences cell adhesion and migration. We compared the effects of increased pressure on the adhesion and motility of GD25 $\beta 1$ -integrin null fibroblasts transfected with wild-type $\beta 1A$ -integrin, S785A or TT788/9AA (phosphorylation-deficient), or T788D (constitutively phosphomimetic) mutants. GD25 $\beta 1$ null cells adhered less than wild type $\beta 1A$ cells, suggesting adherence by non-integrin mechanisms. Preventing Ser-785 or Thr 788/789 phosphorylation reduced adhesion, suggesting that phosphorylation regulates adhesiveness. Substituting Asp for Thr788 stimulated adhesion on both substrates. Pressure decreased migration in all lines and on all matrixes, the most in wild type $\beta 1A$ integrin cells and only slightly in $\beta 1A$ TT788/9AA cells. In comparison, another physical force, repetitive deformation, increased migration in the $\beta 1A$ integrin T788D, S785A and wild type cells on fibronectin and decreased migration on collagen. Deformation did not affect the migration of GD25 $\beta 1$ -integrin null or TT788/9AA cells. ERK blockade neither altered basal migration nor prevented pressure inhibition while the cellular deformation response on fibronectin was altered. $\beta 1$ -integrin phosphorylation regulates cellular adhesion and the deformation effects on motility. The pressure induced motility response is independently regulated.

Keywords

Wound healing; mechanotransduction; pressure; fibroblasts; cellular deformation; deformation and $\beta 1$ integrin

Introduction

The intestinal mucosa experiences diverse forces in normal and diseased states. The small intestine mixes and propels the chyme by peristaltic and segmental contractions, pendular

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movements, and villous motility. The liquid luminal contents are largely non-compressible and interact repetitively with the gut mucosa in complex ways as they pass along the villi. These forces all alter intra-luminal pressure and strain, which along with other physiologic forces may support the normal gut mucosal cytoarchitecture. Such biophysical forces stimulate intestinal epithelial proliferation and modulate intestinal epithelial differentiation in vitro, and activate tyrosine kinase signaling within the gut mucosa in vivo (1).

The intestinal mucosa is constantly subjected to injuries that it must heal to maintain normal function. This physiologic repair system is also required for recovery from pathologic injury such as the chronic ulceration and inflammation observed in ulcerative colitis or Crohn's disease, and is likely deficient when the mucosal barrier deteriorates in sepsis (1). Experimental evidence supports this with the observation that anastomoses rupture at lower pressures in a rat inflammatory bowel disease model, suggesting impaired healing (2). These conditions may be associated with altered contractile rhythms, altered villous motility, decreased mucosal deformation from luminal contents and increased intraluminal pressure.(1). Failure of mucosal healing is critical in such syndromes and has been implicated in their pathogenesis. (3,4)

In vitro, repetitive deformation promotes intestinal epithelial proliferation and differentiation when the enterocytes are cultured on collagen or laminin substrates, but inhibits proliferation and promotes epithelial sheet migration on fibronectin substrates (5). However, the effects of physical forces on the biology of the intestinal mucosa in vivo are less clear, although repetitive deformation stimulates mucosal tyrosine kinase activity in anesthetized rats (6). Previous work from our laboratory suggests that increased extracellular pressure increases epithelial proliferation and decreases migration on collagen and fibronectin substrates. These variations in pressure and strain have been shown to affect wound healing in vitro and in-vivo (5,7).

Fibroblasts are the predominant cell type found in wounds. The migration and adhesion of fibroblasts, transformed from local mesenchymal cells, are integral parts of the wound healing process. Fibroblasts are usually present in the wound within 24 hours and predominate by the tenth postoperative day (8). They attach to the clot matrix in the wound, multiply, and secrete glycoproteins, mucopolysaccharides, and other matrix proteins, including collagen, the primary structural protein of the body. In addition, fibroblasts produce intracellular contractile proteins as they differentiate into myofibroblasts, which are present in the wound by the fifth day and contract like smooth muscle cells to pull the edges of the wound together. Fibroblasts also synthesize collagen, the primary structural protein of the body. Integrin modulation is believed to be the mechanism by which these fibroblast move into wounds.

Integrin modulation may also be sensitive to external mechanical stimuli, such as pressure and strain. How physical forces are translated into biological responses remain poorly understood but the positioning of integrin receptors as a direct bridge between the extracellular matrix and the internal cell cytoskeleton supports integrins as key transducers of these signals (9,10). Focal adhesion formation and cytoskeletal stiffening following application of force to beads coated with a $\beta 1$ -integrin ligand has demonstrated the ability of integrins to transfer external loads across the plasma membrane. (11). Mechanical strain stimulates conformational activation of integrins (12) as well as $\beta 1$ -integrin clustering (13). Furthermore, shear force has been shown to positively correlate with focal adhesion assembly and stabilization (9,14).

We have previously reported that a pathophysiologically relevant (15–80 mmHg) increase in extra cellular pressure stimulates colon cancer cell adhesion to matrix proteins, endothelial cell monolayers, and surgical wounds in vivo by a $\beta 1$ -integrin-dependent mechanism (15). We therefore hypothesized that the effects of physical forces such as extracellular pressure and repetitive strain on cellular migration and adhesion are mediated by $\beta 1$ integrin subunit phosphorylation. We tested this in a $\beta 1$ -integrin-null GD25 murine fibroblast line and its stably

transfected β 1-integrin-expressing derivatives: GD25- β 1A (wild type), GD25- β 1A_{S785A}, GD25- β 1A_{T788/9A} and GD25- β 1A_{T788D}. We exposed these cells to cyclic strain at 10 cycles/minute and 80 mmHg increased pressure for 12 hours, choosing a strain frequency similar in order of magnitude to that observed during peristalsis or villus motility and a pressure more consistent with the increased pressure seen in pathological states. In further studies we investigated the role of ERK in mediating these effects because ERK has mediates the effects of strain in many cell types.

MATERIALS AND METHODS

Cell culture

The β 1-integrin-null GD25 murine fibroblast line and its stably transfected β 1-integrin-expressing derivatives GD25- β 1A, GD25- β 1A_{S785A}, GD25- β 1A_{T788/9A} (kindly provided by Dr. M. Mulvey, University of Utah) and GD25- β 1A_{T788D} (kindly provided by Dr. S. Johansson, Uppsala University) have been previously described (16–18).

Pressure application

Pressure was applied using an airtight Lucite box with an inlet valve for gas application and an outlet valve connected to a manometer. The box was pre-warmed to 37°C to prevent internal temperature and pressure fluctuations. Temperature was maintained within $\pm 2^\circ\text{C}$ and pressure within ± 1.5 mmHg of desired levels. Variation in Po_2 and pH of the culture medium was insignificant (6).

Matrix Pre-coating and Medium Treatments

6-well Petri dishes for pressure studies and 6-well amino-coated Flexwell I plates for strain studies were pre-coated with 12.5 g/mL collagen I or tissue fibronectin (Sigma Chemical Co, St Louis, MO) at saturating concentrations (5). Cells were seeded at 500,000/well and grown to confluence. ERK activation by pressure or strain was blocked by 20 mmol/L of the MEK antagonist PD98059 (Calbiochem, La Jolla, CA) for 30 minutes before the application of pressure or strain to assess wound closure with inhibited signaling. Control cells in these studies were treated with a 0.1% DMSO vehicle control.

Strain Application

Once the cell monolayers were confluent, Flexwell plates were placed in a cell culture incubator (5% CO_2 , 37°C) and the membranes were repetitively deformed, utilizing a computer-controlled vacuum manifold (FX3000; Flexcel, McKeesport, PA), by -20 kPa vacuum at 10 cycles/min, producing an average 10% strain on the adherent cells during periods of deformation. Non-uniformity of strain in the center of the flexible wells was addressed by placing a Plexiglas ring in the center and cells were plated around the periphery of the ring where strain is relatively uniform. Previous studies have shown that the cells remain adherent under these conditions and experience parallel elongation and relaxation (5).

Strain and Pressure Motility Measurements

GD25 WT and mutants were grown in monolayers on 6 well deformable membranes dishes or normal 6 well matrix-coated dishes pre-coated with fibronectin or collagen I. They were then subjected to 10% deformation at 10 cycles/min or 80 mmHg pressure for 24 hours after induction of a small uniform circular wound. The wound model, as previously described by other investigators (5) was created using a 1000- μL pipet tip (1.5-mm diameter) attached to a vacuum source, and suction was applied for approximately 1 second. At each time interval, the remaining wound area in each wound, an index of migration, was calculated after

visualization on a Kodak Image Station (Perkin Elmer, Boston, MA) and compared with migration without strain or pressure.

Cell adhesion assay

Cells were incubated with 5 μ M Calcein AM (Invitrogen, Carlsbad, CA) in PBS for 15 minutes at 37°C. Cells were then washed, re-suspended in growth medium, and allowed to adhere to bacteriologic plates (2.5×10^5 cells/well) pre-coated with 0.78–25 μ g/mL fibronectin for 30 minutes at 37°C under ambient or increased pressure (+15 mm Hg) conditions (19). After 30 minutes, non-adherent cells were washed away with warm PBS and cell adhesion was determined by relative fluorescence per well using a FLx800™ fluorescence microplate reader (BioTek, Winooski, VT).

Statistical analysis

All data is represented as mean $\pm$ SEM. Statistical analysis was by either paired Student t test or Wilcoxon matched-pairs signed-ranks test as appropriate. A 95% confidence interval was set a priori as the desired level of statistical significance.

RESULTS

Pressure inhibits GD25 migration on collagen independently of β 1-integrin mutation

The migration of the various fibroblast mutants were assessed by circular wound closure. Migration of the GD25 cells on collagen was the most rapid in cells expressing wild type β 1A integrin ($45.80 \pm 1.07\%$, $n=6$, $p<0.05$), slowest in β 1A integrin-null cells ($11.8 \pm 0.15\%$, $n=6$, $p<0.05$) and intermediate in cells expressing the non-phosphorylatable β 1A_{TT788/9AA} or β 1A_{S785A} integrin constructs ($24.02 \pm 1.29\%$ and $29.53 \pm 0.54\%$ respectively, $n=6$, $p<0.05$) or the T788-D mutation ($15.77 \pm 0.42\%$, $n=6$, $p<0.05$) that mimics β 1A integrin phosphorylation. Increased pressure (80 mmHg) slowed migration in all lines, but most markedly in cells expressing wild type β 1A integrin ($16.96 \pm 1.16\%$, $n=6$, $p<0.05$) and only slightly in β 1A null cells ($1.55 \pm 0.17\%$, $n=6$, $p<0.05$) (Figure 1). ERK blockade neither altered basal differences in migration among the lines nor prevented inhibition by pressure (Figure 2a).

Pressure inhibits GD25 migration on fibronectin independently of β 1-integrin mutation

The migration of the various fibroblast mutants were assessed using the pipet circular wound method previously discussed. Migration of the GD25 cells on fibronectin was the most rapid in cells expressing GD25- β 1A_{T788D} integrin ($76.47 \pm 1.17\%$, $n=6$, $p<0.05$), slowest in the non-phosphorylatable β 1A_{TT788/9AA} cells ($13.46 \pm 0.41\%$, $n=6$, $p<0.05$) and intermediate in cells expressing the wild type β 1A integrin ($56.1 \pm 0.77\%$, $n=6$, $p<0.05$), the β 1A integrin-null cells ($32.00 \pm 0.74\%$, $n=6$, $p<0.05$), or the β 1A_{S785A} ($53.24 \pm 1.39\%$, $n=6$, $p<0.05$). Increased pressure (80 mmHg) slowed migration in all lines, but most markedly in cells expressing GD25- β 1A_{T788D} integrin ($57.63 \pm 0.64\%$, $n=6$, $p<0.05$) and only slightly in non-phosphorylatable β 1A_{TT788/9AA} cells ($1.26 \pm 0.43\%$, $n=6$, $p<0.05$) (Figure 1). ERK blockade neither altered basal differences in migration among the lines nor prevented inhibition by pressure (Figure 2b). Surprisingly, migration on fibronectin and collagen did not correlate at all with adhesion.

Strain-stimulated cellular migration was decreased on a collagen extracellular matrix in a manner dependent on the β 1-integrin mutation present in the cell line

Migration of the GD25 cells on collagen was the most rapid in cells expressing wild type GD25 integrin (50.05 ± 0.81 , $n=6$, $p<0.05$), slowest in the null β 1A cells ($12.82 \pm 0.26\%$, $n=6$, $p<0.05$) and intermediate in cells expressing the β 1A- β 1A_{T788D} integrin ($36.83 \pm 0.24\%$, $n=6$, $p<0.05$), the β 1A_{TT788/9AA} integrin cells ($17.27 \pm 1.46\%$, $n=6$, $p<0.05$), or the β 1A_{S785A} (33.43 ± 0.70

%, $n=6$, $p<0.05$). Cyclic strain at 10 cycles/min decreased migration in the wild type GD25- $\beta 1A$, $\beta 1A_{T788D}$ and the $\beta 1A_{S785A}$ integrin mutant cell lines (3.03 ± 0.72 , 1.71 ± 0.58 , 1.81 ± 0.54 respectively, $N=6$, $p<0.05$ for all) but did not affect motility of the non-phosphorylatable $\beta 1A_{TT788/9AA}$ or $\beta 1A$ null cell lines (Figure 3). The MEK inhibitor PD98059 did not decrease basal migration or eliminate the strain effect in the effected cell lines (Figure 4a).

Strain-stimulated cellular migration was increased on a fibronectin extracellular matrix in a manner dependent on the $\beta 1$ -integrin mutation present in the cell line

Wound closure of the GD25 cells on fibronectin was the most rapid in cells expressing GD25- $\beta 1A_{T788D}$ integrin (87.38 ± 0.169 , $n=6$, $p<0.05$), slowest in the null $\beta 1A$ cells (13.3 ± 0.4 %, $n=6$, $p<0.05$) and intermediate in cells expressing the wild type $\beta 1A$ integrin (50.05 ± 0.73 %, $n=6$, $p<0.05$), the $\beta 1A_{TT788/9AA}$ integrin cells (42.15 ± 1.27 %, $n=6$, $p<0.05$), or the $\beta 1A_{S785A}$ (41.24 ± 0.62 %, $n=6$, $p<0.05$). Cyclic strain at 10 cycles/min increased migration in the wild type GD25- $\beta 1A$, $\beta 1A_{T788D}$ and the $\beta 1A_{S785A}$ integrin mutant cell lines (2.91 ± 1.00 , 11.09 ± 0.17 , 5.91 ± 0.46 respectively, $N=6$, $p<0.05$ for all) but did not alter wound closure in the non-phosphorylatable $\beta 1A_{TT788/9AA}$ or $\beta 1A$ null cell lines (Figure 3). Inhibition of ERK by the MEK kinase inhibitor PD 98059 decreased the basal migration and prevented any effect of strain on migration in any cell line studied (Figure 4b).

Pressure-stimulated cell adhesion requires phosphorylation of $\beta 1$ -integrin at T788/9

We utilized the GD25 $\beta 1$ -knockout murine fibroblast line and four stably transfected derivatives: GD25- $\beta 1A$, GD25- $\beta 1A_{S785A}$, GD25- $\beta 1A_{TT788/9AA}$ and GD25- $\beta 1A_{T788D}$. $\beta 1$ -integrin expression was reconstituted in the GD25 cells with either the wild-type $\beta 1A$ splice variant or one of three $\beta 1A$ mutants containing alanine or aspartic acid substitutions at S785, T788/9 and T788 phosphorylation sites as previously described (17,20). We assessed the effect of the various phosphorylation site mutations on pressure-mediated GD25 cell adhesion to fibronectin. A 30 minute exposure to 15 mmHg increased pressure enhanced GD25- $\beta 1A$ cell adhesion to collagen by $29.5 \pm 4\%$ ($n = 6$; $P < 0.03$) and to fibronectin by $33 \pm 9\%$ ($n=5$, $P<0.05$) compared with cells maintained under ambient conditions. The GD25- $\beta 1A_{S785A}$ transfectants displayed a $22.7 \pm 7\%$ ($n = 6$; $P < 0.05$) increase in adhesion to collagen and a $30.0 \pm 12\%$ ($n=5$; $P<0.05$) increase in adhesion to fibronectin (Figure 5) in response to increased extracellular pressure. However, the GD25 ($\beta 1$ -null) cells, GD25- $\beta 1A_{TT788/9AA}$ and GD25-- $\beta 1A_{T788D}$ transfectants failed to display any pressure-mediated adhesion effect on fibronectin or collagen.

Discussion

Fibroblast migration into wounds is vital for the healing of all but the most superficial epithelial wounds, and myofibroblast migration is also important for wound contraction and closure. Phosphorylation of the β -subunit cytoplasmic domain of α/β -integrin heterodimers is thought to functionally regulate integrin activity and thus would be expected to influence migration and adhesion (9). The present study confirms that phosphorylation of the $\beta 1$ integrin threonine 788/9 subunit is important in modulating motility as well as adhesion, but suggests that the inhibition of motility by increased extracellular pressure occurs independently of $\beta 1$ phosphorylation, while the modulation of motility by strain does require $\beta 1$ phosphorylation. This contrast extends as well to the intracellular signals that mediate these effects, since the inhibition of motility by pressure occurs despite ERK blockade, while the modulation of motility by strain is ERK-dependent. Of note, these results also extend to fibroblasts our previous observation in intestinal epithelial cells (5) that repetitive deformation stimulates migration across fibronectin but inhibits motility on a collagen substrate.

Other reports had suggested that GD25 fibroblasts lacking the $\beta 1$ integrin subunit cannot adhere to collagen substrates (18). This was clearly not true in our hands. These cells adhered well to collagen I and migrated across it. Pressure inhibited cell motility across collagen even in the absence of $\beta 1$ expression. These results certainly do not argue against the important role of $\beta 1$ integrin heterodimers in adhesion to collagen, and indeed support this concept because adhesion and motility were further influenced by re-expression of wild type or mutated $\beta 1$ integrin subunit. However, they do suggest that fibroblast adhesion to collagen may also be mediated by non- $\beta 1$ integrins or non-integrin adhesion receptors, beyond the scope of the present study.

Regardless of the contribution of non- $\beta 1$ adhesion to background unstimulated adhesion at ambient pressure, $\beta 1$ integrin subunit phosphorylation clearly modulates pressure-stimulated adhesion, to collagen as well as to fibronectin. Fibroblasts resting on a collagen ECM and exposed to 15 mmHg demonstrate an increase in cellular adhesion in the GD25 $\beta 1$ wild type and S785A cell lines. The effects of modulating $\beta 1$ integrin phosphorylation on adhesion to fibronectin are consistent with our previous observations as is the apparent matrix-independence of the stimulation of adhesion by pressure in epithelial cancer cells (9). Consistent with these results, site-directed substitution of chicken $\beta 1$ -integrin S785 to methionine, mimicking a de-phosphorylated residue, in F9 and GD25 cells interferes with cell attachment to laminin (21), while the TT788/9AA double-substitution gives rise to an altered extracellular conformation which is defective in mediating cell attachment to fibronectin. (20).

However, neither basal nor force-stimulated adhesion clearly correlated with motility in these studies. Cell motility is a complex process involving lamellipodial extension at the leading edge of the cell, adhesion to the extracellular substrate, generation of intracellular traction forces, and ultimately cell movement, pulling loose the trailing edge (5). Our results suggest that an increased or constant phosphorylation of the $\beta 1$ -integrin subunits causes an increased adhesion to the underlying matrix, while a constitutively dephosphorylated state of the $\beta 1$ -integrin subunits may inhibit adhesion to the underlying matrix. For motility to occur, the cell must have the ability to strongly attach at the leading edge but then disengage from the matrix at the trailing edge. Motility likely requires that adhesion be closely regulated not only as to the strength of the binding force but also with reference to its location at the leading or trailing edge of the cell. In addition, the generation of intracellular force by the myosin motor represents another target for stimuli such as cyclic strain that can thus modulate motility independently of adhesion (5).

Although adhesion does not correlate with motility, fibroblast motility was indeed influenced by physical forces such as pressure and deformation, but the mechanisms by which pressure and deformation modulate motility appear to differ. The inhibition of fibroblast motility by pressure is independent of ERK, while the modulation of motility by deformation requires ERK. The pressure effects are matrix-independent, while strain effects are matrix-dependent. The patterns of dependence on $\beta 1$ -integrin phosphorylation also differ in that increased or constant phosphorylation of the $\beta 1$ -integrin subunits increases pressure stimulated migration while having no effect on pressure stimulated adhesion. Although one might initially expect all physical forces to act similarly, it is perhaps no more surprising that various forces influence cell biology differently than that various cytokines do. Indeed, this is observed in the volume and shape changes of chondrocyte organelles during tissue compression and deformation (22). Human periodontal ligament fibroblasts subjected to either cyclic tensional or compressive forces in vitro also have demonstrated differences in extracellular matrix synthesis and degradation (23).

The matrix-dependence of the effects of deformation on fibroblast motility is striking, and consistent with what we have previously observed in intestinal epithelial cells (5,24). Fibronectin is an acute phase reactant that increases in the plasma settings of acute illness, while tissue fibronectin is usually sparse, but accumulates abundantly into the tissue in settings of chronic injury or inflammation (8). Thus, these results suggest that fibronectin may alter the fibroblast phenotypic response to strain to promote motility and thus wound healing in settings of chronic injury. The promotion of wound healing by tension has previously been noted clinically (5). In contrast, the inhibition of fibroblast motility by pressure appears to escape this matrix-regulation. It is interesting in this regard that form-fitting pressure-applying devices are frequently used to attempt to ameliorate hypertrophic scar or keloid formation.

In summary, the relationship between $\beta 1$ integrin phosphorylation, cellular adhesion and cellular migration is complex, and both adhesion and migration are differently regulated by physical forces such as deformation and pressure. The mechanisms by which the described force-activated signaling pathways of pressure and cyclic deformation regulate cell migration and adhesion are still not entirely clear. However, the $\beta 1$ -integrin T788/9 residues and the regulatory kinases that phosphorylated them may prove useful therapeutic targets to modulate fibroblast motility in abnormal wound healing.

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Figure 1.

On collagen and fibronectin, wound closure is universally reduced in the parental GD25 β 1-integrin null and all transfected GD25 cell lines when exposed to 80 mm Hg increased pressure versus ambient pressure control (* $p < 0.005$; $n = 6$ each).

Figure 2. Pressure decreases wound closure on a (A) collagen and (B) fibronectin substrate, to the same extent, after treatment with the dimethyl sulfoxide (DMSO) vehicle control or after ERK blockade by the upstream MEK inhibitor PD98059 when compared to ambient pressure control (* $p < 0.005$; $n = 6$ each).

Figure 3.

Wound closure on a fibronectin substrate is increased in GD25 β 1 wild type, T788D and S785A cell lines (2.91 ± 1.00 , 11.09 ± 0.17 , 5.91 ± 0.46 respectively, $*p < 0.05$, $N=6$ for all) when exposed to cyclic deformation at 10 cycles/hour but wound closure on a collagen substrate is decreased in GD25 β 1 wild type, T788D and S785A cell lines (3.03 ± 0.72 , 1.71 ± 0.58 , 1.81 ± 0.54 respectively, $*p < 0.05$, $N=6$ for all) when exposed to cyclic deformation at 10 cycles/hour versus ambient control.

Figure 4. (A) ERK blockade by the upstream MEK inhibitor PD98059 versus DMSO control did not alter the decreased migration in GD25 β 1 wild type, T788D and S785A cell lines on a collagen substrate when exposed to cyclic deformation (* $p < 0.05$; $n = 6$ each). (B) The same ERK blockade by PD98059, however, abolished migration in response to cyclic deformation in GD25 β 1 wild type, T788D and S785A cell lines on a fibronectin substrate when compared to the DMSO control (* $p < 0.05$; $n = 6$ each).

Figure 5.

Stably transfected fibroblast cell lines on a collagen and fibronectin substrate were exposed to either ambient or 15 mmHg increased pressure conditions for 30 minutes and assessed for adhesion. There was a significant increase in adhesion in the GD25- β 1A wild type ($29.5 \pm 4\%$, $*p < 0.03$, $n = 6$) and the GD25- β 1A_{S785A} ($22.7 \pm 7\%$, $*p < 0.05$, $n = 6$) cell lines on collagen and a significant increase in adhesion in the GD25- β 1A wild type ($33 \pm 9\%$, $p < 0.05$, $n = 5$) and the GD25- β 1A_{S785A} ($30.0 \pm 12\%$, $p < 0.05$, $n = 5$) cell lines on fibronectin.