

Substrates Elicit Different Patterns of Intracellular Signaling Which in Turn Cause Differences in Cell Adhesion

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(Received 2 February 2010; accepted 21 April 2010; published online 4 May 2010)

Associate Editor Jason M. Haugh oversaw the review of this article.

Abstract—Although intracellular signal transduction is relatively well understood, how the cells can distinguish among thousands of micro-environments is not. This study proposes that a systems level view of intracellular signaling is necessary to interpret differences in cell interactions with different substrates. Human umbilical vein endothelial cells were exposed to 10 substrates (9 adsorbed extracellular matrix proteins and uncoated polystyrene), and the activities of 4 intracellular signaling kinases were quantified for cells on each substrate as a function of time. Principal component analysis demonstrates that each substrate elicits a different pattern of signaling. Mean activity of the 4 kinases can distinguish 44 of 45 possible pair-wise comparisons among the substrates. Partial Least Squares Regression Analysis is used to hypothesize causal relationships between signaling activity and cell adhesion or β_1 -integrin expression. Inhibition studies generally confirm that ERK (extracellular signal-related kinase) and JNK (c-Jun N-terminal kinase) cause increased adhesion and β_1 -integrin expression. Inhibition of IKK ($I\kappa B$ kinase), on the other hand, showed no statistically significant effect on β_1 -integrin expression and led to significant decreases in cell adhesion—confirming a causal link but opposite of the hypothesized relationship (that inhibition of IKK would increase adhesion).

Keywords—Signal transduction, Extracellular matrix, Endothelial cells, Systems biology, Biocompatible materials, Principal component analysis, Least-squares analysis.

INTRODUCTION

One of the underlying tenants of biomaterials science is that cells behave differently when interacting with different materials. Much of the research in

developing new materials or adapting existing materials seeks to elicit desirable behaviors from cells in contact with biomaterials; however, understanding of why cells respond in the ways they do is limited. The design of biomaterials falls into two major categories. First, materials are chosen from existing synthetic materials (e.g., poly(methyl methacrylate), poly(L-lactic acid), expanded poly(tetrafluoro ethylene)) for their mechanical or other properties (e.g., degradation rate, optical transparency, ease of implantation, attachment strength, etc.) and then cultured with cells to determine the cell behavior they elicit.²⁰ *Post hoc* modification with proteins, peptides, roughness, and a variety of other surface modifications is often applied and cellular response retested.^{11,32,38} The second major strategy is to make biomimetic materials by mimicking natural materials—particularly their mechanical properties and protein composition. The goal is to achieve cellular response similar to the mimicked material by matching the important details such as protein composition as closely as possible. It is becoming evident that there is a possible way to merge these two strategies to achieve synergy between the ease of synthesis of the first strategy and desirable cell behavior of the second strategy.

This synergistic approach requires understanding how cells interpret cues from materials and convert those signals, via cellular signal processing, into the changes in gene expression leading to desired or undesired responses. Cell biology provides a great deal of information that can be used as a starting point in developing this understanding. For example, cell adhesion to solid substrata is known to involve integrins which can initiate signaling cascades involving kinases such as mitogen-activated protein kinase (MAPK).^{4,23,35} Other signals can come from cell surface receptors known as receptor tyrosine kinases because, when bound to ligand, they become phosphorylated on

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tyrosine residues and then act as kinases to initiate intracellular signaling cascades.²³ Mechanical forces can have direct effects on intracellular signaling as well as signal through these kinase cascades.^{19,27}

These pieces of information provide a great deal of information; however, it is not clear how the cell can differentiate among thousands or even millions of different combinations of micro-environmental cues at various concentrations or levels of each cue. For example, integrins are known to signal through several kinase cascades.^{9,34} Despite the belief that there must be a reason for the different types of α and β subunits (19 α and 8 β subunits are currently known), there is very little literature describing differences in signaling among these different heterodimers.¹³ If there were no such differences, then a cell would have a difficult time distinguishing among surfaces coated with Collagen I (binds predominantly $\alpha_1\beta_1$ and $\alpha_2\beta_1$), Laminin ($\alpha_6\beta_1$ and $\alpha_6\beta_4$), and Fibronectin ($\alpha_5\beta_1$ and $\alpha_v\beta_3$),^{2,36} yet there are many examples of cells that behave very differently when cultured on surfaces coated with these substrates.²⁶ How does the cell respond differently to one of these proteins vis-à-vis another if all integrin family members signal through the same pathways?

The rationale for this study is that this question can only be answered using a systems approach to studying intracellular signaling in response of cells to biomaterials. In other words, if MAPK activation were to be studied for any of the three substrates mentioned (Collagen I, Laminin, Fibronectin), one would expect MAPK to be activated in all three. However, they may not activate this pathway to the same extent or with the same temporal pattern. If several pathways known to be affected by integrin signaling are studied in this way, we hypothesize that different extracellular matrix (ECM) protein substrates will elicit different levels of activation of each of these pathways resulting in a unique pattern of signal activation for each type of ECM.

Here we study this by culturing human umbilical vein endothelial cells (HUVECs) on 9 different ECMs (Collagen I, C1; Collagen IV, C4; Gelatin, G; Laminin, L; Matrigel, M; Fibronectin, Fn; Vitronectin, Vn; Heparan Sulfate Proteoglycan, HS; and Chondroitin Sulfate Proteoglycan, CS) and uncoated tissue culture polystyrene (PS). HUVECs are used both because they are relevant to biomaterials via their anti-thrombotic nature and because they are naturally found at two-dimensional interfaces. These ECM components are chosen to represent various types of ECM components as well as to use the most common substrates used in cell culture and biomaterials. The activity of four kinases, extracellular signal-related kinase (ERK1/2, a member of the MAPK family), c-Jun N-terminal kinase (JNK), Akt (also known as protein kinase B),

and I κ B kinase (IKK), were quantified at several time points for cells cultured on each substrate. These kinases are chosen because they are nodal points in the signal transduction network and because they are known to be important either in integrin signaling or endothelial function.

These measurements resulted in a matrix of data consisting of 10 columns (cells cultured on 9 ECMs plus polystyrene) and 60 rows (12 time points plus calculated mean, max, and integrated activity with respect to time for each of the four kinases). This matrix was analyzed using principal component analysis (PCA), a matrix analysis technique which uses singular value decomposition to isolate Principal Components (PCs) providing information about the correlation of signal activation with ECM substrate.^{14,16} Briefly, PCA uses singular value decomposition to decompose a matrix of data into three matrices: scores, eigenvalues, and loadings.⁷ The eigenvalue matrix only has values along the diagonal, and these eigenvalues are ordered such that the largest is in the first row/column, second largest in the second row/column, and so forth. This results in the first row of the scores matrix and the first column of the loadings matrix capturing maximal information content about the variance contained in the original data matrix. Using PCA, information about the ECM component on the substrate (contained in the scores matrix) can be correlated with activity of 4 kinases at various time points or calculated mean, maximum, or integrated activity as a function of time (contained in the loadings matrix). We group the mean activities of the 4 kinases, which were found to correlate strongly with the first 3 PCs, into high, medium, or low activity; and then we show that almost all of the patterns of signaling are different. Based on these results, we detail how the cell can potentially use these four signaling pathways to distinguish among each of these 10 substrates based on patterns of intracellular signaling.

We then study whether these differences in intracellular signaling cause differences in cell adhesion, a real and complex example of a desired cell behavior. We also study β_1 -integrin expression as a molecular mediator of adhesion. The β_1 -integrin is the most common integrin subunit, and it pairs with many α subunits to form hetero-dimers capable of binding to a wide array of ECM components.¹³ As such, it is the single most important integrin subunit in cell adhesion events overall. Part of our goal in studying expression of this molecule is to assess whether achieving correlation between intracellular signaling and expression of a single gene product is more meaningful than correlation between intracellular signaling and a complex, multi-factorial cell behavior such as cell adhesion.

To study correlation between intracellular signaling and these cellular behaviors, we use Partial Least Squares (PLS) Regression Analysis. PLS is a matrix analysis tool related to PCA. PLS adds an additional matrix of data and, when the eigenvalues are rank-ordered, a least-squares regression is used to maximize variance captured in the signaling data matrix and also variance captured in this additional output matrix. Here we perform PLS using either cell adhesion vs. ECM substrate composition or β_1 -integrin expression vs. ECM substrate composition as the output matrix. We show that PLS can elucidate correlations between patterns of cell signaling and patterns of cell adhesion and β_1 -integrin expression.

Although PLS only results in correlations between cell signaling and cell behaviors, these correlations serve as hypotheses of causative relationships.¹⁴ For example, here we show a correlation between ERK activity and cell adhesion and between ERK activity and β_1 -integrin expression; thus, we hypothesize that ERK activity *causes* increased cell adhesion and β_1 -integrin expression. We test these hypotheses by inhibiting these signaling pathways and measuring cell adhesion and β_1 -integrin expression under the inhibition of the signaling pathways studied (ERK, JNK, Akt, IKK). If the hypothesis proves valid, we would expect to observe decreased cell adhesion and β_1 -integrin expression under inhibition of ERK. We also show correlations with JNK activity which is also hypothesized to cause increased cell adhesion and β_1 -integrin expression and IKK activity which is hypothesized to cause decreased cell adhesion and β_1 -integrin expression. Inhibition of Akt is studied to test the null hypotheses for this signaling pathway (i.e., that this pathway does not cause differences in cell adhesion and β_1 -integrin expression).

These studies can not only provide detailed information about this one cell behavior for this one cell type; they can also establish proof-of-principle for a general method for using systems biology techniques to connect changes in biomaterial properties with changes in cell behavior through changes in the effect of the biomaterial on intracellular signaling. This information can provide the basis for design of bioactive materials based on *a priori* knowledge of the cell behaviors desired, the intracellular signaling activities necessary to achieve those behaviors, and the micro-environmental cues necessary to achieve that pattern of intracellular signaling. If we are successful in understanding the pattern of intracellular signaling necessary to elicit desired cell behaviors, then it may be possible to design synthetic, bioactive materials that elicit the pattern of signaling required to achieve the desired cell behavior(s). This process potentially enables true rational design of bioactive materials.³⁷

MATERIALS AND METHODS

Cell Culture

Primary pooled HUVECs were purchased from Cascade Biologicals. Cells were grown to confluence in T-75 flasks in MCDB 131 media containing 20% bovine growth serum, 1 mg/mL penicillin–streptomycin (100 mg mL⁻¹), 2 μ L mL⁻¹ Heparin sulfate (8 KU mL⁻¹) and 2 μ L mL⁻¹ endothelial cell growth factor (10 mg mL⁻¹). Passage two or three cells were used for all experiments.

Preparation of ECM-Coated Surfaces and Contact with HUVECs

ECM components gelatin (G), collagen I (C1), collagen IV (C4), laminin (L), matrigel (M), fibronectin (Fn), vitronectin (Vn), chondroitin sulfate (CS) and heparan sulfate (HS) were used to coat 12-well tissue culture plates. An uncoated well (i.e., polystyrene—PS—surface) was used as an additional surface. Laminin was isolated from mouse EHS tumor according to the protocol of Kleinman *et al.*¹⁸ Porcine skin type-A gelatin, bovine kidney heparan sulfate, and human placenta collagen IV were purchased from Sigma. Rat tail collagen I, matrigel and human fibronectin were purchased from BD Biosciences. Bovine plasma vitronectin was purchased from Calbiochem. Chondroitin sulfate type A was purchased from Wako Chemicals. All ECM components were diluted in 0.15 M sodium carbonate 0.35 M sodium bicarbonate pH 9.6 buffer. ECM components were added to 12-well plate at the concentration of 6 μ g cm⁻² either to match manufacturers' recommendations (gelatin, collagen I, collagen IV, fibronectin, Matrigel, laminin) or to be consistent with other ECMs in the absence of a manufacturer's recommendation (heparan sulfate, chondroitin sulfate). Vitronectin was used at 0.5 μ g cm⁻² to match its manufacturer's recommendation, and this concentration is likely to be saturating due to being 1000-fold greater surface density than is required for adhesion.¹² ECM coated plates were incubated for 2 h at room temperature and then stored overnight at 4 °C.

Plates were washed with phosphate buffered saline (PBS). HUVECs were trypsinized and an aliquot counted using a hemocytometer after trypan blue stain. Approximately 200,000 cells were plated per well of a 12-well plate in MCDB 131 medium. To ensure that all cells contacted the surface quickly (close to time = 0 min), plates were centrifuged at 770 \times g for 3 min. They were subsequently cultured in a 5% CO₂ incubator at 37 °C for the desired length of time such as 5, 10, 15, 30, 45, 60, 75, 90 or 120 min, 24, 48 or

72 h. For 48 and 72 h time points, the media was changed daily. At every time point, media was removed from the wells and plates were quickly frozen in a liquid N₂ dewar. 12-well plates were stored at -80 °C until used in the experiment.

Immunoprecipitation and Kinase Assays

Cell lysate was prepared according to published protocols.^{3,15} Briefly, HUVECs were lysed in ice-cold 50 mM Tris-HCl buffer pH 7.5 containing 150 mM NaCl, 2 mM EGTA, 1 mM DTT, 20 mM sodium pyrophosphate, 30 mM sodium fluoride, 200 μM sodium orthovanadate, 1 mM benzamidine, 1% triton X-100, and 10 μL mL⁻¹ protease inhibitor cocktail. Cell debris was cleared by centrifuging at 13000×g for 15 min at 4 °C. Supernatant was collected into fresh tubes, and protein amount was determined by bicinchoninic acid (BCA) assay kit (Pierce Protein Research Products, Thermo Scientific). Desired amount of protein lysate was used in immunoprecipitation (IP), see Table 1.

Each kinase was immunoprecipitated using an antibody specific for the kinase. Anti-ERK1/2 (Cat no: 06-182) and Anti-Akt antibodies (Cat no: 05-591) were purchased from Millipore. Anti-JNK (Cat no: sc-474) and Anti-IKK (Cat no: sc-7607) antibodies were purchased from Santa Cruz Biotechnology. Protein A or protein G coated 8-well strips were purchased from Pierce Biotechnology. These strips were washed three times in 50 mM Tris pH 7.5 buffer containing 150 mM NaCl and 0.05% Triton X-100 (TBST). The antibodies were diluted (10 μg mL⁻¹) in TBST buffer containing 1% bovine serum albumin (BSA). 100 μL of diluted antibody solution was added to protein A/G coated wells. After overnight incubation, the wells were washed in TBST containing 1% BSA. Total cell lysate (100 μg for ERK, 200 μg for IKK, 400 μg for Akt and JNK) was added to each well and incubated overnight at 4 °C.

Kinase assays were performed according to Janes *et al.* with minor modifications.^{3,15} IP wells were washed two times with TBS (150 mM NaCl, 50 mM Tris, pH 7.5) and once with kinase wash buffer (20 mM Tris-HCl pH 7.5, 15 mM MgCl₂, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM DTT). The

wells were resuspended in 20 μL kinase wash buffer and warmed to 37 °C. Then 20 μL of substrate (40 μg of myelin basic protein for ERK (M1891, Sigma), 3 μg ATF2 for JNK (12-367, Millipore), 20 μM IκB peptide for IKK (p226-0001, Biomol International), 20 μM Aktide for Akt (#502303369, Fisher)) and 20 μL kinase assay buffer (kinase wash buffer, 0.4 μM protein kinase A inhibitor (12-151, Fisher), 4 μM protein kinase C inhibitor (12-121, Fisher), 4 μM calmidazolium, 100 μM cold ATP, 10 μCi [γ-³²P]ATP) were added to wells. The kinase reaction was allowed to proceed for 30–180 min (see Table 1) at 37 °C and then terminated by addition of 100 μL 75 mM H₃PO₄ or 20 mM EDTA. The exact condition for each kinase assay is summarized in Table 1.

The reaction mix was transferred to phosphocellulose filter plates (SLFG 025 50, Fisher) and vacuum-filtered. Each well was washed ten times with 75 mM H₃PO₄ and then two times with 70% ethanol. The filters were then transferred to scintillation vials and measured by liquid scintillation counter. Incorporated radioactivity (CPM) was normalized by dividing all CPM by the average counts of all samples taken on that day of experimentation (to minimize the effect of variation of radioactivity of [γ-³²P]ATP stock). Measurements were performed in triplicates. Mean activity, maximum activity and area under the curve (AUC) were calculated. Of the 1440 data points, 47 points (approximately 3%) were excluded because the point was greater than 9 standard deviations from the other 2 replicates and more than 50% different than the mean of the other two replicates. Of the 120 means, 4 were excluded (approximately 3%); 2 were excluded due to the criterion above, and an additional 2 were excluded because 4 of the time-points upon which they were based had been excluded.

Principal Component Analysis

PCA analysis was applied to these data. The data (mean of 3 replicates) were inserted into a matrix with kinase activities at each time point as columns of the matrix and ECM components as the rows (data matrix is available upon request to the authors). The PLS Toolbox (Matlab, Eigenvector Inc.) then preprocesses the data by mean centering (setting each column's

TABLE 1. Kinase assay conditions.

Kinase	Lysate in IP (μg)	Substrate	Amount	Reaction time	Termination
ERK	100	MBP	40 μg	30 min	75 mM H ₃ PO ₄
JNK	400	ATF2	3 μg	3 h	75 mM H ₃ PO ₄
IKK	200	IκB peptide	20 μM	1 h	20 mM EDTA
Akt	400	Aktide	20 μM	1 h	75 mM H ₃ PO ₄

mean to zero) and variance scaling (setting each column's variance to one) each column to prevent magnitude of values of different measurement types from weighting particular columns more heavily. The PLS Toolbox then applies an algorithm to perform singular value decomposition to decompose the data matrix into a scores matrix, eigenvalue matrix, and loadings matrix. The eigenvalues are ordered from high (1st eigenvalue) to low (nth eigenvalue), where n is the rank of the data matrix, and the rows/columns of the scores and loadings matrices are reordered accordingly. The meanings of the eigenvalues, scores, and loadings are discussed in the "Results" section. PCA assumes that the data results from linear combinations of important effects; however, calculated values such as those used here (mean, maximum, AUC) can allow PCA to elucidate some non-linear effects. 3 PCs were analyzed because the first 3 PCs capture most (>50%) of the variance for the 4 kinases measured. Also, the eigenvalues showed an inflection point at 3 PCs indicating that this is a local optimum for the number of PCs.

Adhesion Assay

Approximately 20,000 cells were plated per well of a 96-well plate and then were incubated for 1 h at 37 °C. The media was removed by inverting the plates, and each well was washed once with PBS and then fixed with 3% paraformaldehyde for 20 min. The fixed cells were washed with PBS and stained using 2% toluidene blue (in PBS) for 1 h at room temperature. The cells were washed 6–7 times with PBS followed by incubation with 1% SDS in PBS for 5 min on an orbital shaker (30 rpm). The absorbance of the toluidene blue released from the cell membrane was measured at 670 nm using a plate reader (Fluostar Omega, BMG Labtech). Cell adhesion is normalized to adhesion of HUVECs on poly-lysine surfaces. Three replicates were performed for each condition.

Flow Cytometry for Measuring β_1 -Integrin Expression

Approximately 200,000 cells per well were plated in a 12-well plate and then were cultured for 24 h. Cells were washed once with PBS and detached using cell dissociation reagent (C 1419, Sigma, MO), resuspended in 1 mL of M131 containing 1% BSA and counted using a hemocytometer. Primary antibody (anti- β_1 -integrin conjugated to FITC, CD2901, Invitrogen) was added at a concentration of 10 μ L/10⁶ cells to the cell suspension and incubated at 4 °C for 1 h. After incubation the cells were washed three times (M131 media with 1% BSA), centrifuged at 700 \times g, then fixed with 1% paraformaldehyde in PBS for 15 min. 200 μ L of 1% BSA in PBS was added to each

sample and stored overnight at 4 °C. Fluorescence activated cell sorting (FACS) analysis was performed on a FACScalibur (Becton Dickinson, NJ). Results are expressed in terms of geometric mean of fluorescence which is a measure of the integrin number. Four replicates of each sample were performed.

Partial Least Squares Regression Analysis

PLS was performed, according to the protocol of Janes *et al.*, to correlate kinase activity with adhesion and β_1 -integrin expression.^{14,16} The matrix used for PCA (see above) was inserted into a matrix of data (x-block). An output matrix (y-block) containing the cell behavior (cell adhesion or β_1 -integrin expression) as the one column and the 10 different ECM substrates as rows are also included. The column is mean centered and variance scaled. The PLS Toolbox in Matlab (Eigenvector, Inc) performs singular value decomposition of the signaling data matrix to obtain three matrices: scores, eigenvalues, and loadings.⁷ Eigenvalues are ordered such that the maximum variance of both the x-block and y-block are captured by the first row/column of the scores/loadings matrices and so forth for the next row/column. The first row/column of the scores/loadings matrices are known as Latent Variable 1 (LV1), the second row/column as LV2, and so forth. Interpretation of the LVs is described in "Results" section.

Inhibition Assay

The adhesion assay and FACS for β_1 -integrin expression were performed in the presence of inhibitors of ERK (10 μ M of U0126, Promega), JNK (5 μ M of SP600125, Sigma, MO), IKK (10 μ M PS1145 dihydrochloride, Sigma, MO) and Akt (20 μ M of LY294002, Promega). Adhesion and β_1 -integrin assays were performed as described above with uninhibited samples as controls. Adhesion for each sample is normalized to the mean adhesion for its uninhibited control. Statistical differences are shown by Student's *t* tests ($p < 0.05$) between uninhibited control and inhibited samples.

Data Analysis

Analysis of Variance (ANOVA) was performed on the mean activity of each kinase on each of the surfaces. A modified Fisher's least significant difference (LSD) analysis was used to group these mean activities where the $LSD = \sqrt{2(MSE)/n}$, MSE is the mean squared error within groups from the ANOVA, and $n = 3$ (number of replicates per group). Heparan sulfate was excluded from the JNK analysis of mean

activity (denoted by asterisk in Fig. 4) because its results had large variance relative to the other surfaces for JNK measurements. ANOVA ($p < 0.05$) with *post hoc* Fischer's LSD ($p < 0.1$) is used to group the ECMs for the cell adhesion and β_1 -integrin expression. Inhibition assay results use Student's *t* tests ($p < 0.05$) to compare uninhibited control with each inhibited sample.

RESULTS

Kinase Assay

The normalized kinase assay data are shown in Fig. 1. These 4 plots, one for each kinase, contain 10 series each, one for each ECM substrate, detailing the activity of the kinases for ECs cultured on that substrate with respect to time. This is the typical method for analyzing data of this type; however, in this case it is not very revealing for several reasons. One reason is the large amount of data. It is very difficult to compare

among 10 different curves simultaneously so drawing global conclusions about these data is difficult. Secondly, we can only analyze one plot at a time so we can only see how ECM changes signaling through one kinase at a time. Although there are differences in kinase activity elicited by different ECM substrates, it is difficult to use these plots to do more than qualitatively describe differences in signaling elicited by each ECM. For this reason, we use PCA to aid in data analysis.

Principal Component Analysis: Variance Captured by PCs

PCA was applied to the data matrix of normalized kinase activity for 12 time points, mean, maximum, and AUC for each kinase (60 columns) for all 10 substrates (10 rows). PCA decomposes this data matrix into three matrices (scores, eigenvalues, and loadings) with the eigenvalues ordered from highest (1st row/column) to lowest (*n*th row/column). Thus, the first row of the scores matrix and the first column of the

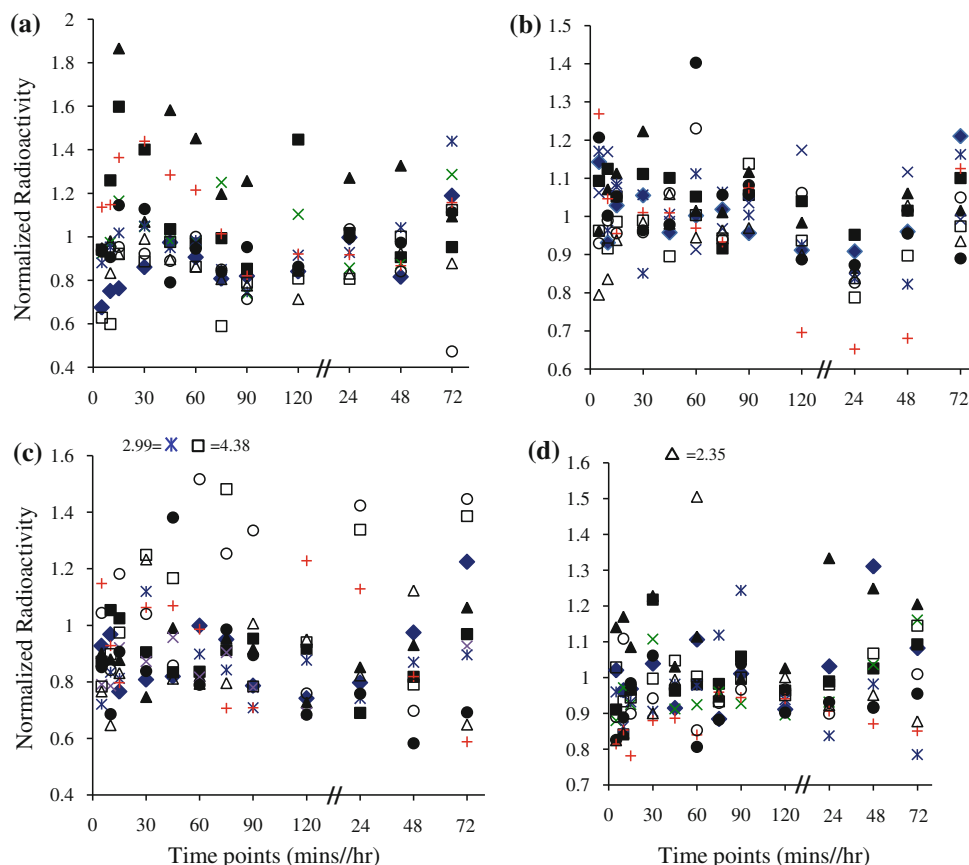


FIGURE 1. Normalized radioactivity vs. time for (a) ERK, (b) JNK, (c) IKK and (d) Akt activity on the 9 ECMs (G, filled squares; collagen I, filled triangles; collagen IV, green "x"; laminin, blue asterisks; Matrigel, filled circles; fibronectin, red plus; vitronectin, open triangles; chondroitin sulfate, open squares; heparan sulfate, open circles) and polystyrene (blue diamonds). X-axis is minutes (0–120) and hours (24–72). Three values were well above the range and are marked accordingly above figure (c) and (d) at their respective time points.

loadings matrix are defined as the 1st principal component (PC1) and capture the most variance (information content) of the original data matrix that any one row/column pair can capture. The 2nd row and column of the scores and loading matrices respectively captures the 2nd most variance and so forth.

Three principal components (PCs) were chosen because these three PCs capture most (58.7%) of the variance in the data and because an inflection in the eigenvalues indicates that this is a local optimum. The variance plot (Fig. 2) summarizes the percentage of variance in kinase activity for each time point or calculated value that is captured by each PC. For example, PC1 (blue bars in Fig. 2) captures 75–80% of the variance associated with the mean, maximum, and AUC of ERK signaling. PC1 also captures a substantial percentage of variance for ERK time points between 15 and 90 min. This shows that differences in ERK activity explains the most about differences in signaling among the ECs cultured on the 10 substrates. It appears that JNK activity is the second most informative in explaining differences because PC1 captures more than 50% of the variance associated with JNK time points at 10, 15, and 30 min as well as the mean and AUC calculated values for JNK.

The second PC (PC2) primarily captures the variance associated with Akt activity (green bars in Fig. 2) which includes a substantial percentage of most Akt time points and approximately 65% of the variance associated with the mean activity of Akt. Thus Akt seems to provide the next most information regarding differences among substrata. Finally, PC3 (red bars in

Fig. 2) includes variance associated with IKK activity—approximately 35% of the variance associated with the mean activity of IKK and a substantial percentage of variance associated with activity at 60 min and longer time points. PC3 also includes substantial information about intermediate time points (60–90 min) for JNK and Akt; however, the mean activities of these kinases are captured mostly by PC1 and PC2.

Principal Component Analysis: Correlation of Signaling with Substrates

The scores and loadings plots for each PC (Fig. 3) provide more detailed information about how the signaling of each kinase correlates with variation of ECM substrate. Each PC consists of a row vector and a column vector consisting of scalar coefficients for each substrate composition (scores) or kinase activity time point or calculated value (loadings). The absolute value of the coefficient indicates how strongly correlated the composition or kinase activity is with that PC, and the sign (+ or –) of the coefficient indicates a positive or negative correlation.

Scores for PC1 (Fig. 3a) show that collagen I and gelatin (and possibly collagen IV) are positively correlated with PC1, vitronectin and chondroitin sulfate are negatively correlated with PC1, and the others (polystyrene, Matrigel, laminin, fibronectin, heparan sulfate) are neutral with PC1. The loadings plot for PC1 (Fig. 3b) shows that both ERK and JNK means are positively correlated with PC1, and these were the two kinases whose variance was most captured by PC1 (see discussion of Fig. 2 above). Comparing with statistical groupings of the mean activity of ERK (Fig. 4), we find that collagen I and gelatin have high activity of ERK and that vitronectin and chondroitin sulfate have low activity of ERK. This confirms that mean ERK activity correlates positively with PC1. Since PC1 explains the most variance in these data (PC1 = 26%, PC2 = 19%, PC3 = 14%), ERK explains more variance in signaling in response to variation of ECM substrate than the other kinases measured. However, ERK cannot be the entire story for PC1 because fibronectin, whose ERK activity is high, does not have a high score for PC1 and conversely for polystyrene and heparan sulfate. These differences can be understood by also examining JNK mean activity (Fig. 4) which shows that fibronectin exhibits low JNK activity and that polystyrene and heparan sulfate have medium JNK activity. Collagen IV, which is near the 60% confidence limit used to distinguish positive correlation in Fig. 3a, has high activity of JNK. Although IKK mean is correlated with PC1, more of its variance is captured by PC3 so it is more straightforward to see

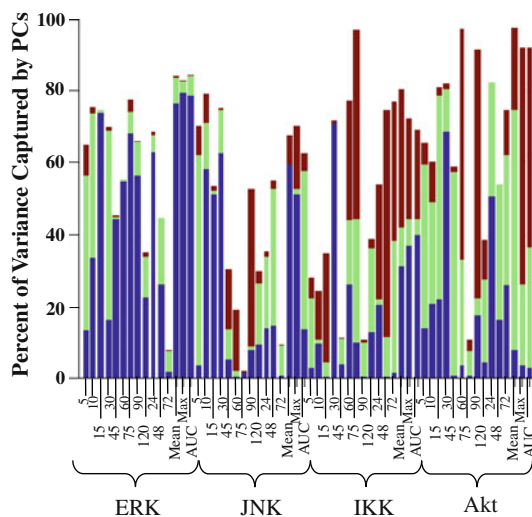


FIGURE 2. Percent of variance captured by each PC for each kinase time point (12 time points for each, from left to right on x-axis) and calculated values (Mean, Max, and AUC). PC1 (blue bars), PC2 (green bars) and PC3 (red bars) combine to total percent of variance captured for each time point.

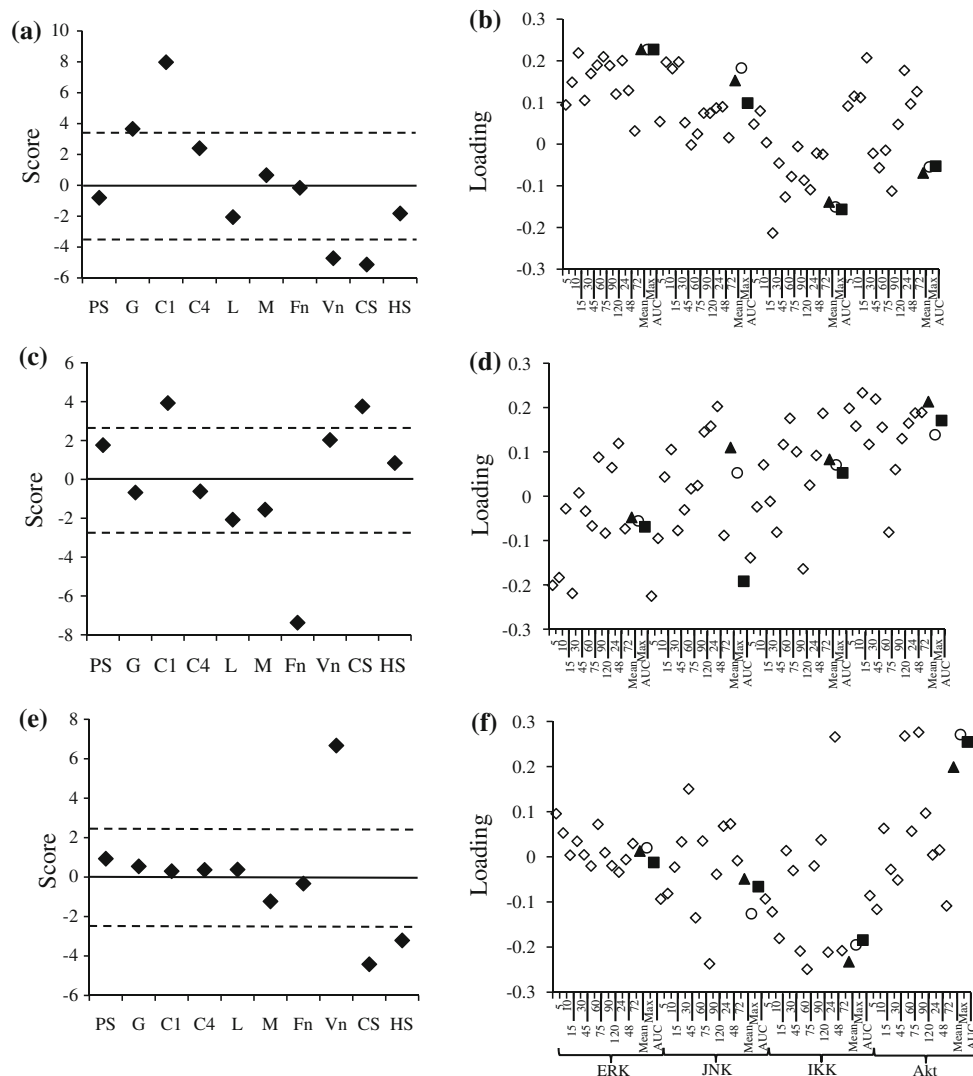


FIGURE 3. Scores and loadings plots for PC1 (a, scores; b, loadings), PC2 (c, scores; d, loadings), and PC3 (e, scores; f, loadings). Y-axes are weighting coefficients with zero representing no correlation. Dashed lines at non-zero values represent 60% confidence intervals for non-zero weighting coefficients. X-axes of loadings plots consist of kinase activity time points (diamonds), mean (filled triangles), maximum (open circles), and AUC (filled squares) for each kinase.

IKK's correlation with composition in PC3 (Figs. 3e and 3f).

Scores for PC2, Fig. 3c, show that collagen I and chondroitin sulfate (and possibly vitronectin) are positively correlated with PC2, fibronectin is negatively correlated, and the other ECMs are neutral. Based on information from the variance plot (Fig. 2), we expect this to be predominantly a function of Akt signaling, and the loadings plot for PC2 (Fig. 3d) indicates that Akt activity is positively correlated with PC2. Figure 4 shows that polystyrene and collagen I have high mean Akt activity; whereas, fibronectin, Matrigel, laminin and heparan sulfate exhibit low mean Akt activity. Mean Akt activity explains the correlation of collagen I and fibronectin with PC2, but polystyrene, laminin, heparan sulfate,

and Matrigel are neutral with PC2 so other signals must also be important in PC2. It appears from Fig. 3d that this additional information is captured in time points (early ERK, late JNK, and late IKK) rather than mean values of activity for these kinases.

Scores for PC3, Fig. 3e, indicate that differences in signaling among vitronectin (positively correlated) and chondroitin sulfate and heparan sulfate (negatively correlated) are primarily due to IKK signaling. The loadings for PC3 (Fig. 3f) show that IKK is negatively correlated with PC3. The same trend can also be seen in Fig. 4 where chondroitin sulfate has high mean activity of IKK (heparan sulfate is medium but ranked 2nd greatest IKK activity) and vitronectin exhibits low activation of IKK.

Grouping Based on Mean Activity

The above analysis using PCA demonstrates that much of the variance in the signaling data can be captured by mean kinase activity. Figure 4 shows the ECMs ranked from least (left) to greatest (right) normalized mean activity for each kinase. As stated in the “Methods”, Fisher’s LSD was performed to group

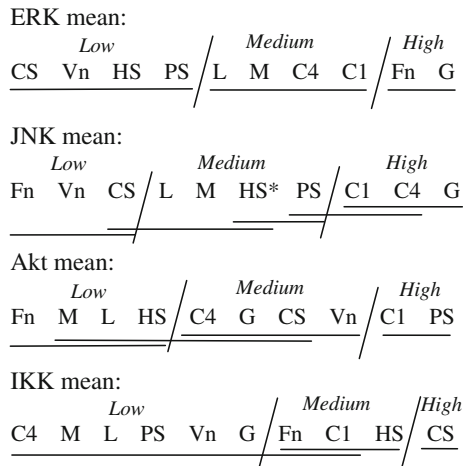


FIGURE 4. Groupings of mean kinase activity for each substrate ranked from least (left) to most (right) established by modified Fisher’s least significant difference—horizontal lines denote surfaces which are not statistically different. “Low”, “Medium”, and “High” are defined only to facilitate discussion. * HS was excluded from statistical analysis of JNK due to high variance.

these ECMs into “high”, “medium”, and “low” activities where “low” and “high” are defined as being statistically different.

When viewed piecemeal, these data are interesting but do not appear to be particularly impactful. However, when the pattern of signaling is viewed as a whole (as seen in Table 2), it becomes evident that ECMs elicit different patterns of activity of these four kinases in combination. For example, collagen I elicits a pattern of medium ERK, high JNK, high Akt, and medium IKK activity; and this pattern is not elicited by the other ECMs studied. Similarly, high IKK, low ERK and low JNK correspond to chondroitin sulfate.

Another way of viewing these data is even more revealing—Table 3 shows all pair-wise comparisons between ECM substrates with the kinases whose activities are different between the two substrates noted in the table. For example, if we compare gelatin and fibronectin, JNK and Akt distinguish these as JNK is high on gelatin but low on fibronectin and Akt activity is medium on gelatin but low on fibronectin. It is interesting to note that, with only mean activity of 4 kinases, cells are able to distinguish 44 out of the 45 pairs of ECMs (98% of the pairs were distinguished). The pair that does not show statistical significance is Matrigel/laminin.

Although any further conclusions based on these data are currently speculative, it is interesting to note a few points about these data. First, it appears that ERK signaling groups the substrates into predominantly structural (high) and non-structural (low) molecules.

TABLE 2. Patterns of signaling observed on each of the ECM surfaces.

	Polystyrene	Gelatin	Collagen I	Collagen IV	Laminin	Matrigel	Fibronectin	Vitronectin	Chondroitin sulfate	Heparan sulfate
ERK	Low	High	Med	Med	Med	Med	High	Low	Low	Low
JNK	Med	High	High	High	Med	Med	Low	Low	Low	Med
Akt	High	Med	High	Med	Low	Low	Low	Med	Med	Low
IKK	Low	Low	Med	Low	Low	Low	Med	Low	High	Med

TABLE 3. Pair-wise differences between ECMs based on mean kinase activity.

	Heparan sulfate	Chondroitin sulfate	Vitronectin	Fibronectin	Matrigel	Laminin	Collagen IV	Collagen I	Gelatin
Polystyrene	AI	EJAI	JA	EJA	EJA	EJA	EA	E	EJA
Gelatin	EI	EJ	EJ	JA	EJ	EJ	E	EA	
Collagen I	EA	EJAI	EJA	EJA	JA	JA	A		
Collagen IV	EI	EJAI	EJ	EJA	J	J			
Laminin	EI	EI	EJA	EJ	n/s				
Matrigel	EI	EI	EJA	EJ					
Fibronectin	E	EAI	EA						
Vitronectin	AI	EI							
Chondroitin sulfate	EI								

Letters denote which mean activities are different: E = ERK, J = JNK, A = Akt, I = IKK.

Second, ERK and Akt are able to distinguish between collagen I and gelatin which are chemically identical but different in conformation—collagen I is an in-register triple helix whereas gelatin is denatured and reassembled as not-in-register triple helices. Moreover there are only minor differences in the chemical structure of collagen I/gelatin which is one long collagenous domain, Gly-Xxx-Pro, vs. collagen IV which has two non-collagenous domains which are fairly short.⁴⁰ However, collagen IV is distinguished from collagen I by Akt; whereas, ERK is able to differentiate between gelatin and collagen IV. Third, vitronectin and fibronectin appear to signal differently despite being thought to be very similar.^{8,21} ERK signaling is different (fibronectin = high, vitronectin = low), and Akt activities are different (fibronectin = low, vitronectin = medium). Finally, it is interesting to note that the cells do not distinguish between Matrigel and laminin; Matrigel is a combination of Laminin, collagen IV, and a heparin sulfate proteoglycan so this may not seem surprising.³¹

Quantification of Cell Behavior

Figure 5 shows HUVEC adhesion to the 10 substrates—9 ECMs and polystyrene. ANOVA is performed ($p < 0.05$), and Fisher's LSD ($p < 0.01$) is used to group the levels of adhesion into “high”, “medium”, and “low” to facilitate discussion (Fig. 5b).⁶ Gelatin

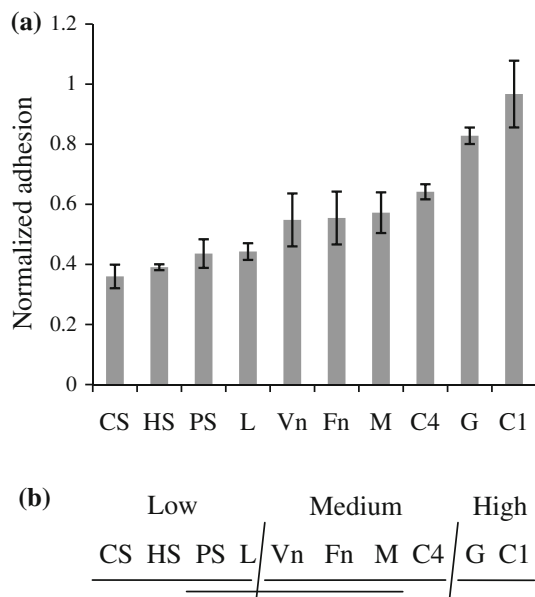


FIGURE 5. Cell adhesion varies with substrate composition. (a) Cell adhesion, normalized to poly-lysine coated substrates, for each substrate. Error bars are standard error of the mean (SEM), $n = 4$. (b) Groupings based on Fischer's LSD ($p < 0.1$) where bars below substrates denotes samples that are statistically similar.

and collagen I are classified as “high” adhesion surfaces; and chondroitin sulfate, heparan sulfate, polystyrene and laminin are classified as “low” adhesion surfaces. Vitronectin, fibronectin, Matrigel and collagen IV are classified as “medium”.

Figure 6 shows β_1 -integrin expression (geometric mean of fluorescence) measured using FACS of HUVECs cultured on the 10 different substrates. The number of β_1 -integrins present on these cells varies with ECM composition ($p < 0.05$, ANOVA). The ECMs are grouped as “high”, “medium” and “low” by Fisher's LSD ($p < 0.01$) in the same way as was performed for the adhesion data. Collagen IV, collagen I and gelatin are grouped as “high” β_1 -integrin expression surfaces; whereas, heparan sulfate, polystyrene, chondroitin sulfate, laminin and vitronectin are considered to be “low” β_1 -integrin expressing surfaces. Fibronectin and Matrigel are categorized as “medium”.

The trends for adhesion and β_1 -integrin expression are very similar with gelatin and collagen I being highly adhesive and high β_1 -integrin expressers; whereas, heparan sulfate, polystyrene, chondroitin sulfate and laminin are the least adhesive and exhibit the lowest levels of β_1 -integrin expression. The rank orders of vitronectin, fibronectin, Matrigel, and collagen IV are identical in the two assays; however, there are slight differences into which statistical grouping these substrates belong.

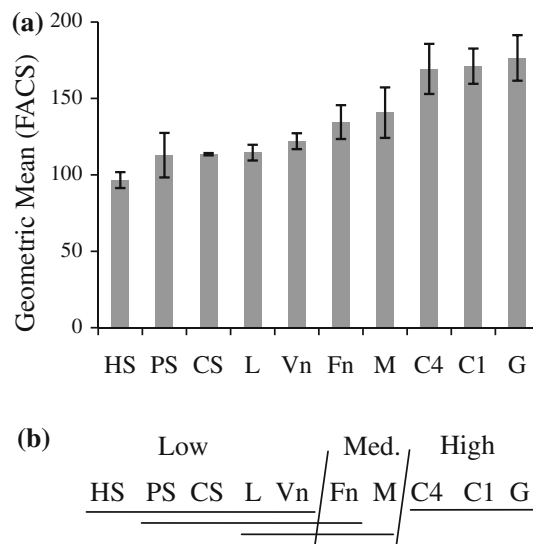


FIGURE 6. β_1 -integrin expression varies with substrate composition. (a) Geometric mean of FACS for each substrate. Error bars are SEM ($n = 4$). (b) Groupings based on Fischer's LSD ($p < 0.1$) where bars below substrates denotes samples that are statistically similar.

*Correlating Cell Adhesion with Intracellular
Cell Signaling*

The HUVEC adhesion to each of the 9 ECMs and polystyrene (Fig. 5) was correlated with activities of ERK, JNK, IKK and Akt using PLS regression analysis.^{7,16} As discussed in the “Methods”, PLS uses the kinase activity matrix in which the columns are the activities of the 4 kinases at each time-point plus calculated values (mean, maximum, and AUC) and the rows are the 10 substrate compositions. This “x-block” is joined with a “y-block” representing some output with which the x-block is to be correlated. In this case we perform PLS twice, once with adhesivity as the single column in the y-block and again with β_1 -integrin expression as the single column in the y-block.

When PLS is performed using adhesivity as the y-block, the first row of the “scores” matrix and the first column of the “loadings” matrix, known as latent variable 1 (LV1), capture 26% of information about the variation in the signaling data and 89% of the information in the adhesion data. In other words, if only this row and column were multiplied together,

26% of the variance in the signaling data (x-block) would be recreated, and the scores alone recreate 89% of the variance in the adhesion data—8 out of the 10 substrates are rank ordered the same as Fig. 5a.

The scores plot (Fig. 7a) shows that gelatin and collagen I are positively correlated with LV1, vitronectin and chondroitin sulfate are negatively correlated with LV1, and the others are less strongly correlated with LV1. The loadings plot (Fig. 7b) shows that ERK at early timepoints (5, 10, 15, 30, 45, 60 min), ERK calculated values (mean, maximum, AUC), JNK at early timepoints (5, 10, 15, 30, 45 min), JNK mean activity, and JNK AUC are positively correlated with LV1. Several of the IKK values (30, 45, 60 min, IKK mean, IKK max and IKK AUC) are negatively correlated with LV1. Although a few of the Akt timepoints are positively correlated with LV1, Akt is mostly uncorrelated with LV1.

Above (PCA results), it was found that taking the mean activity for each kinase provided a useful metric of the overall activity. Figure 4 shows that collagen I and gelatin have high mean activity of JNK and gelatin

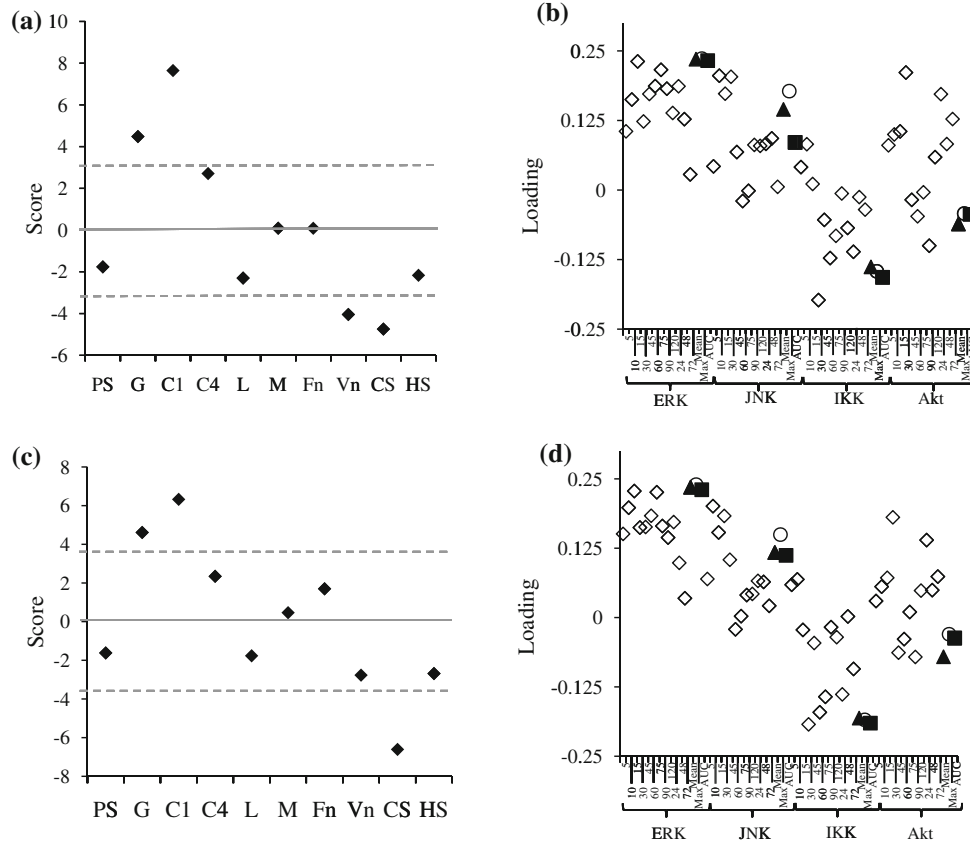


FIGURE 7. PLS results for cell adhesion (a and b) and β_1 -integrin expression (c and d). Scores coefficients vs. substrate composition are shown for cell adhesion (a) and β_1 -integrin expression (c). Loadings coefficients for each kinase time-point (diamonds) and calculated value (mean = triangles, maximum = circles, AUC = squares) are shown for cell adhesion (b) and β_1 -integrin expression (d).

has high activity of ERK which are positively correlated with LV1. Collagen I has intermediate activity of IKK so this does not provide much information; however, chondroitin sulfate has high IKK activity and a low score on LV1. Chondroitin sulfate and heparan sulfate also have low activities of ERK, and chondroitin sulfate has low JNK activity so these effects are confounded when only interpreting scores and loadings from PLS analysis. Finally, reviewing the rank-ordered mean activities of Akt does not reveal any discernable trends in regards to this PLS analysis with cell adhesion.

In a similar manner, we correlated β_1 -integrin expression on HUVECs with the activities of the kinases. Figure 7 shows the scores (Fig. 7c) and loadings (Fig. 7d) plots of LV1 for PLS with β_1 -integrin expression as the y-block. LV1 captures 25% of information of the signaling data and 86% of the variation in the β_1 -integrin expression. From the scores plot (Fig. 7c), collagen I and gelatin are positively correlated, chondroitin sulfate is negatively correlated, and the other substrates are less strongly correlated with LV1. From the loadings plot (Fig. 7d), ERK (all ERK time points, mean, maximum, and AUC) and JNK activities at short time (5, 10, 15, and 30 min time-points) as well as JNK mean and AUC are positively correlated with LV1; whereas, IKK activities at intermediate times (30, 60, 75, 120 min, and 24 h) as well as IKK mean and maximum activities are negatively correlated with LV1. As described above, when comparing these correlations to the mean activities measured for HUVECs cultured on the 10 substrates (Fig. 4), the positive correlations of LV1 with ERK and JNK mean activities are corroborated by JNK activity being high for gelatin, collagen I, and collagen IV and ERK being high for gelatin. The negative correlation with IKK is also matched by collagen IV and gelatin exhibiting low activity of IKK. Vitronectin, chondroitin sulfate, and heparan sulfate all have low mean activity of ERK, and vitronectin and chondroitin sulfate have low mean activity of JNK. As before, chondroitin sulfate has high mean activity of IKK.

Table 4 summarizes these results. Note that, in general, high mean activities of ERK and JNK and

low mean activity of IKK correlate with high adhesion and β_1 -integrin expression, and vice versa.

Inhibition Assay

The results from the PLS analysis provide correlation among the signaling of ERK, JNK, and IKK with cell adhesion and β_1 -integrin expression; however, this does not establish causation. Based on cell biology knowledge, that these intracellular signaling pathways are used to transmit sensation of micro-environmental cues from cell surface receptors to the nucleus in order to affect changes in gene expression and cell behavior, we hypothesize that increased ERK and JNK activity and decreased IKK activity *cause* increased cell adhesion and increased β_1 -integrin expression.⁹ To test this hypothesis, we performed the cell adhesion and β_1 -integrin expression assays in the presence of inhibitors of these three kinases as well as an inhibitor for Akt as a control since we do not hypothesize there to be a causative relationship between Akt activity and cell adhesion or β_1 -integrin expression.

Figure 8 shows HUVEC adhesion to the 10 surfaces when HUVECs were inhibited with inhibitors for ERK (U0126), JNK (SP600125), IKK (PS1145 di-hydrochloride) and Akt (LY294002). ERK inhibition leads to lesser adhesion for HUVECs cultured on collagen I, collagen IV, and Matrigel ($p < 0.05$). These are three of the five substrates with the greatest ERK activity; gelatin and fibronectin are the other two, and fibronectin ERK inhibited vs. uninhibited is nearly significant ($p \approx 0.06$). When JNK is inhibited, adhesion is significantly decreased on polystyrene, gelatin, vitronectin, and chondroitin sulfate. Collagen I JNK inhibited vs. uninhibited and fibronectin JNK inhibited vs. uninhibited are nearly significant ($p \approx 0.06$ and $p \approx 0.1$, respectively). This trend suggests that JNK is important to cell adhesion and that, regardless of initial level of JNK activity, decreasing activity tends to decrease cell adhesion. In other words, gelatin has high activity and vitronectin has low activity, and inhibition of JNK decreases cell adhesion for both substrates.

IKK is hypothesized to have an inverse relationship with cell adhesion; thus, we might expect inhibition of

TABLE 4. Summary of correlation among mean activities of ERK, JNK, and IKK with cell adhesion and β_1 -integrin expression.

	Polystyrene	Gelatin	Collagen I	Collagen IV	Laminin	Matrigel	Fibronectin	Vitronectin	Chondroitin sulfate	Heparan sulfate
ERK, mean	Low	High					High	Low	Low	Low
JNK, mean		High	High	High			Low	Low	Low	
IKK, mean	Low	Low		Low	Low	Low		Low	High	
Adhesion	Low	High	High		Low				Low	Low
β_1 -Integrin	Low	High	High	High	Low			Low	Low	Low

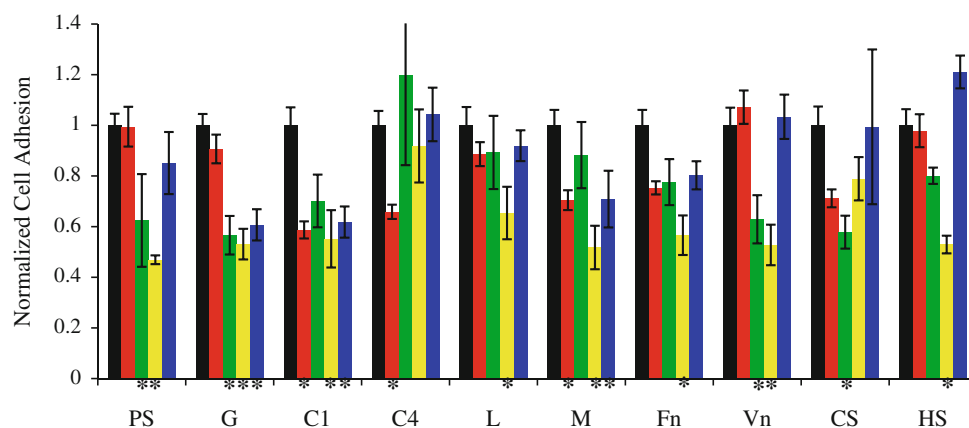


FIGURE 8. Inhibition studies for cell adhesion. Cell adhesion, each sample normalized to its uninhibited control, is measured for each substrate with no inhibitor (black), ERK inhibitor (red), JNK inhibitor (green), IKK inhibitor (yellow), or Akt inhibitor (blue). Asterisks below the bar indicate statistically significant decreases, *t* test compared with uninhibited control of the same substrate ($p < 0.05$). Error bars are SEM ($n = 4$).

TABLE 5. Summary of inhibition studies of cell adhesion and β_1 -integrin expression.

	Polystyrene	Gelatin	Collagen I	Collagen IV	Laminin	Matrigel	Fibronectin	Vitronectin	Chondroitin sulfate	Heparan sulfate
ERK	N/*	N/N	-/-	-/-	N/N	-/N	N/-	N/N	N/N	N//N
JNK	-/*	-/-	N/-	N/-	N/+	N/N	N/N	-/N	-/N	N/N
IKK	-/*	-/N	-/N	N/N	-/N	-/N	-/N	-/N	N/N	-/N
Akt	N/*	-/N	-/N	N/N	N/+	-/N	N/N	N/N	N/+	N//N

The symbol before the slash denotes change in cell adhesion, symbol after the slash indicates change in β_1 -integrin expression ($p < 0.05$). “-” indicates a decrease, “+” indicates an increase, “N” indicates no change.

* Polystyrene was not included in the β_1 -integrin expression inhibition study.

IKK to increase cell adhesion. Contrary to this expectation, we observe inhibition of IKK leading to decreased cell adhesion on polystyrene, gelatin, collagen I, laminin, Matrigel, fibronectin, vitronectin, and heparan sulfate. Based on these data, IKK is clearly causally linked to cell adhesion. The “Discussion” section provides more insight into the possible meaning of the discrepancy between these data and the hypothesis generated by PLS analysis. Finally, Akt was hypothesized not to be causally linked to cell adhesion; however, cell adhesion of cells on gelatin, collagen I, and Matrigel are decreased when Akt is inhibited. This indicates a possible link between Akt signaling and cell adhesion even though this was not noted by the PLS analysis. Table 5 summarizes the effects of inhibition of these kinases on cell adhesion.

We also measured the β_1 -integrin expression of cells under the inhibition of these kinases. Figure 9 shows that inhibition of ERK decreases β_1 -integrin expression for cells cultured on collagen I, collagen IV, and fibronectin. These are three of the four substrates exhibiting the greatest mean activity of ERK. Inhibition of JNK decreases β_1 -integrin expression on gelatin, collagen I, and collagen IV. These are all three of

the substrates exhibiting high mean activity of JNK. Inhibition of JNK also increases β_1 -integrin expression for cells cultured on laminin. Inhibition of IKK has no effect, although the expression on Matrigel and vitronectin are almost statistically significant increases ($p \approx 0.09$ and $p \approx 0.11$, respectively). Finally, inhibition of Akt results in a significant increase of β_1 -integrin expression when cells are cultured on laminin and chondroitin sulfate (heparan sulfate is also near to significance, $p \approx 0.07$).

DISCUSSION

There are many examples of cells behaving differently when cultured on different materials or in different micro-environments. Much of the work in the field of biomaterials involves bringing cells into contact with materials, either *in vitro* or *in vivo*, and observing the behavior of the cells. This has led to an abundance of data showing that various micro-environmental cues lead to differences in cell behaviors of interest.^{1,24,30,33} The key question we seek to answer here is: how? How do these variations in micro-environment cause

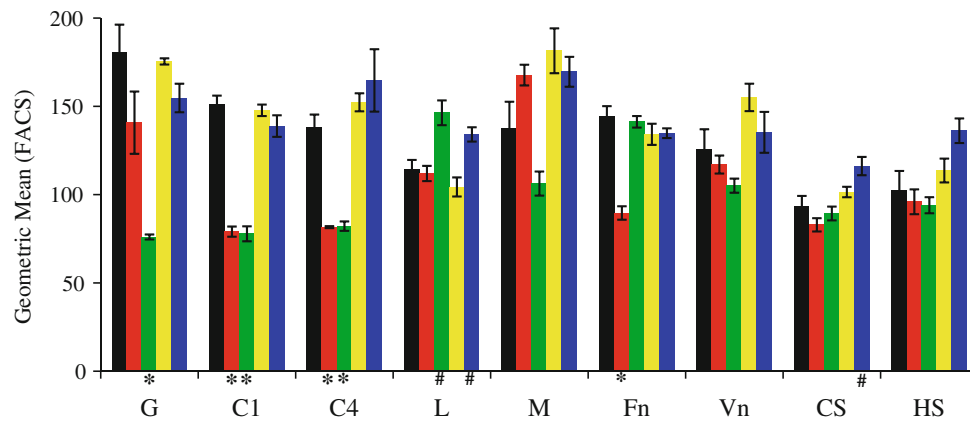


FIGURE 9. Inhibition studies for β_1 -integrin expression. β_1 -Integrin expression is measured for each substrate with no inhibitor (black), ERK inhibitor (red), JNK inhibitor (green), IKK inhibitor (yellow), or Akt inhibitor (blue). Symbols below the bar indicate statistically significant decreases (*) and increases (#), *t* test compared with uninhibited control of the same substrate ($p < 0.05$). Error bars are SEM ($n = 3$).

differences in cell behavior? Here we start with a specific formulation of this question: how does variation in substrate protein cause differences in cell adhesion strength?

This question is broken into two parts. First, how does the signaling machinery of the cell distinguish among substrate proteins? Second, do these different patterns of intracellular signaling activity *cause* the observed differences in cell behavior?

How Does the Signaling Machinery of the Cell Distinguish Among Substrate Proteins?

Cell biology literature includes many studies linking a particular integrin or signaling pathway with various cell behaviors so this may seem to be an answered question; however, existing information cannot explain how subtle differences in substrate (e.g., collagen I vs. collagen IV) can lead to differences in signaling. Additionally this information exists piecemeal by signaling pathway or integrin type so it would be exceedingly difficult to compile these studies into a systems level view of how a cell determines to which substrate it is bound. In this study we show that different ECM molecules elicit different patterns of activity of four intracellular signaling kinases. This serves as a working hypothesis that this is how cells interpret differences in this micro-environmental cue (substrate composition).

If this hypothesis is valid, it provides insight into how one cell type might be able to distinguish among thousands or more possible combinations and levels of various micro-environmental cues including ECM composition, mechanical compliance of the matrix, mechanical forces applied to the cell, types and concentrations of cytokines present (including gradients),

cell-cell signaling either through contact or autocrine/paracrine signaling, and other factors (e.g., nutrient concentrations, endocrine signaling, etc.). How does a cell distinguish amongst all of these possible micro-environments with a very limited number of intracellular signaling pathways? Here we show that a combination of the mean kinase activities of only 4 kinases can mostly distinguish among 10 ECM substrates. Using a linear view of signaling, we might think that 4 kinases could distinguish among 4 different substrates. Here we categorized these 4 kinases by high/low mean activities generating 2^4 or 16 possible combinations. Viewed this way, it is not only clear how the cells can distinguish among 10 different ECMs, it seems somewhat unsurprising. This is the power of a systems view of signaling which shows that the number of different possible combinations of micro-environmental cues distinguishable by the cell is λ^n where λ is the different pieces of information included in each signaling molecule and n is the number of signaling molecules containing independent information.

Here we only study 4 kinases ($n = 4$) and assume that the important information is included in the mean activity which we categorize as high or low ($\lambda = 2$). It is likely that there are other kinases which we have not measured here that are important ($n > 4$) and that each kinase includes other information that is important such as maximum value, integrated activity with respect to time (AUC), and temporal details such as whether activity is high or low at short time ($\lambda > 2$). Even with modest increases in these values, the number of combinations of micro-environmental cues amongst which a cell can differentiate reaches to thousands.

In this study we have chosen to study ERK and JNK because they are known to be involved in signaling from integrins and Akt and IKK because they

are known to be important in endothelial function thus important in understanding endothelial behavior. Although it may be important to add additional kinases in future studies to understand particular cell behaviors, it is a strength of this study that only 4 kinases can capture differences in signaling among almost all pairs of these 10 different substrates. One weakness of this study is that criteria for statistical tests needed to be relaxed due to variance in signaling data and being limited to 3 replicates per time-point. Standard deviation in the data was on average 0.28 (with a range of normalized means from 0.75 to 1.61). It is likely that a study with greater number of replicates could achieve significance with more standard statistical criteria ($p < 0.05$); however, even this underpowered study required 1440 data points so such a study would be expensive and time consuming. Some efficiency might be gained by measuring fewer time-points, but enough need to be measured to always quantify the peak in activity.

Previous work in this field has focused on the activation of pathways by individual extracellular matrices. There are some examples of comparisons between or among different ECM proteins. For example, Hahn *et al.* showed that endothelial signaling in response to flow is different depending on whether the substrate is fibronectin, basement membrane (i.e., Matrigel), or collagen I.¹⁰ Reyes *et al.* have shown that neither a peptide from collagen I, GFOGER, nor a segment of fibronectin, FnIII₇₋₁₀, is sufficient to achieve phosphorylation of focal adhesion kinase (FAK); however, when combined, these molecules lead to FAK phosphorylation.²⁹ The study presented here examines a wider range of ECM proteins and a wider range of intracellular signaling cascades to understand the system-wide signaling elicited by different ECM proteins.

This work represents just a small sample of the thousands of possible micro-environments leading to what we hypothesize to be different patterns of signaling. The field of biomaterials now readily accepts that mechanical properties of the matrix and mechanical forces applied to cells are essential to understanding and controlling cell behavior.^{17,25,27} Growth factors and other soluble molecules binding to cell-surface receptors introduce another set of combinations.²⁸ Cell-to-cell signaling through cell-cell adhesion molecules and mechanical forces applied to one another further enriches the number of combinations. Also, whereas in this study we only adsorbed one ECM protein at a time, ECM proteins are usually found in combinations in the body so this further increases the number of possible micro-environments. Adding in topographical features (such as size of features and alignment), gradients (of cytokines or ECM proteins), temporal variation of these factors,

variations among cell types, and other factors potentially increases the number of possible micro-environments into thousands of combinations.

Do Different Patterns of Intracellular Signaling Activity Cause Differences in Cell Behavior?

Here we study cell adhesion because it is one of the primary cell behaviors necessary to achieve bioactivity, and we study endothelial cells both for their relevance to blood-contacting materials as well as the fact that their natural state is to line a two-dimensional surface (the blood vessel wall).²² We directly measure cell adhesion, and we also measure a molecular mediator of cell adhesion, the β_1 -integrin subunit. The cell behavior desired is cell adhesion, but this is a complex phenomenon involving expression of several gene products, formation of super-molecular assemblies such as focal adhesions, cell spreading through cytoskeletal rearrangement, and other processes.³⁹ In undertaking this work, we were concerned that it might not be straightforward to relate cell adhesion directly with a pattern of intracellular signaling, so we also measured a single gene product that we considered to be the single most important molecule to cell adhesion—the β_1 -integrin subunit. As described above, this subunit is the most prevalent integrin subunit, but we should note that integrins with this subunit do not bind to all of the ECM components studied here—particularly vitronectin, chondroitin sulfate, and heparan sulfate.^{5,8} Despite this, the trends of cell adhesion strength and β_1 -integrin expression are very similar.

The PLS analysis revealed correlation between signaling (high activities of ERK and JNK and low activity of IKK) and cell adhesion and the same correlation between signaling and β_1 -integrin expression. This led to a hypothesis that these signals *cause* increased cell adhesion and β_1 -integrin expression. Specifically we hypothesized that inhibition of ERK or JNK would decrease cell adhesion and β_1 -integrin expression, and we hypothesized that inhibition of IKK would increase cell adhesion and β_1 -integrin expression. We hypothesized that inhibition of Akt would have no significant effect.

When these studies were performed, we found that the cell behavior of β_1 -integrin expression matched the hypotheses fairly closely (conclusion shown schematically in top half of Fig. 10). Three of the four substrates eliciting the greatest ERK activity and the three substrates eliciting the greatest JNK activity were all affected by inhibition of these kinases to decrease their β_1 -integrin expression. It was unexpected that there were no significant differences observed when IKK was inhibited, but the Matrigel and vitronectin substrates were near to significance ($p \approx 0.09$ and $p \approx 0.11$,

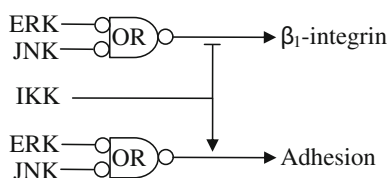


FIGURE 10. Schematic depicting proposed causal relationship between pattern of signaling and β_1 -integrin expression and cell adhesion.

respectively) and these would be increases if significant. Thus, there may be a causal link between IKK activity and lesser β_1 -integrin expression. All of these results are either consistent with the generated hypotheses or, in the case of IKK, not inconsistent. However, the increased expression for JNK inhibition of laminin samples and Akt inhibition of laminin and heparan sulfate samples was unexpected. This mostly serves to indicate that even the gene expression of one molecule is complex and cannot be completely explained by the mean activity of 4 kinases, but it seems that even this crude metric can explain much of the behavior observed.

Cell adhesion is a much more complex phenomenon, and at the outset of this study it was not clear whether signaling would directly correlate with such a complex phenomena much less whether that link would be causal in a straightforward way. The results from this study seem to indicate that correlation with signaling is straightforward. Hypothesizing causal relationships from this correlation was likewise straightforward, but the results from the inhibition study are somewhat more difficult to interpret than those for β_1 -integrin expression. ERK inhibition causes decreases in adhesion of cells on three of the five substrates eliciting the greatest ERK activity and may have decreased adhesion on a fourth (fibronectin, $p \approx 0.06$). JNK inhibition decreases adhesion on 4 substrates and may have decreased adhesion on 2 other substrates ($p \approx 0.07$ and 0.1). These results seem to show a causal link between ERK and JNK activity and cell adhesion.

However, inhibition of IKK also led to decreases in adhesion of cells cultured on 8 of the 10 substrates—despite expecting the opposite relationship (conclusion shown schematically in the bottom half of Fig. 10). The most likely explanation for this is that the original signaling data were confounded by the high IKK activity substrate (chondroitin sulfate) also having low ERK and low JNK activities. Interesting, though, is that the PLS analysis did correctly hypothesize a causal relationship between IKK signaling and adhesion, but the proportionality of the response predicted was inverse. Also, although Akt was not expected to affect cell adhesion, inhibition of Akt led

to increased adhesion on 3 substrates and near significance ($p \approx 0.07$) on a fourth.

These results seem to show that using these systems biology tools to generate hypotheses regarding causal links between signaling and cell behaviors is possible and worked fairly well. As we expected, the hypotheses generated for the direct link to expression of a single gene product (β_1 -integrin) were more accurate than were the hypotheses to the more complex phenomenon of cell adhesion. However, if we had only studied β_1 -integrin expression, we would have mistakenly believed that IKK had no effect on that phenomenon (or perhaps an increase); whereas, it is in reality the most potent negative response we observed when studying the complex phenomenon of cell adhesion. Observing some samples unaffected by ERK or JNK inhibition or some samples affected by Akt inhibition seems to indicate that basing this entirely on mean activity is a crude metric which leaves some parts of the phenomenon unexplained. As discussed above, including more pieces of information per kinase or more signaling molecules (λ^n) can increase the information content available to explain these events.

Systems Biology as a Tool for Biomaterials Science

Why go to all of this effort? It is clear that, for this simple cell behavior and only 10 substrate compositions, it would have been much easier to only measure data for Figs. 5 and 6 without performing this systems biology analysis. There are two major reasons why we believe that the field of biomaterials must go beyond such descriptive experiments.

First, there are thousands to millions of combinations of micro-environmental cues, and the field of biomaterials needs a framework to understand how these cues interact to produce cell behaviors. Descriptive studies of cell adhesion can yield very different results merely by changing the cell culture media, cell type, or other similarly mundane variations in micro-environment; however, most studies change only one of these factors at a time—recent work by Garcia and co-workers shows an elegant example of synergistic effects in signaling when varying only two factors.²⁹ This is good science; but, unless the experimental conditions exactly match the conditions in the application we envision, these experiments provide little understanding upon which to base rational design of materials that elicit desired cell behaviors under the specific conditions required for the application at hand. Intracellular signaling has the potential to be a rather generic descriptor of how a cell interprets its micro-environment and how it converts that interpretation into changes in gene expression and ultimately cell behavior. Thus, understanding cell behavior

through systems biology tools may allow us to understand how various micro-environmental cues, by affecting many of the same signaling pathways, can combine to achieve the richness of variation in cell behaviors observed.

Secondly, biomaterials engineers almost never want to achieve a single desired cell behavior but instead want to achieve several desired cell behaviors simultaneously. In the purely descriptive mode of biomaterials science, the data are often not clear or sometimes contradictory in regards to how to achieve several cell behaviors simultaneously. By understanding how patterns of intracellular signaling combine to cause desired cell behaviors, it may become clear how to achieve multiple behaviors simultaneously. Additionally, by understanding the link between biomaterial properties and the pattern of signaling generated, these same techniques may elucidate how to use known techniques (e.g., conjugation of peptides to a surface, controlling mechanical properties, release of growth factors, etc.) to achieve the pattern of signaling required to cause the combination of cell behaviors desired.

The present study is a long way from achieving these lofty goals; however, this study does show limited proof-of-principle of a generalizable method that can be used to work toward this eventual goal of understanding how biomaterials cause differences in intracellular signaling and how those intracellular signals in turn cause differences in cell behaviors.

CONCLUSIONS

Culturing HUVECs on ten different substrates (9 adsorbed ECM proteins and uncoated polystyrene) elicits different patterns of mean activity of 4 intracellular signaling kinases (ERK1/2, JNK, Akt, and IKK). Of 45 possible pair-wise comparisons, 44 are shown to be different—only Matrigel and laminin do not elicit different patterns of signaling. This can explain why cells behave differently on these different substrates. We then used PLS to discover causal relationships between intracellular signal activity and cell behaviors. Using PLS, we hypothesized that ERK and JNK activities caused increased cell adhesion and β_1 -integrin expression and that IKK activity caused decreased cell adhesion and β_1 -integrin expression. Inhibition studies generally confirm these hypotheses for ERK and JNK. Inhibition of IKK, on the other hand, showed no statistically significant effect on β_1 -integrin expression (although two samples were nearly significant increases) and led to significant decreases in cell adhesion—confirming a causal link but opposite of the hypothesized relationship (that inhibition of IKK would increase adhesion).

ACKNOWLEDGMENTS

This research was supported by the NIH (R21 EB004386), the American Heart Association, and the Arizona Biomedical Research Commission (#0917). The authors thank Dr. Kevin Janes for generous assistance in helping us reproduce his kinase assay protocols, helping us learn to use the PLS Toolbox, and in assisting in experimental design. We also thank Dr. Christine Pauken who supervises our cell culture facility, Dr. Lawrence Mandarino for allowing us to use his scintillation counter, and Dr. Yung Chang for allowing us to use her FACScaliber.

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