

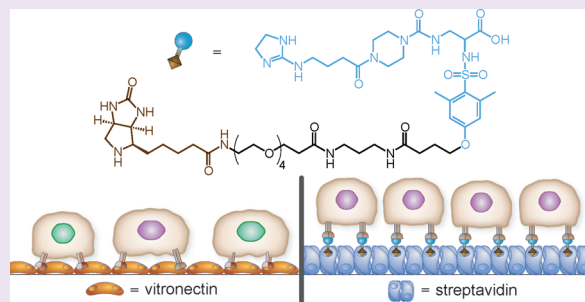
Small-Molecule-Modified Surfaces Engage Cells through the $\alpha_v\beta_3$ Integrin

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Supporting Information

ABSTRACT: Integrins play myriad and vital roles in development and disease. They connect a cell with its surroundings and transmit chemical and mechanical signals across the plasma membrane to the cell's interior. Dissecting their roles in cell behavior is complicated by their overlapping ligand specificity and shared downstream signaling components. In principle, immobilized synthetic peptides can mimic extracellular matrix proteins by supporting integrin-mediated adhesion, but most short peptide sequences lack selectivity for one integrin over others. In contrast, synthetic integrin antagonists can be highly selective. We hypothesized that this selectivity could be exploited if antagonists, when immobilized, could support cellular adhesion and activate signaling by engaging specific cell-surface integrins. To investigate this possibility, we designed a bifunctional (RGD)-based peptidomimetic for surface presentation. Our conjugate combines a high affinity integrin ligand with a biotin moiety; the former engages the $\alpha_v\beta_3$ integrin, and the latter allows for presentation on streptavidin-coated surfaces. Surfaces decorated with this ligand promote both cellular adhesion and integrin activation. Moreover, the selectivity of these surfaces for the $\alpha_v\beta_3$ integrin can be exploited to capture a subset of cells from a mixed population. We anticipate that surfaces displaying highly selective small molecule ligands can reveal the contributions of specific integrin heterodimers to cell adhesion and signaling.



Virtually every cell in the human body, with the exception of erythrocytes, produces a member of the integrin family of receptors.¹ This family of cell-surface adhesion molecules provides a pivotal connection between a cell and its surroundings. Integrins are noncovalent heterodimeric transmembrane proteins composed of one α and one β subunit.² In humans, there are 18 α subunits and 8 β subunits that form 24 unique $\alpha\beta$ combinations.¹ Both of the subunits contribute to the recognition of insoluble extracellular matrix (ECM) proteins.³ Integrin–ECM interactions are stabilized through integrin clustering.^{4,5} Cytoplasmic proteins such as talin, vinculin, and paxillin concentrate at these adhesion complexes to regulate kinases, including focal adhesion kinase (FAK) and Src.⁴ Through these kinases, integrins mediate a variety of cellular processes including anchorage, motility, and cell shape. Integrin engagement also controls cell survival, proliferation, and differentiation.⁴ Different integrin heterodimers activate many of the same downstream signaling kinases;⁴ however, differences among integrin intracellular domains and associated transmembrane proteins can result in integrin-specific signaling events.^{4,6} Discriminating between integrin-specific signaling events is difficult because of integrins' overlapping ligand specificities.^{7,8} Monoclonal antibodies recognizing specific integrins are valuable tools for dissecting integrin function, but these agents typically act by blocking the binding and

signaling of specific integrins. Materials that can engage specific integrins and thereby recruit them for adhesion and signaling would complement antibody approaches. We envisioned combining modern surface fabrication methods with highly selective integrin ligands to probe and exploit integrin function.

Our strategy requires access to integrin ligands that target specific heterodimers selectively. The tripeptide arginine-glycine-aspartic acid (RGD)⁹ was identified as the minimal recognition sequence for a group of integrins.¹⁰ This sequence is found within several ECM proteins including fibronectin, vitronectin, fibrinogen, von Willebrand factor, thrombospondin, laminin, entactin, tenascin, osteopontin, and bone sialoprotein; thus, RGD is a general integrin binding motif.^{8,11} Subsequently, synthetic peptides derived from ECM proteins or phage display screens have been appended to many diverse materials to support cell adhesion.^{11,12} In most applications explored to date, linear RGD-containing peptides are employed. For dissecting integrin function, however, RGD-containing peptides have two drawbacks. First, the affinity of these integrins for unstructured linear RGD is weak; the GRGDSP hexapeptide derived from fibronectin is 1000 times less effective at

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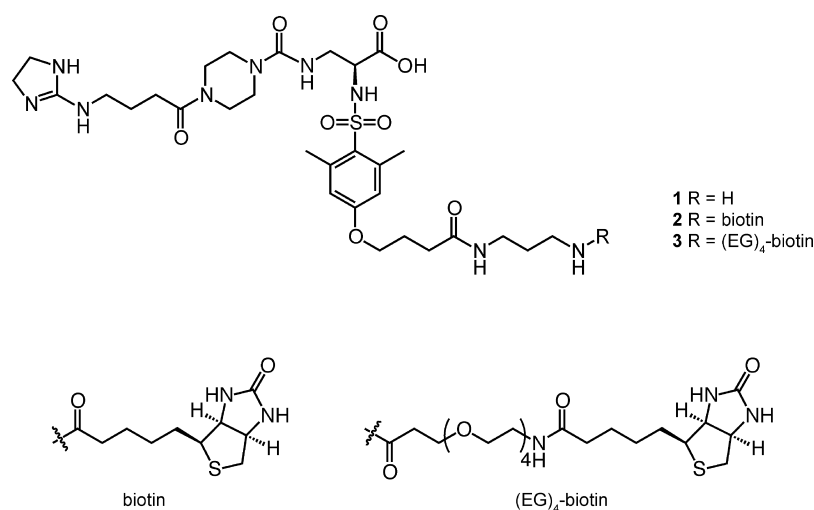


Figure 1. Chemical structures of the bifunctional compounds used for surface production. The parent compound 1 is an RGD-based small molecule, which possesses a linker bearing an amine group for conjugation to other moieties. Amine 1 can be functionalized to append biotin (2) or a biotin with an oligo(ethylene glycol) ((EG)₄) linker (3).

supporting cell adhesion than the full-length protein.¹³ The second is that the tripeptide RGD is recognized by one-third of all integrins ($\alpha_{\text{IIb}}\beta_3$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, $\alpha_v\beta_1$, $\alpha_5\beta_1$, and $\alpha_8\beta_1$).¹¹ The ability of these integrins to bind RGD is not indicative of shared function. Genetic knockout studies suggest that the functions of many integrins are distinct.¹⁴ For instance, both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins recognize RGD, but the activity of intracellular kinases in response to integrin-mediated adhesion depends on whether the cells express $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrin.¹⁵ These studies offer incentive to devise new strategies to probe integrin function.

Synthetic small molecules have been found that engage select integrins.¹⁶ One factor that affects integrin specificity for RGD is the conformation of the ligand.^{17–19} Preorganization of key functional groups can yield ligands that bind their target with high affinity and greater specificity.²⁰ Accordingly, small molecules targeting several integrins including $\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha_{\text{IIb}}\beta_3$, and $\alpha_5\beta_1$ have been generated.^{21–24} These small molecules can act as potent integrin antagonists and tumor-targeting agents.^{21,25} We have previously employed one such RGD-based peptidomimetic for selective targeting of tumor cells expressing $\alpha_v\beta_3$ integrin.^{26,27} Although designed to antagonize integrin functions, we postulated that these small molecules should support cell adhesion and signaling when appended to a surface. In this way, surfaces could be tailored to recruit specific integrin functions.

A limited number of specific integrin-targeting small molecules have been tested for their ability to support cell adhesion when immobilized on a surface.^{28–31} It is unknown, however, if the resulting synthetic surfaces mediate adhesion through a specific integrin. Moreover, no such surface or material has been shown to activate integrin-mediated signaling.^{28–31} Surfaces that activate integrin-mediated signaling could selectively promote signal transduction pathways downstream of a specific integrin. Moreover, surfaces presenting highly selective small molecules could be useful for capturing desired cell types from a heterogeneous population. Thus, we set out to investigate the properties of surfaces modified with a selective integrin ligand, as they could yield insights that complement those obtained from traditional loss-of-function studies.

RESULTS AND DISCUSSION

Design and Synthesis of Bifunctional Conjugates. To test the utility of highly specific, synthetic integrin antagonists in adhesion, we devised a modular method for ligand immobilization. The ligand we tested is a synthetic antagonist targeting $\alpha_v\beta_3$ integrin. This compound is a variation of an inhibitor identified by Degradó and co-workers.³² We previously developed a route to append a linker terminating in an amino group (compound 1).²⁷ The amine can be coupled to additional functional units to afford bifunctional compounds.²⁷ We envisioned that a biotinylated version of the $\alpha_v\beta_3$ antagonist would yield a bifunctional compound that could be presented on readily available polystyrene plates coated with streptavidin.³³ Streptavidin-coated surfaces can display adhesion ligands at a density of approximately 5.2 pmol cm⁻²,³⁴ which is within the range of other methods for immobilizing adhesion ligands to polystyrene.³⁵ This strategy has also been used to generate defined, peptide-substituted surfaces for propagating human pluripotent stem cells.³⁶ We therefore synthesized a bifunctional molecule with two distinct motifs: the $\alpha_v\beta_3$ integrin targeting nonpeptidic small molecule³² and a biotin moiety.

A key parameter in implementing this immobilization strategy is the length of the linker separating the integrin-binding and biotin groups. Inspection of the streptavidin–biotin complex suggests the binding pocket is buried.³³ To explore the spacer requirements, we generated bifunctional ligands that differ in the length of the spacer separating the integrin-binding and biotin groups (Figure 1). Both compounds were derived from amine 1.²⁷ This synthetic building block²⁷ was exposed to a biotin derivative bearing a succinimidyl ester (NHS-biotin) to yield conjugate 2 (Figure 1). Similarly, we prepared bifunctional compound 3 with a longer spacer between the two binding moieties (Figure 1).³⁷

Cell Adhesion. We evaluated streptavidin-coated surfaces presenting the biotinylated small molecules in a cell adhesion assay. We employed M21 cells, a melanoma line with high levels of $\alpha_v\beta_3$ integrin.²⁶ These cells did not adhere to streptavidin-coated surfaces (Figure 2, panel a), nor did they bind to surfaces functionalized with compound 2, which bears the shorter linker (Figure 2, panel a). We postulated that the

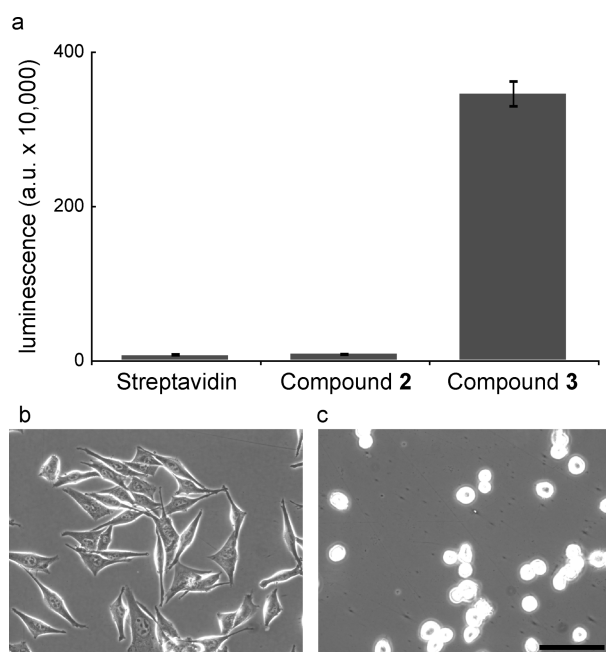


Figure 2. Surfaces presenting the bifunctional small molecule 3 support cell adhesion. (a) M21 cells bind to the indicated surfaces as measured by a luminescence assay. Error bars represent the standard deviation ($n = 3$ technical replicates). (b, c) Micrographs of M21 cells cultured on surfaces presenting 3 (b) or a biotinylated heparin-binding peptide (FHRRIKA) (c). Scale bar, 100 μm .

accessibility of the integrin binding group was the problem. Consistent with this idea, surfaces generated with compound 3, which has a longer connector separating the biotin and the integrin-binding group, support cell adhesion (Figure 2, panel a). Thus, linker length is a critical factor for ligand accessibility on streptavidin-coated surfaces, and the linker in compound 3 possesses the requisite properties.

Cell spreading can be an important determinant of cell survival;^{4,38} therefore, we investigated the extent of cell spreading on surfaces modified with compound 3. The resulting surfaces mediate both cell attachment and spreading (Figure 2, panel b). To compare the influence of integrin engagement with other modes of adhesion, we plated cells on a

streptavidin-coated surface modified with the heparin-binding peptide FHRRIKA derived from bone sialoprotein.³⁹ This peptide supports cell attachment by binding to cell surface glycosaminoglycans (GAGs); therefore, cell adhesion is independent of integrin engagement. Although cells attached to this surface, they did not spread (Figure 2, panel c). These data reveal that our RGD-based small molecule supports robust cell adhesion. We next examined the key issue of ligand specificity.

Evaluating the Specificity of the Integrin-Ligand Presenting Surfaces. The peptidomimetic group in our bifunctional ligand was designed to target the $\alpha\beta_3$ integrin.³² To assess the specificity of the conjugate, we used surface plasmon resonance (SPR) experiments. The bifunctional ligand 3 was immobilized on streptavidin-functionalized sensor chips, and the resulting surface was exposed to several recombinant, soluble integrin heterodimers. We compared the binding of $\alpha\beta_3$ with that of the closely related $\alpha\beta_5$ integrin and another RGD-recognizing integrin, $\alpha_5\beta_1$. Of these integrins, only $\alpha\beta_3$ integrin interacted with the surface; no binding of $\alpha\beta_5$ or $\alpha_5\beta_1$ integrins was detected (Figure 3, panel a). These data highlight the excellent $\alpha\beta_3$ integrin selectivity of the small molecule ligand.

To determine whether cell binding to the surface was mediated by the $\alpha\beta_3$ integrin, we used an antibody blocking experiment. Antibodies against either $\alpha\beta_3$ integrin or β_1 integrin were tested for their abilities to inhibit all adhesion of the M21 melanoma cell line. We postulated that the activity of the β_1 integrin would be especially diagnostic because it forms heterodimers with a variety of α subunits, many of which recognize the RGD motif.² For comparison, cell adhesion to surfaces coated with fibronectin and vitronectin also was assessed, because these proteins interact with a spectrum of integrins.^{8,40} As expected, adhesion to the protein-coated surfaces was inhibited to some extent by both antibodies. Adhesion to vitronectin was inhibited to a greater extent by the $\alpha\beta_3$ blocking antibody, and adhesion to fibronectin was inhibited to a greater extent by the β_1 blocking antibody (Figure 3, panel b). In contrast, adhesion to surfaces presenting the small molecule ligand was unaffected by the β_1 blocking antibody but was almost completely ablated by the $\alpha\beta_3$ blocking antibody (Figure 3, panel b). To control for any

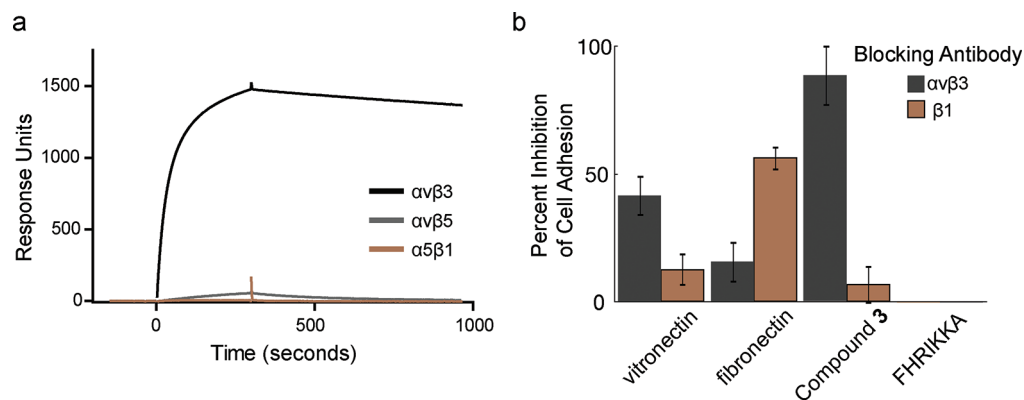


Figure 3. Adhesion to the surface functionalized with small molecule 3 is mediated by the $\alpha\beta_3$ integrin. (a) Binding of recombinant $\alpha\beta_3$, $\alpha\beta_5$, or $\alpha_5\beta_1$ integrin to 3 was assessed using surface plasmon resonance spectroscopy. (b) Percent inhibition of M21 cell binding to the indicated surfaces in the presence of antibodies that block either the $\alpha\beta_3$ or the β_1 integrins as measured by a luminescence assay (100 minus the average ratio of the mean luminescence of cell lysates plated in the presence of a blocking antibody versus those without antibody). Error bars represent the standard deviation ($n = 3$ biological replicates).

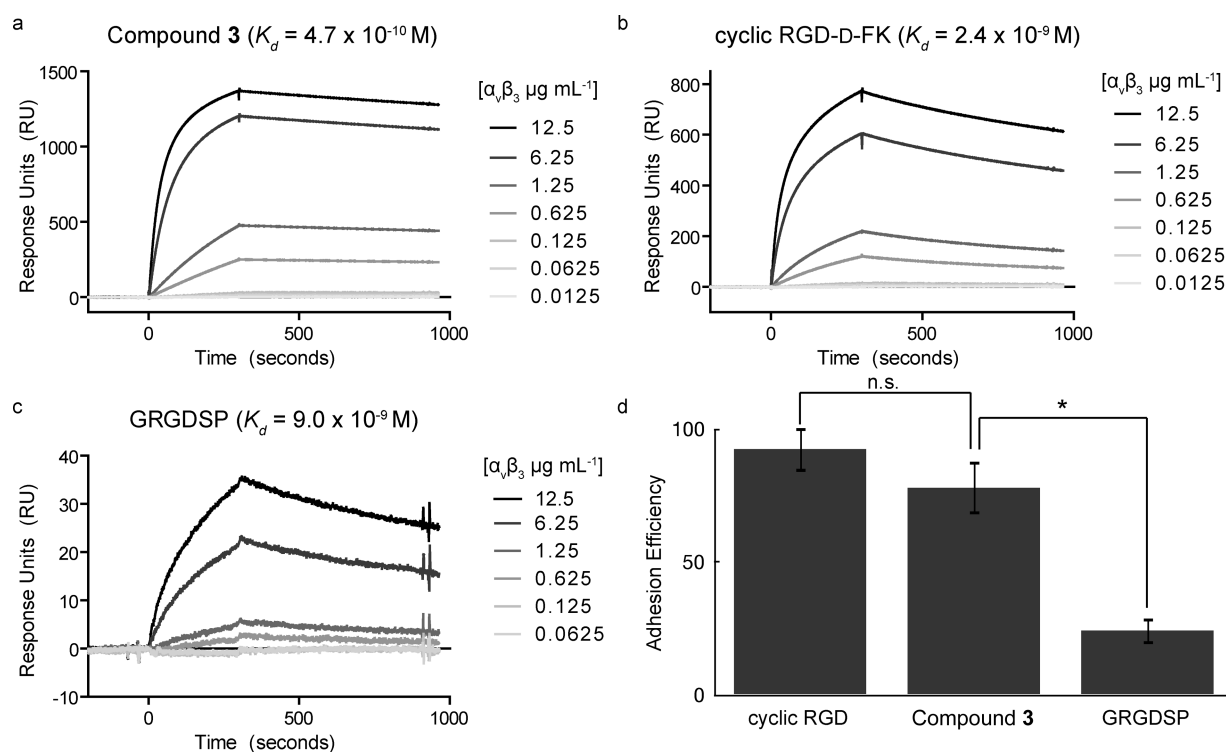


Figure 4. Comparison of synthetic integrin ligands. (a–c) SPR sensorgrams showing the binding of recombinant $\alpha_v\beta_3$ integrin to streptavidin-functionalized flow cells presenting (a) the small molecule ligand 3, (b) cyclic RGD-D-FK, or (c) the linear peptide GRGDSP. A streptavidin-functionalized flow cell saturated with biotin was used as a control. (d) Percentage of cells binding to indicated surfaces relative to cells binding to vitronectin-coated surfaces as measured by a luminescence assay (average ratio of the mean luminescence of cell lysates for cells bound to vitronectin-coated surfaces after 18 h *versus* the mean luminescence of cell lysates for cells bound to the indicated surfaces). Error bars represent the standard deviation ($n = 3$ biological replicates). Statistical analysis was performed using a two-tailed Student's *t* test. Statistically significant *P* values < 0.01 are indicated with an asterisk. The abbreviation n.s. denotes a value that is not statistically significant.

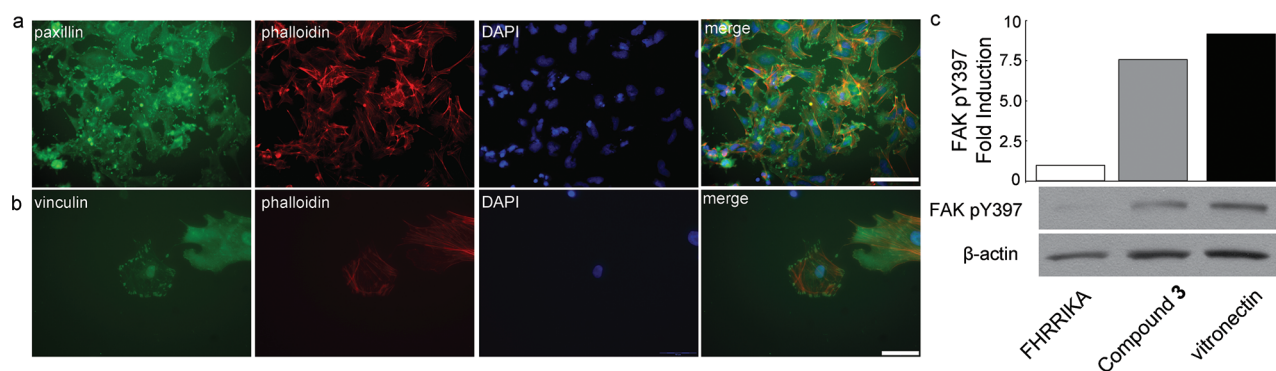


Figure 5. Surfaces displaying the small molecule 3, which binds the $\alpha_v\beta_3$ integrin, activate signaling. (a, b) M21 cells cultured on a surface presenting 3 were stained for paxillin (a, green) or for vinculin (b, green) and counterstained with phalloidin (red) and DAPI (blue). Scale bars, 100 μm (a), 50 μm (b). (c) Histogram depicting change in focal adhesion kinase (FAK) phosphorylation (pY397) for cells cultured on the indicated surfaces; values were normalized to β -actin levels. Immunoblot analysis for cells cultured on indicated surfaces using a phospho-specific antibody against FAK pY397 and an antibody against β -actin.

integrin-independent effects, we plated cells treated with the antibodies onto surfaces presenting a GAG-binding peptide. The blocking antibodies did not inhibit cell interactions with the GAG-binding surfaces (Figure 3, panel b). Together, the data indicate that adhesion to surfaces presenting the small molecule relies upon $\alpha_v\beta_3$ integrin engagement.

Comparison of Integrin Ligands. Our next objective was to determine whether differences in integrin-binding affinity were manifested in surfaces decorated with different integrin ligands. Specifically, we generated surfaces presenting the small molecule ligand, a short linear RGD-containing peptide,⁹ or a

cyclic RGD derivative that is selective for $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins.⁴¹ From the sensorgrams obtained from the $\alpha_v\beta_3$ integrin interacting with these surfaces, dissociation constants for the ligands were determined (Figure 4, panels a–c). Of the three ligands, our small molecule peptidomimetic had the highest affinity for $\alpha_v\beta_3$ ($K_d = 4.7 \times 10^{-10}$ M). Thus, the small molecule, when presented on a solid substrate, maintains its high affinity for the $\alpha_v\beta_3$ integrin.

The peptide-integrin ligand might be less selective than a cell binding experiment, as cells can exploit multivalent interactions. We therefore used a cell adhesion assay to compare surfaces

displaying the small molecule to those presenting other integrin ligands. M21 cells were allowed to attach overnight, unbound cells were removed by washing, and the remaining bound cells were lysed. The concentration of ATP in the lysate was used as a measure of viable cell number and therefore the number of cells bound. The results indicate that surfaces bearing the cyclic RGD derivative were almost as effective as the extracellular matrix protein vitronectin at supporting adhesion (Figure 4, panel d). As expected, the percentage of cells bound to the selective small-molecule-substituted surfaces was slightly less than that observed with cyclic RGD-modified substrates (Figure 4, panel d). The surfaces presenting the promiscuous and low affinity ligand RGD peptide were the least effective at supporting cell adhesion (Figure 4, panel d). In general, surfaces with high affinity ligands were the most adhesive. Substrates capable of interacting with multiple cell surface receptors (vitronectin, cyclic RGD derivative, linear RGD), however, can compensate for their lower affinity. The discrepancy among the synthetic ligands' binding affinities for recombinant $\alpha_3\beta_3$ integrin (Figure 4, panels a–c) and their effectiveness as cell adhesion ligands (Figure 4, panel d) suggests a role for both integrin-ligand affinity and selectivity.

Integrin Activation. The previous results indicate that substrates displaying the small molecule ligand mediate cell adhesion; however, they do not address whether those tailored surfaces activate integrin signaling. A hallmark of integrin signaling is the formation of focal adhesions and actin stress fibers.⁴ Focal adhesions can be detected by staining for the adaptor proteins paxillin and vinculin, which bind the cytoplasmic tail of integrin β subunits upon integrin activation.⁴ Cells cultured overnight on surfaces presenting compound 3 exhibited punctate staining of paxillin and vinculin as well as the formation of defined actin stress fibers (Figure 5, panels a and b). Thus, the small-molecule-presenting surfaces promote focal adhesion formation.

Focal adhesion formation results from the activation of integrin-responsive kinases.⁴ Integrin-mediated adhesion to the ECM triggers autophosphorylation of focal adhesion kinase (FAK) in the activation loop residue tyrosine 397.⁴² We therefore measured the levels of phosphorylated FAK in cells cultured upon the functionalized surfaces. To benchmark FAK pY397 levels, we seeded cells onto vitronectin-coated surfaces. We also measured FAK pY397 levels for cells grown on surfaces either modified with 3 or presenting a heparin-binding peptide. The latter serves as a useful control, because adhesion through GAGs should not activate integrin signaling. After 3 h, bound cells were lysed; the resulting samples were subjected to immunoblotting to detect FAK pY397. Cells cultured on vitronectin contained high levels of FAK pY397 relative to those cultured on the surfaces modified with heparin-binding peptide (Figure 5, panel c). Interestingly, the level of FAK phosphorylation in cells grown on the small-molecule-coated surfaces was similar to that obtained for cells cultured on vitronectin (Figure 5, panel c). Together, these data demonstrate that surfaces presenting a small molecule integrin ligand can indeed promote the formation of focal adhesions and stimulate kinase activity downstream of the integrins.

Manipulating Mixed Populations of Cells. The selectivity of the small-molecule-decorated surfaces was tested by exposing them to a mixed population of cell types. To generate such a population, we differentiated human embryonic stem (hES) cells. Embryonic stem cells are unique in their ability to self-renew indefinitely and differentiate into many

different cell types.⁴³ To initiate differentiation, hES cells were placed in suspension culture and allowed to form spheroid structures termed embryoid bodies (EBs); these cellular aggregates differentiate in a manner similar to the early embryo.⁴⁴ In their pluripotent state, hES cells do not express $\alpha_3\beta_3$ integrin (Figure 6, panel a), but a subset of differentiated

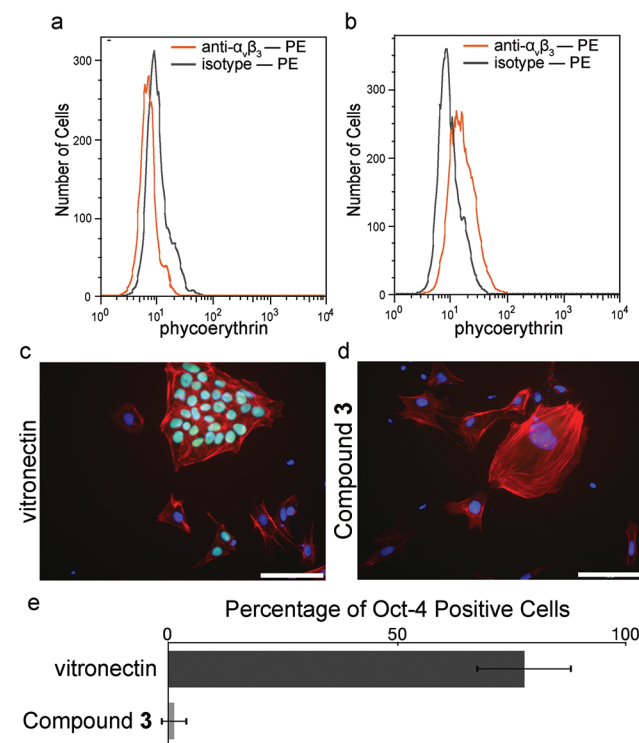


Figure 6. A surface decorated with the $\alpha_3\beta_3$ integrin-binding small molecule 3 supports the adhesion of differentiated cells but not undifferentiated human embryonic stem (hES) cells. (a, b) Flow cytometry analysis of $\alpha_3\beta_3$ integrin levels in the cell population. (a) Human ES cells (WA07) were analyzed by flow cytometry for $\alpha_3\beta_3$ integrin. (b) Differentiated cells derived from embryoid bodies formed from the hES cells were analyzed by flow cytometry for $\alpha_3\beta_3$ integrin. An increase in $\alpha_3\beta_3$ integrin is seen for the differentiated cell population. (c, d) A mixed population of undifferentiated and differentiated cells was seeded onto vitronectin-coated surfaces (c) or surfaces presenting compound 3 (d) and immunostained for Oct-4 (green) and counterstained with phalloidin (red) and DAPI (blue). Scale bars, 100 μm . (e) High-content imaging was used to determine the number of Oct-4 positive cells from the mixed population bound to the indicated surfaces. Error bars represent the standard deviation for 48 images.

cells derived from EBs does (Figure 6, panel a). The differences in cell surface receptors led us to postulate that a synthetic surface could retain cells with specific integrin expression profiles.

We seeded a population of cells containing undifferentiated hES cells and EB-derived cells onto surfaces presenting either our $\alpha_3\beta_3$ ligand or vitronectin. After 48 h, cells were fixed and stained for the marker of pluripotency Oct-4. The presence or absence of the transcription factor Oct-4 marks undifferentiated and differentiated cells, respectively.⁴³ Cells also were counterstained with phalloidin and DAPI; the former identifies actin filaments, and the latter serves to visualize the location of cell nuclei. As expected, vitronectin-coated surfaces support the adhesion of both undifferentiated and differentiated cell types

(Figure 6c). In contrast, surfaces presenting the $\alpha_v\beta_3$ -specific ligand only bind differentiated cells (Figure 6d). High-content imaging of cells cultured on the surfaces decorated with the small molecule further demonstrates the selectivity of the tailored surfaces (Figure 6e). These data emphasize the utility of surfaces that engage specific cell surface receptors. They also indicate derivatives of hES cells can be manipulated on the basis of substratum selectivity.

Discussion. Biocompatible materials can mimic features identified as important from studies of cell–matrix interactions, cellular signaling mechanisms, and developmental pathways.⁴⁵ An additional advantage of using synthetic materials over those that occur naturally is that the former can be tailored to a specific application. We reveal here that a high-affinity, selective small molecule can be used to functionalize surfaces to engage only those cells that produce the target $\alpha_v\beta_3$ integrin. Traditionally, cell adhesive surfaces have been decorated with small synthetic fragments derived from ECM proteins.⁴⁶ Although effective at supporting cell adhesion, these ligands typically lack selectivity for specific cell surface receptors. In contrast to linear peptides, small molecules can exhibit higher affinity, selectivity, and stability and are resistant to enzymatic degradation.¹² Thus, small molecules offer advantages over traditional methods for generating defined, synthetic surfaces and for probing integrin-mediated biological processes.

Our small-molecule-modified surfaces not only mediate cell adhesion but also activate integrin signaling. This activation is significant because crosstalk between specific integrins and growth factor receptors provides important contextual signals during development and regulates cell migration and differentiation.⁴⁷ Integrin $\alpha_v\beta_3$, for example, cooperates with growth factors to promote angiogenesis.⁶ Synthetic strategies are being devised to direct growth factor signaling using insoluble substrata. Recently, we demonstrated that synthetic peptides displayed on a surface could nucleate the transforming growth factor β receptor signaling complex and sensitize bound cells to the soluble growth factor.^{48,49} These surfaces can potentiate specific growth factor signaling to deliver signals with spatial control.⁴⁹ The use of insoluble cues to promote synergistic signals from integrins and growth factors could direct cell behavior and perhaps even cell fate decisions.

Precision control over cell binding and cell signaling would advance the fields of regenerative medicine and stem cell biology. Controlling the differentiation of human pluripotent cells to specific cell types remains a challenge. Typically, complex purification protocols employing fluorescence or magnetic-activated cell sorting are used to enrich for desired cells types or to separate differentiated cells from pluripotent cells. Such separations are required as pluripotent cells have the potential to form teratomas.^{50,51} Our results indicate that surfaces selective for a specific cell-surface protein provide an alternative, rapid, and convenient method for capturing cells from a mixed population. Tailored surfaces could, therefore, augment or perhaps even replace current purification protocols. Unlike traditional methods for cell sorting,⁵² our strategy relies not only on the presence of a specific cell-surface marker, but its functionality as well. Moreover, surfaces presenting combinations of small molecules targeting specific adhesion receptors and growth factor receptors can provide a powerful method to sequester specific cell types and even guide their subsequent differentiation.

To accomplish these strategies, we must expand our ability to target specific cell-surface receptors using synthetic molecules.

Small molecule binders selective for integrins other than $\alpha_v\beta_3$ have been reported. These small molecules target RGD-recognizing integrins ($\alpha_{IIb}\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_v\beta_6$)^{21,53,54} as well as ligands for RGD-independent integrins ($\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_v\beta_7$, $\alpha_1\beta_2$).^{21,22} Although the integrin ligands reported to date were designed to be antagonists, the general, modular strategy outlined here could readily be applied to identify small molecules that promote cell adhesion. Tailored surfaces presenting these highly selective ligands could thus be used to unravel the contributions of specific integrins to cellular adhesion and signaling. Moreover, we anticipate that selective integrin-binding surfaces could also promote specific cell decisions.

METHODS

Synthesis of Bifunctional Conjugates. The synthesis of the key building block **1** has been published.²⁷ Procedures for the synthesis of compounds **2** and **3** are detailed in the Supporting Information. Other bifunctional molecules used in this study included biotin-Ahx-GFHRRIKA-NH₂ (Biomatik), biotin-(OEG)₄-GRGDSP (Anaspec), and cyclo RGD-D-FK-(OEG)₂-(OEG)₂-biotin (Peptides International).

Cell Culture. Human M21 melanoma cells were maintained in RPMI medium containing fetal bovine serum (10%), pen-strep antibiotics (100 U), and glutamax (2 mM) (Gibco). Human embryonic stem cells (WA07) were maintained as described previously.³⁶ Cells were induced to differentiate as embryoid bodies in medium consisting of Iscove's modified Dulbecco's medium, 15% fetal bovine serum, 1% nonessential amino acids (Gibco). Cells were maintained at 37 °C in 5% carbon dioxide. For adhesion experiments, cells were seeded in serum-free mTeSR medium (Stem Cell Technologies).

To display the adhesion ligands on polystyrene, non-tissue-culture-treated plates (BD Falcon) were coated with 10 $\mu\text{g mL}^{-1}$ streptavidin (Prospec) in Hanks' balanced salt solution (HBSS) (Gibco). Wells were washed twice with HBSS and then coated with a 5 μM solution of biotinylated ligand diluted in HBSS.

Adhesion Assays. M21 cells were detached from cell culture flasks using 1 mM EDTA in PBS for 5–10 min. Cells were resuspended in a serum-free medium at a concentration 6000 cells mL^{-1} . Cell suspensions were seeded on to vitronectin-coated surfaces (5 $\mu\text{g mL}^{-1}$, R&D Systems), fibronectin-coated surfaces (2.5 $\mu\text{g mL}^{-1}$, R&D Systems), or streptavidin-coated surfaces (10 $\mu\text{g mL}^{-1}$, Prospec) functionalized with a biotinylated small molecule (5 μM). Blocking antibodies used in this study included the $\alpha_v\beta_3$ (clone LM609, 5 $\mu\text{g mL}^{-1}$, Millipore) and β_1 (clone 6s6, 10 $\mu\text{g mL}^{-1}$, Millipore) blocking antibodies. After 3 h, surfaces were washed 2 times with PBS, and the cells were lysed with radioimmunoprecipitation assay buffer containing 10% CellTiter-Glo (Promega), which is a homogeneous and sensitive method to determine the number of viable cells in culture based on the presence of ATP. The luminescence was measured using an Infinite M1000 plate reader (Tecan).

Microscopy and Immunostaining. Images were collected with a Hamamatsu digital camera mounted onto an Olympus IX81 microscope. Primary antibodies used in this study included antibodies against paxillin (1:250, BD), vinculin (1:250, Sigma), and Oct-4 (1:250, R&D Systems). Cells were fixed with PBS containing 4% formaldehyde for 20 min and then permeabilized and blocked with PBS containing 0.1% Triton X-100 and 2% bovine serum albumin (BSA). Antibodies were incubated in blocking buffer overnight at 4 °C. Secondary staining was performed with Alexa Fluor 488-conjugated antibodies (1:1000, Invitrogen), which were diluted in blocking buffer and exposed to cells for 1 h at RT. Cells were counterstained with Alexa Fluor 594-conjugated phalloidin and 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen). Image overlays were generated using ImageJ software. High-content imaging was performed using a BD Pathway 855 with laser autofocus. Image analysis was performed using BD AttoVision 1.6.2. Nuclear regions of

interest were determined using DAPI stained nuclei and constrained using Oct-4 stained nuclei. Data analysis was performed using BD Image Data Explorer in Microsoft Excel.

Western Blotting. To determine the levels of FAK phosphorylation, M21 cells were transferred to the relevant surfaces in serum-free medium. After 3 h, cells were lysed at a final concentration of 1% Triton-X 100, 1 mM EDTA, 2 mM Na₃VO₄, 2 mM Na₂MoO₄, 2 mM NaF, and 1:100 HALT protease inhibitor mixture (Pierce). Samples were resuspended in SDS-PAGE sample buffer and run on 4–20% gradient polyacrylamide Tris-HCl Ready gel (Bio Rad). For immunoblot analysis, samples were transferred to a 0.45- μ m PVDF membrane (Millipore) in transfer buffer (14.4 g glycine, 3.03 g Tris base, and 10% methanol to 1 L, pH 8.3). Membranes were blocked for 1 h at RT using blocking buffer (TBS containing 0.1% Tween-20 (TBS-T) and 5% nonfat dry milk) before incubating with primary antibody overnight at 4 °C. The primary antibodies used in this study included antibodies against β -actin (Cell Signaling, 1:5000) and phospho-FAK Y 397 (BD, 1:2000) and were diluted in 5% BSA in TBS-T. Blots were rinsed 3 times for 10 min with TBS-T before incubating with horseradish peroxidase (HRP)-conjugated secondary (Jackson Immunoresearch) antibodies diluted in blocking buffer for 1 h at RT. Blots were then rinsed 3 times for 10 min with TBS-T and developed with chemiluminescent substrate (ECL; Pierce) and X-ray film (Thermo). Blots were analyzed using the ImageJ.

Flow Cytometry. To determine the levels of $\alpha_5\beta_3$ integrin, cells were detached with 1 mM EDTA in PBS and resuspended at 4×10^5 mL⁻¹ in integrin binding buffer (IBB) (25 mM Tris HCl, 150 mM NaCl, 1.5% BSA, 5 mM glucose, 1.5 mM MgCl₂, 1.5 mM MnCl₂). Surface marker staining using an $\alpha_5\beta_3$ integrin phycoerythrin (PE) conjugated antibody (BD) was performed on ice in integrin binding buffer for 30 min. After antibody exposure, the cells were washed once with IBB and then analyzed using a FACSCalibur flow cytometer and FlowJo software (Tree Star, Inc.).

Surface Plasmon Resonance Spectroscopy. SPR analysis was performed using a BIAcore 2000 instrument (GE Healthcare) to determine the binding affinities of immobilized biotinylated integrin ligands. Streptavidin-coated SA sensorchips (GE Healthcare) were used according to the manufacturer's instructions and washed with two 60 s pulses of a solution containing 1 M NaCl with 50 mM NaOH prior to peptide immobilization. Biotinylated compounds (10 μ M) were immobilized at a flow rate of 10 μ L min⁻¹ in sample running buffer (HBS-P supplemented with 1 mM MgCl₂, GE Healthcare) using two 60 s pulses to ensure maximum immobilization. A reference channel was functionalized using biotin (Sigma-Aldrich). Sensorgrams were collected using soluble integrins (R&D Systems) injected in running buffer at a flow rate of 10 μ L min⁻¹ with surface regeneration performed using two 60 s pulses of a solution containing 1 M NaCl with 50 mM NaOH. Sensorgram analysis yielded dissociation constants (K_d) by kinetic analysis (simultaneous k_a/k_d) using BIAevaluation Software (GE Healthcare).

■ ASSOCIATED CONTENT

● Supporting Information

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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