



A gene expression-based comparison of cell adhesion to extracellular matrix and RGD-terminated monolayers



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ARTICLE INFO

Article history:

Received 19 September 2014

Received in revised form

3 February 2015

Accepted 6 February 2015

Available online 3 March 2015

Keywords:

Biomimetic material

Fibronectin

Integrin

Self-assembled monolayer

ABSTRACT

This work uses global gene expression analysis to compare the extent to which model substrates presenting peptide adhesion motifs mimic the use of conventional extracellular matrix protein coated substrates for cell culture. We compared the transcriptional activities of genes in cells that were cultured on matrix-coated substrates with those cultured on self-assembled monolayers presenting either a linear or cyclic RGD peptide. Cells adherent to cyclic RGD were most similar to those cultured on native ECM, while cells cultured on monolayers presenting the linear RGD peptide had transcriptional activities that were more similar to cells cultured on the uncoated substrates. This study suggests that biomaterials presenting the cyclic RGD peptide are substantially better mimics of extracellular matrix than are uncoated materials or materials presenting the common linear RGD peptide.

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1. Introduction

To remain viable, many cells must attach to, and spread on, an extracellular matrix (ECM), the insoluble aggregate of numerous proteins and glycosaminoglycans that mediates cell adhesion and provides a host of extracellular cues [1–6]. These cues include several motifs that primarily interact with integrins to mediate adhesion but also include ligands that engage diverse non-adhesion related cell-surface receptors [4–10]. In practice, the large number of bioactive motifs present in the ECM makes it difficult to understand the role that each plays in adhesion and signaling. Even so, materials that are modified with ECM ligands are important in a variety of areas, including biomaterials, where coatings presenting a single biomolecular ligand give control over cell adhesion and subsequent cellular behavior [11–14]. Yet, there remains substantial debate surrounding the extent to which these simplified substrates functionally mimic natural ECM [6,15]. We propose that analyzing gene expression in cells adherent to substrates presenting either matrix proteins or cell adhesive peptides

will give a better understanding of the efficacy of biomimetic peptides serving as ECM mimics in biomaterial modification.

Previous studies have generally compared basic cellular phenotypes – for example, adhesion, spreading, migration, and cytoskeletal structure – on model substrates [11,15–18]. These model substrates typically have a layer of adsorbed protein – such as collagens, laminin, fibronectin, or protein mixtures like Matrigel – on glass or plastic surfaces [5,16,17]. While the protein layers often have well-defined compositions, the cellular responses vary because the adsorbed proteins are present in a heterogeneous distribution with respect to their conformations, orientations, and densities [6,18]. These limitations have motivated the development of model substrates that have just one, or a small number of, biologically active molecules [8–11,19,20]. Of these, self-assembled monolayers (SAMs) have emerged as the best-defined ECM mimics because the monolayers are structurally well-defined and allow for the presentation of peptide ligands against otherwise inert backgrounds. These peptide-modified SAMs have been used in studies of cell adhesion, migration, proliferation, and differentiation, and are well-suited for a broad range of studies involving the interactions of cells with ECM [8,19–22].

The tripeptide Arg-Gly-Asp (RGD) found in the 10th type III domain of fibronectin remains the best-characterized adhesion ligand and the most widely used for modifying culture substrates

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and biomaterials [8,23–27]. This peptide binds approximately one half of the integrin receptor family and on its own supports cell adhesion, spreading, and migration [15]. The RGD peptide can be presented in either a linear form or in a cyclic form and several studies have shown that the latter supports greater spreading, larger traction forces, and a more punctate organization of focal adhesions, indicative of more robust cell adhesion [13–15]. The cyclic peptide mimics the active conformation of the native protein's receptor-bound state and has an approximately 300-fold higher affinity for $\alpha_v\beta_3$ integrin than the corresponding linear peptide [8,28–32]. Further, cells adherent to monolayers presenting the cyclic peptide display a phenotype more like those on fibronectin-coated substrates [12]. To date, however, no studies have employed a systems-level comparison to address the extent to which peptide-modified monolayers, or monolayers initially presenting no bioactive motifs, mimic protein-coated substrates. We hypothesize that the comparison of differential gene expression of cells on these substrates will provide a measure of the biological response of cells on model substrates and the extent to which the various substrates are functional mimics of commonly used matrix-coated substrates. We further hypothesize that cells cultured on substrates presenting the cyclic RGD peptide will demonstrate more similarity in global gene expression to fibronectin-cultured cells than those cultured on linear RGD.

2. Materials and methods

2.1. Materials

General laboratory chemicals and reagents were purchased from VWR. Cell culture reagents, and antibodies were purchased from Life Technologies. Octadecanethiol, fibronectin, collagen IV, collagen II, and laminin were purchased from Sigma–Aldrich. Formaldehyde was purchased from Ted Pella, Inc.

2.2. Peptide synthesis

Cyclic RGD (RGDfC) and cyclic RDG (RDGfC) (f denotes a phenylalanine residue having the D-configuration at the alpha carbon) were synthesized as previously described on 2-chlorotriethyl chloride resin [32]. After lyophilization, the peptides were dissolved in 0.1% TFA in water and stored at $-20\text{ }^\circ\text{C}$ until further use. Linear RGD (Ac-GRGDSC-NH₂) and linear RDG (Ac-GRDGSC-NH₂) were synthesized using standard Fmoc SPPS on MBHA-Fmoc-rink amide resin. For the final coupling, acetylated glycine was used. After lyophilization, the peptide was dissolved in 0.1% TFA in water and stored at $-20\text{ }^\circ\text{C}$ until further use.

2.3. Preparation of monolayers

Self-assembled monolayers were prepared as described previously [14]. Briefly, titanium (40–50 Å) and then gold (220–500 Å) were evaporated onto glass coverslips using an electron beam evaporator (Thermionics) at a rate of $0.2\text{--}0.4\text{ nm s}^{-1}$ with a pressure of 1.0×10^{-6} Torr.

Fibronectin, collagen IV, collagen II, and laminin coated gold substrates were prepared by soaking slides in an ethanolic solution of octadecanethiol for 1 min to generate an octadecanethiol monolayer, then rinsed and dried under air. These surfaces were then incubated with either 25 $\mu\text{g}/\text{mL}$ of fibronectin, 8 $\mu\text{g}/\text{cm}^2$ collagen IV, 8 $\mu\text{g}/\text{cm}^2$ collagen II, or 10 $\mu\text{g}/\text{mL}$ laminin in $1 \times$ PBS (pH 7.4) for 1 h at $37\text{ }^\circ\text{C}$. Octadecanethiol surfaces not pre-coated with protein were incubated in $1 \times$ PBS (pH 7.4) for 1 h at $37\text{ }^\circ\text{C}$.

Self-assembled monolayers were also prepared by incubating gold-coated slides in an ethanolic solution of symmetric tri(ethylene glycol)-terminated disulfide (EG₃) and asymmetric maleimide and tri(ethylene glycol)-terminated disulfide for 12–16 h at room temperature. The disulfide reagents were used at a concentration of 1 mM with the maleimide-terminated group present at relative fractions of 1%. Cyclic RGD, cyclic RDG, linear RGD, or linear RDG, at concentrations of at least 10 μM , were each immobilized to maleimide-presenting monolayers for 1 h at $37\text{ }^\circ\text{C}$. The substrates were characterized by SAMDI mass spectrometry. All substrates were rinsed with $1 \times$ PBS (pH 7.4) prior to cell seeding.

2.4. Cell culture

HT1080 fibrosarcoma cells (CCL-121; ATCC) were maintained in low glucose DMEM, supplemented with 10% FBS, 100 U penicillin, 100 μg streptomycin and 2 mM L-glutamine at $37\text{ }^\circ\text{C}$ at 5% CO₂. Cells were seeded at a density of $0.75\text{--}1.0 \times 10^4$ cells/cm² and allowed to spread for 48 h in serum-supplemented media.

2.5. Immunofluorescence

Cells were fixed with 4% formaldehyde in $1 \times$ PBS (pH 7.4), blocked in 1% bovine serum albumin (BSA), 2.5% goat serum, 2.5% non-fat milk, and 0.1% Triton X-100 in $1 \times$ PBS (pH 7.4). Actin and cell nuclei were labeled by incubation with Alexa 488-phalloidin and Hoechst 33342. Microscopy was performed on a C2+ laser confocal microscope (Nikon Instruments, Inc.) and image analysis was performed using Fiji software [33]. Cell Counter plugin was used to quantify cell numbers in each field of view using stained nuclei. The area fraction of fluorescently labeled actin was measured from thresholded images, and cell area was calculated for each field of view using corresponding cell counts. Thresholding was done so as to exclude the background but encompass all actin, using constant settings for all images analyzed. Statistical significance was determined using an Analysis of Variance (ANOVA) model with Tukey's multiple comparison test and Bartlett's test for equal variances using GraphPad software.

2.6. RNA extraction

RNA was extracted using TRIzol reagent (Life Technologies) according to protocol. 10 μg of RNase free glycogen (Life Technologies) was added to each sample prior to isopropanol precipitation to improve yield. Washed RNA pellets were suspended in 25 μL of RNase free water. Samples were concentrated and further purified using RNeasy MinElute Cleanup Kit (Qiagen) according to protocol and stored at $-80\text{ }^\circ\text{C}$. RNA quality was checked using a NanoDrop 2000c Spectrophotometer (Thermo Scientific) and by gel electrophoresis. RNA extracts with a 260/280 wavelength ratio less than 2.00 or a concentration less than 250 ng/ μL were excluded from further analysis.

2.7. RT-qPCR

Total RNA (20.7 ng) was reverse transcribed using SuperScript VILO cDNA Synthesis Kit for RT-PCR or SuperScript VILO Master Mix (Life Technologies). Real-time PCR was performed using TaqMan Fast Advanced Master Mix and TaqMan Gene Expression Assays (Life Technologies). qPCR was performed using the ABI PRISM 7900HT in the Genomics Core at Northwestern University. Analysis was performed using SDS and ExpressionSuite Software (Life Technologies).

2.8. Illumina gene expression analysis

RNA quality was checked by Agilent 2100 Bioanalyzer by the Genomics Core at Northwestern University. RNA expression profiling was performed in quadruplicate using the Illumina HumanHT-12v4 Expression BeadChip, providing coverage of over 35,000 genes and expressed sequence tags. Raw data processing and identification of differentially expressed genes was performed by the Bioinformatics Core at Northwestern University and Chipster [34]. Raw signal intensities of each probe were obtained using Illumina's BeadStudio data analysis software and imported to the lumi package of Bioconductor for data transformation and normalization [35–37]. Differentially expressed genes were identified using an Analysis of Variance (ANOVA) model with empirical Bayesian variance estimation on the basis of a statistically significant (adjusted p value <0.05) and 1.5 fold change (up or down) in expression level [38]. Genes involved in cell migration, adhesion, or cytoskeleton were identified using GO pathways in Chipster and KEGG pathways.

3. Results

3.1. Design and preparation of substrates

We used self-assembled monolayers (SAMs) for all experiments in this work, as they give structurally well-defined and reproducible surfaces for the study of cell adhesion (Fig. 1) [39]. To prepare self-assembled monolayers, we first evaporated titanium and then gold onto glass coverslips. We then immersed the gold-coated slides in a solution of octadecanethiol (ODT) to give hydrophobic monolayers that could subsequently be modified by adsorption of fibronectin, collagen IV, collagen II, or laminin protein to generate protein-coated substrates. We used the octadecanethiolate monolayer as a model of cell culture substrates that are not pre-coated with ECM prior to the attachment of cells, but recognize that this surface chemistry does not fully mimic all versions of cultureware, including tissue culture plastics that have a net negative charge. Extensive prior work, including the use of surface plasmon resonance spectroscopy, has shown that matrix proteins (and virtually all proteins) adsorb rapidly and effectively irreversibly to the methyl-terminated monolayers [40–42].

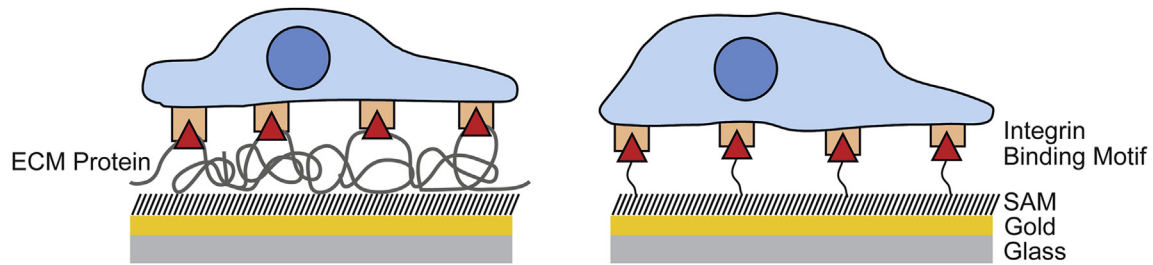


Fig. 1. Cells were cultured on a self-assembled monolayer (SAM) on gold-coated glass substrates. Cell adhesion was compared for monolayers that were either coated with an adsorbed layer of extracellular matrix protein coating (left) or were modified by covalent attachment of RGD peptides (right). In both cases, cell adhesion was mediated by binding of integrin receptors (square) to the proteins or peptides on the monolayer (triangle).

To generate peptide-presenting substrates, we also immersed gold coated slides in a solution of disulfide molecules terminated in tri(ethylene glycol) and maleimide groups such that the latter was present at a relative density of 1%. The presence of the tri(ethylene glycol) groups on these monolayer substrates carries particular significance, as they are effective at preventing the non-specific adsorption of protein and therefore ensure that cell adhesion is mediated through interactions with the immobilized ligands [19–21]. We immobilized either linear RGD (IRGD) or cyclic RGD (cRGD) peptides to the monolayer by way of a reaction between a cysteine thiol group on the peptide and a maleimide group on the monolayer (Fig. 2) [14,43]. The linear peptide includes the serine residue next to the aspartate, as this residue has been shown to improve binding to the integrin receptors [44]. The cyclic peptide contains a D-phenylalanine residue in this position, again as this residue has been shown to improve affinity of the ligand for integrin receptors [29]. As control substrates, we prepared monolayers presenting the scrambled peptide sequence, RDG, which does not bind integrin receptors, and therefore does not support cell adhesion [45]. We characterized the resulting monolayers using MALDI-TOF MS (SAMDI-MS) to

confirm complete immobilization of the peptide (Supp. Fig. 1) [43].

3.2. Analysis of cell phenotype

We used HT1080 cells in this work because this line has been frequently used in studies of cell adhesion and because it expresses a broad array of adhesion receptors, including $\alpha_v\beta_3$ and $\alpha_5\beta_1$, which are known to bind to the RGD sequence [46–50]. Many studies of cell adhesion allow cells to attach to the substrate in the absence of serum, in part because serum proteins can adsorb to the substrate and block or interfere with the matrix–integrin interactions. Because we are using monolayers that are effective at preventing the non-specific adsorption of protein, we allowed cells to attach in the presence of serum [19,21]. In this way we avoided the additional variability in the experimental conditions associated with changing compositions of the media. Hence, we seeded HT1080 cells on all substrates at equal densities and cultured the cells in serum-supplemented media (Fig. 3A). We cultured the cells for 48 h prior to analysis; this time is common in studies of cell attachment and spreading and allows characterization of the initial response of cells

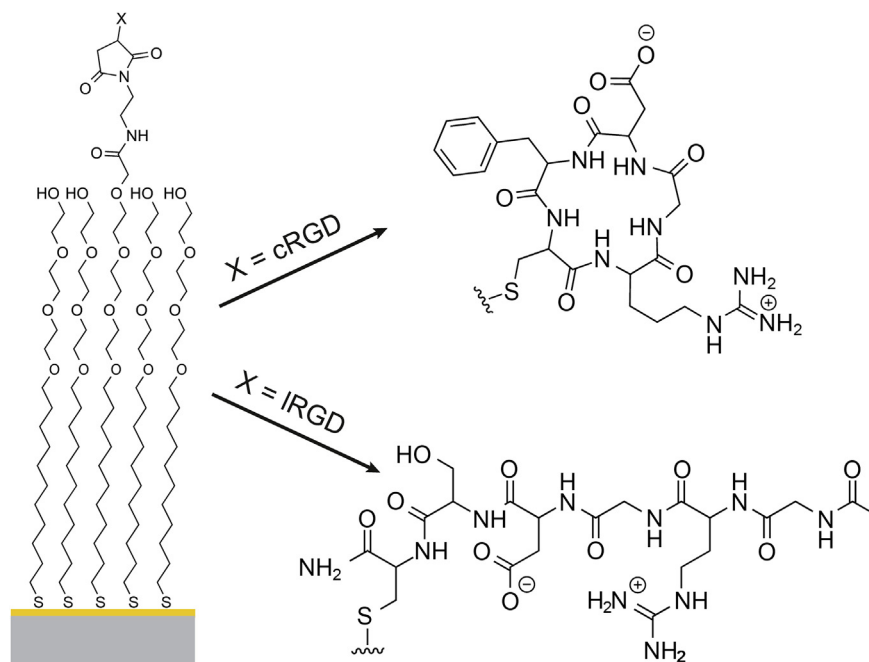


Fig. 2. This work used self-assembled monolayers presenting maleimide groups against a background of tri(ethylene glycol) groups, to which either a cyclic peptide (cRGD) or linear peptide (IRGD) was immobilized by way of a cysteine residue in the peptide.

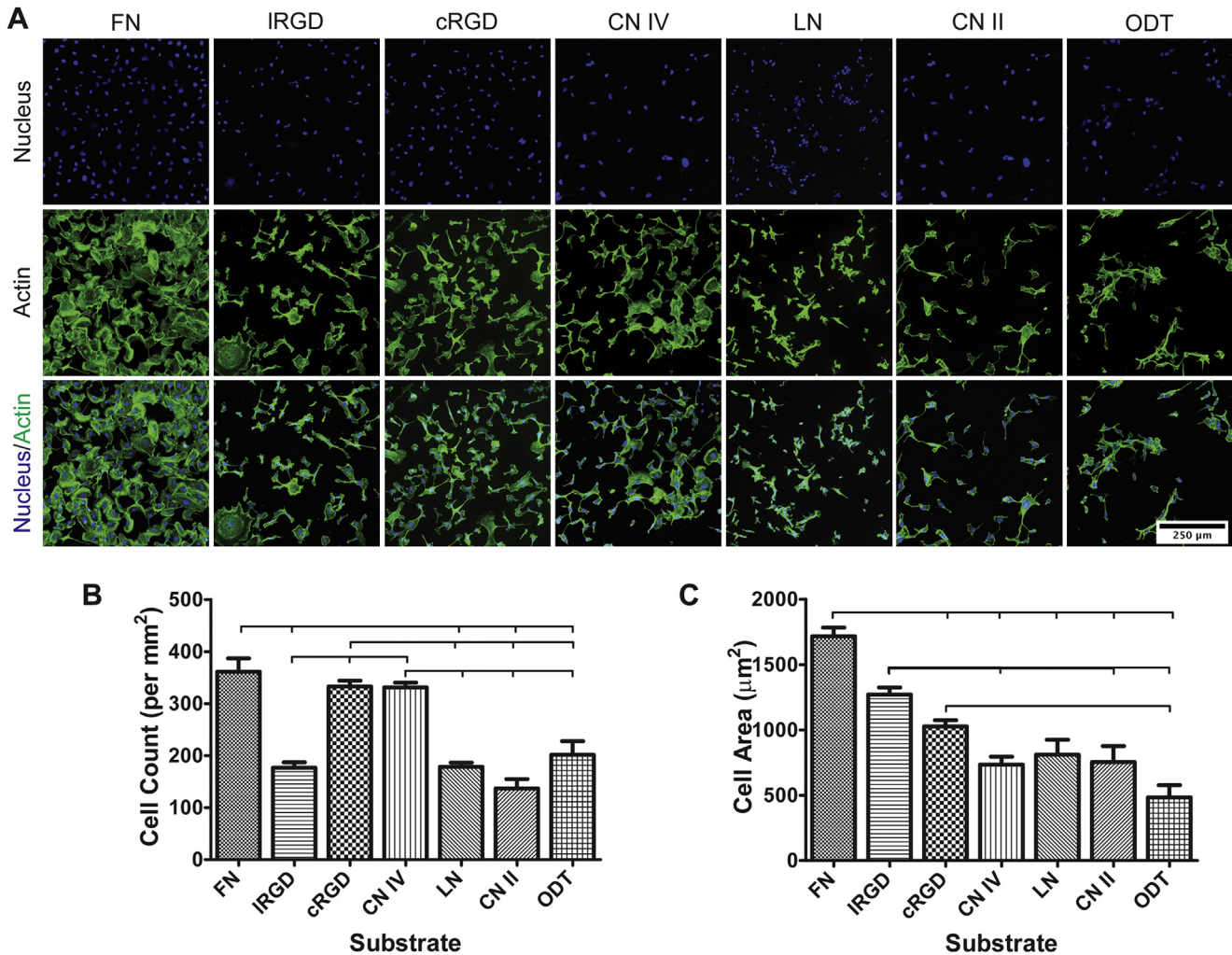


Fig. 3. (A) HT1080 cells were cultured on self-assembled monolayers presenting fibronectin protein (FN), linear RGD peptide (IRGD), cyclic RGD peptide (cRGD), collagen IV protein (CN IV), laminin protein (LN), collagen II protein (CN II), or octadecanethiol monolayer (ODT). Cells were fixed and stained to label the nuclei (blue) and actin (green). The number of cells that attached to each substrate per unit area is shown in panel B and the average projected area of adherent cells are shown in panel C. Statistical significance ($p < 0.01$) is denoted by bars. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to the different matrices. We then fixed the cells and fluorescently labeled the nuclei and microfilaments in order to examine differences in cell spreading and morphology in five independent fields of view. We observed that cells on fibronectin and collagen IV, as well as cells on monolayers presenting IRGD and cRGD, generally remained adhered during fixation. A significant fraction of cells on collagen II, laminin, and octadecanethiol substrates were easily removed. We therefore took care in the handling and rinsing of substrates to avoid the direct application of buffers to the cell-coated surfaces, minimizing cell loss for the subsequent analysis of cell phenotype. We found that essentially no cells adhered to the monolayers presenting the scrambled peptides cRDG and IRDG, and therefore excluded these experiments from the subsequent analysis. We determined the density of cells on each substrates and found that cells on fibronectin, cRGD, and collagen IV substrates attached at densities greater than 300 cells/mm² compared to cells cultured on IRGD, collagen II, laminin, and octadecanethiol substrates which attached at densities of less than 200 cells/mm² ($p < 0.01$) (Fig. 3B). We also analyzed cell spreading and found that cells on fibronectin and IRGD substrates had the largest areas, with area greater than 1200 µm², whereas cells cultured on cRGD, collagens II and IV, laminin, and octadecanethiol substrates had areas less than 1050 µm² ($p < 0.01$) (Fig. 3C).

3.3. Quantitative gene expression using RT-qPCR

We next compared the mRNA expression levels of selected genes in adherent HT1080 cells cultured on the various substrates using real-time reverse transcriptase polymerase chain reaction (RT-qPCR). We focused on the expression of three ECM component transcripts – fibronectin (*FN1*), collagen IV (*COL4A5*), and laminin (*LAMA4*) – that represent the three major epithelial ECM molecules, and three integrin receptors – α_v (*ITGAV*), α_2 (*ITGA2*), and β_5 (*ITGB5*) – that demonstrate some specificity for fibronectin, collagen II/laminin, and collagen IV, respectively [51,52]. *GAPDH* served as a reference gene transcript to normalize expression levels across samples. We cultured the cells as described above then lysed them with TRIzol reagent, eliminating samples that degraded during extraction and cleanup, thereby minimizing DNA and protein contamination while ensuring a suitable concentration of RNA for further analysis. We determined the relative quantification (RQ) values for the expression of each mRNA transcript in cells on each of the substrates relative to those on fibronectin (Table 1).

Cells cultured on the octadecanethiolate monolayers that did not have an adsorbed layer of ECM protein displayed the greatest

Table 1

Changes in gene expression measured by RT-qPCR, relative to fibronectin, of cells cultured on self-assembled monolayer substrates presenting linear RGD peptide, cyclic RGD peptide, collagen IV, laminin, collagen II, or octadecanethiol.

Biological group	Target	RQ	P-value
cRGD	<i>FN1</i>	1.574	0.143
	<i>COL4A5</i>	1.755	0.007
	<i>LAMA4</i>	1.688	0.074
	<i>ITGA2</i>	1.843	0.007
	<i>ITGAV</i>	0.946	0.892
	<i>ITGB5</i>	1.451	0.182
IRGD	<i>FN1</i>	7.967	0.008
	<i>COL4A5</i>	5.461	0.177
	<i>LAMA4</i>	3.479	0.180
	<i>ITGA2</i>	4.188	0.212
	<i>ITGAV</i>	1.738	0.293
	<i>ITGB5</i>	3.022	0.134
CN IV	<i>FN1</i>	1.046	0.716
	<i>COL4A5</i>	0.815	0.209
	<i>LAMA4</i>	0.835	0.674
	<i>ITGA2</i>	1.054	0.633
	<i>ITGAV</i>	0.922	0.976
	<i>ITGB5</i>	0.698	0.448
CN II	<i>FN1</i>	1.764	0.044
	<i>COL4A5</i>	2.07	0.004
	<i>LAMA4</i>	1.153	0.742
	<i>ITGA2</i>	2.789	0.066
	<i>ITGAV</i>	1.283	0.240
	<i>ITGB5</i>	2.936	0.154
LN	<i>FN1</i>	0.710	0.853
	<i>COL4A5</i>	6.038	0.068
	<i>LAMA4</i>	2.656	0.188
	<i>ITGA2</i>	6.358	0.074
	<i>ITGAV</i>	1.256	0.469
	<i>ITGB5</i>	3.235	0.196
ODT	<i>FN1</i>	14.416	0.022
	<i>COL4A5</i>	8.152	0.173
	<i>LAMA4</i>	3.868	0.030
	<i>ITGA2</i>	3.594	0.027
	<i>ITGAV</i>	2.789	0.001
	<i>ITGB5</i>	1.397	0.145

changes relative to cells cultured on a fibronectin-coated monolayer, with a 15-fold increase in mRNA expression of fibronectin ($p < 0.05$) (Fig. 4A) and a 4-fold increase in expression of laminin ($p < 0.05$) (Fig. 4C). Expression of integrins α_2 and β_5 showed 3-fold

($p < 0.005$) (Fig. 4D) and 4-fold increases ($p < 0.05$) (Fig. 4E) in mRNA expression, respectively, relative to fibronectin-coated substrates.

Cells cultured on substrates coated with laminin and collagens II and IV displayed few changes in gene transcript expression levels compared to those cultured on fibronectin-coated substrates (Table 1). Specifically, cells cultured on collagen IV and laminin substrates showed no statistically significant differences in expression levels for any of the gene transcripts compared to cells on fibronectin substrates. Cells cultured on collagen II-coated substrates, however, showed significant changes in mRNA expression of fibronectin and collagen IV, with increases of 76% ($p < 0.05$) and 107% ($p < 0.005$), respectively (Table 1).

Cells cultured on monolayers that presented the cRGD peptide showed similar expression levels of the selected mRNA transcripts compared to cells on the fibronectin-coated monolayers, with the exceptions of the collagen IV (Fig. 4B) and integrin α_2 (Fig. 4D) mRNA transcripts, which showed slight increases of 76% ($p < 0.01$) and 84% ($p < 0.01$), respectively. In comparison, cells cultured on IRGD substrates revealed larger changes in expression for all mRNA transcripts. While only an 8-fold increase of fibronectin expression (Fig. 4A) demonstrated statistical significance ($p < 0.01$), the magnitude of change in mRNA expression for each of the remaining gene transcripts in cells adherent to IRGD substrates displayed similar trends to those adherent to octadecanethiol substrates.

3.4. Global gene expression profile of cells on fibronectin vs RGD substrates

This transcript analysis reveals that cells adherent to monolayers presenting cRGD show more similarity to those on fibronectin-coated monolayers than do those cultured on IRGD-presenting monolayers. To more thoroughly compare these peptide-presenting substrates to fibronectin matrix, we performed global gene expression profiling using an Illumina HT-12v4 microarray chip. We isolated RNA from adherent HT1080 cells cultured on monolayers presenting fibronectin, cRGD, or IRGD as previously described. Changes in gene expression were determined using fibronectin as a reference.

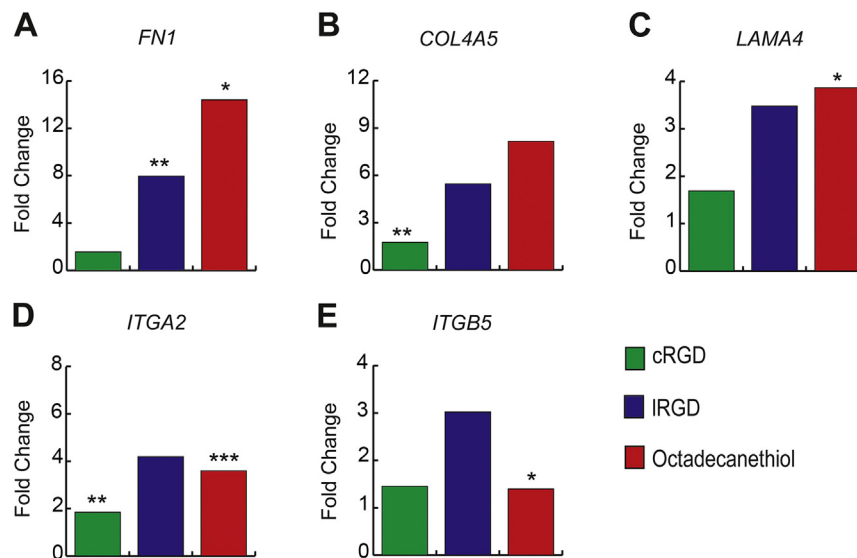


Fig. 4. The changes in gene expression for fibronectin (*FN1*, A), collagen IV (*COL4A5*, B), laminin (*LAMA4*, C), integrin α_2 (*ITGA2*, D), and integrin β_5 (*ITGB5*, E) are shown as fold-change for cells cultured on cyclic RGD (cRGD, green), linear RGD (IRGD, blue), and octadecanethiol (ODT, red) relative to fibronectin cultured cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We first compared gene expression in cells cultured on fibronectin-coated and cRGD-presenting substrates. From the approximately 35,000 gene transcripts analyzed, we identified a total of 944 differentially expressed genes that demonstrated statistically significant ($p < 0.05$) changes in gene expression. Of these only 88 genes showed a greater than 50% change in expression level. When comparing cells cultured on IRGD-presenting substrates to fibronectin-coated substrates we identified 2788 statistically significant differentially expressed genes, of which 285 genes showed a greater than 50% change in expression. While we observed overlap in the genes with statistically significant changes in expression between the RGD substrates, cells cultured on IRGD substrates typically exhibited a higher magnitude change in expression for these genes (Supp. Table 1). Between the cRGD and IRGD substrates, a total of 286 differentially expressed genes ($p < 0.05$) had greater than 50% change in expression, 36 of which are categorized either as ECM, cell-ECM adhesion, or cytoskeletal proteins, highlighting their dependence on substrate composition (Fig. 5, Supp. Table 2) [48,49,53].

On a larger scale, the global gene expression profiles revealed clear trends for genes coding for proteins that mediate interactions between the cytoskeleton and the ECM, particularly integrins and focal adhesion proteins. Most integrin receptors were expressed at higher levels on monolayers presenting either cRGD or IRGD as compared to those on fibronectin substrates, including integrins α_2

(*ITGA2*), α_{11} (*ITGA11*), and β_1 (*ITGB1*) (Fig. 6A). These integrins bind to non-RGD sequences in a broad spectrum of ECM proteins, including collagens, laminin, and fibronectin [54–58]. In contrast, integrins known to bind RGD sequences, including integrins α_V (*ITGAV*), α_5 (*ITGA3*), and β_3 (*ITGB3*), were unchanged in cells cultured on cRGD and IRGD relative to fibronectin. Transcripts for focal adhesion proteins were mostly expressed at lower levels with the notable exception of focal adhesion kinase (*PTK2*), a key protein for mechanotransduction signaling (Fig. 6A).

Next, we examined the expression of genes associated with the extracellular matrix, especially those coding for ECM component proteins and matrix metalloproteinases (MMPs), which are known to alter the composition and structure of the extracellular matrix, potentially affecting integrin signaling and integrin expression. Both ECM component proteins and MMPs generally displayed increases in gene expression, with notable increases in expression of prevalent native ECM proteins, collagen IV (*COL4A5*), laminin (*LAMA4*), and fibronectin (*FN1*) (Fig. 6B). However, the number of genes with statistically significant changes in expression of ECM component proteins and MMPs was greater for cells cultured on IRGD substrates than cells cultured on cRGD substrates. Notably, MMP-1 (*MMP1*), and five ECM component proteins – laminin α_4 (*LAMA4*), laminin γ_2 (*LAMC2*), collagen VI (*COL6A1* and *COL6A3*), and collagen VII (*COL7A1*) – demonstrated greater than 50% changes in expression in cells cultured on IRGD substrates relative to those on fibronectin substrates. For cells cultured on cRGD substrates, only MMP-4 (*MMP4*) and ECM component protein, laminin α_4 (*LAMA4*), showed greater than 50% changes in expression relative to cells on fibronectin substrates.

Finally, we observed clear patterns of change in gene transcripts of cytoskeletal proteins. Specifically, we observed a down regulation of gene transcripts associated with microfilaments, intermediate filaments (vimentin, keratin, etc.), and microtubules, in cells cultured on both cRGD and IRGD, relative to cells cultured on fibronectin, whereas genes associated with myosin motor proteins displayed a trend of upregulation (Fig. 6C). Notably, beta actin (*ACTB*), vimentin (*VIM*), and the majority of tubulin associated genes showed statistically significant decreases in expression on both RGD substrates, whereas myosin light chain kinase (*MYLK*) showed a significant increase in expression, relative to fibronectin substrates, for monolayers presenting either IRGD or cRGD.

4. Discussion

4.1. Peptide mimics of extracellular matrix

The materials used for culturing cells in the laboratory, and to a lesser extent in medical devices, are commonly modified with an extracellular matrix protein to promote cell adhesion. While this strategy improves cell adhesion relative to uncoated materials, it frequently fails to offer satisfactory control over the biological activity induced by the adsorbed protein matrix. This limitation arises in part because the adsorbed proteins are present in a distribution of orientations and because they are denatured to various extents. Further, impurities introduced during protein preparation can alter the composition of the bioactive coating [59,60]. A promising strategy that addresses these challenges is the immobilization of short peptide motifs to a material, as peptides generally have unstructured conformations that are not strongly affected by immobilization. There remains significant debate as to whether surfaces presenting a single short peptide can serve as functional mimics of ECM. Most studies that compare peptide-modified materials to extracellular matrix materials have measured cell adhesion, spreading, and cytoskeletal structure, but these phenotypic measures can be insensitive to cellular activities and signaling pathways

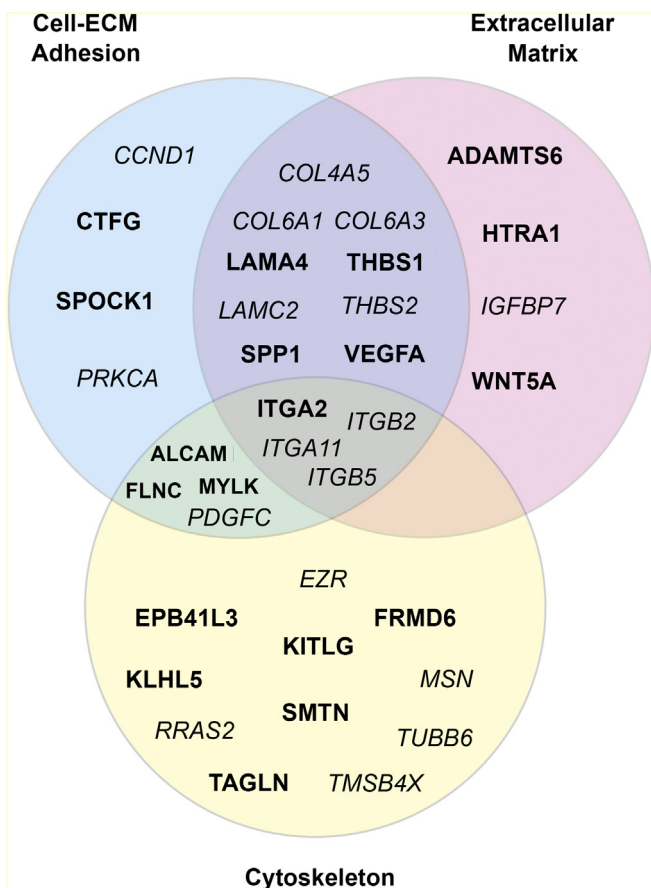


Fig. 5. Statistically significant ($p < 0.05$) genes with greater than 50% changes in expression were categorized by involvement in pathways related to Cell-ECM Adhesion (blue), Extracellular Matrix (red), or Cytoskeleton (yellow). Genes expressed in cyclic RGD only, linear RGD only, or both cyclic RGD and linear RGD are indicated by standard, italics, or bold typeface, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

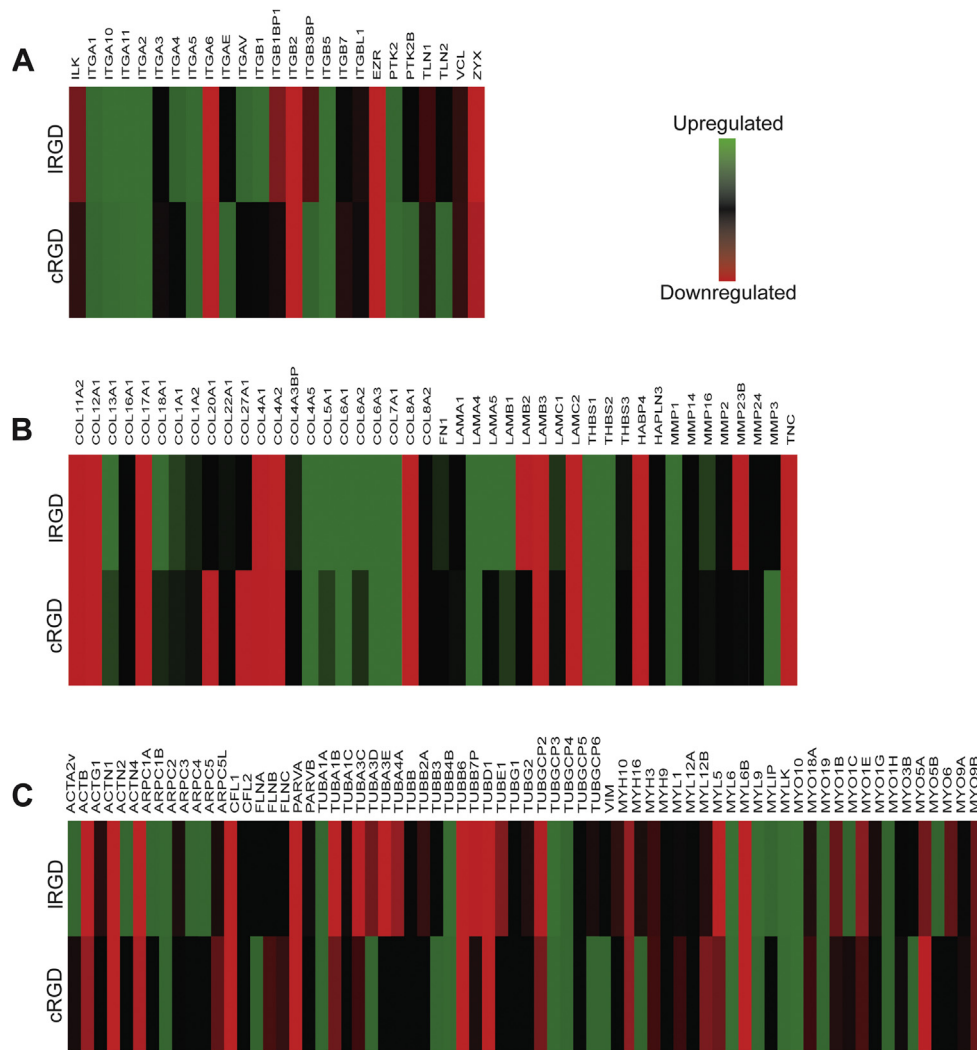


Fig. 6. All genes associated with cell adhesion (A), extracellular matrix (B), and cytoskeleton (C) pathways were grouped and shaded according to direction and degree of change in gene expression. Increased gene expression is indicated by green, decreased gene expression is indicated by red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that are important to cell viability [14,22,45,61]. In this study, we employed large-scale gene expression profiling to provide a more comprehensive comparison of biological activity on both protein and peptide-modified substrates after 48 h and to explore the extent to which model substrates can serve as functional mimics of ECM for HT-1080 epithelial cells. We expect that this trend will apply to the culture of other fibronectin-dependent cell lines, but the present example does not allow us to generalize the findings. This work does, however, provide a protocol for comparing the effectiveness of model substrates with the traditional matrix-coated substrates.

4.2. A peptide-modified monolayer is better than an unmodified substrate

We found that the monolayers presenting RGD peptides – in either the linear or cyclic conformation – are far better mimics of fibronectin-coated substrates than are hydrophobic substrates that are not coated with an ECM protein for a two day culture period. Specifically, we measured expression levels of six key adhesion transcripts and found that HT-1080 cells cultured on uncoated octadecanethiolate monolayers displayed a striking up-regulation

of extracellular matrix protein gene transcripts when compared to cells on fibronectin substrates, suggesting that these cells were actively remodeling their microenvironment. Indeed, one measure of the effectiveness of an ECM mimic is the extent to which cells actively engage in remodeling their ECM [62,63]. This observation is significant because many researchers still use uncoated substrates for cell culture and these uncoated substrates can be expected to significantly perturb cellular phenotypes in the early phase of culture when cellular activities are in a high state of flux due to matrix remodeling [64,65]. In contrast, cells adherent to cRGD-terminated monolayers displayed gene expression patterns that were significantly more similar to those in cells adherent to fibronectin-coated substrates. We further note that we are observing changes in gene expression after 48 h of culture, and that many studies use culture conditions that are substantially longer. Hence, the changes we observe in gene expression likely do not translate to similar changes in protein synthesis—for example the morphologies of cells on the different matrices are similar—but expect that this trend would hold in longer term cultures. The monolayers presenting an RGD peptide against an inert background are more effective substrates for short-term cell culture and cell adhesion studies compared to uncoated substrates.

4.3. cRGD is a better mimic of fibronectin than is IRGD

Prior studies investigating cell adhesion, spreading, and migration of cells cultured on fibronectin compared to substrates modified with the RGD peptide have revealed significant differences between the peptide substrates and the matrix-coated substrates [22,66–68]. Fewer studies have explicitly compared cell adhesion on substrates modified with either linear or cyclic RGD peptides. Those studies that have compared the different RGD conformations to each other generally find that the efficiency of cell adhesion, the degree of cell spreading, and the organization of the cytoskeleton are greater on the cyclic peptide, which has been attributed to a higher affinity of the cyclic peptide for the integrin receptor. In one such example, we showed that cells adherent to monolayers presenting the cyclic peptide exhibit a more even distribution and a greater number of focal adhesions, leading to increased cell adhesion [69]. Our present results similarly show that monolayers presenting the cyclic peptides serve as a more effective mimic for fibronectin than do those presenting the linear peptides. This observation suggests that the poor performance of RGD modified materials in previous studies may be attributed to the use of a low affinity peptide ligand and not to the use of a simple peptide ligand alone. We therefore suggest that cRGD should always be used in place of IRGD for modifying materials with biomimetic peptide coatings, particularly for promoting a more desirable initial attachment and spreading phenotype.

Our findings reveal a general trend of increased integrin and ECM protein expression on both RGD-presenting monolayers relative to fibronectin-coated substrates. We find that increased integrin expression is correlated to the increased expression of the ECM protein to which that integrin binds. For example, the $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ integrin binding receptors that bind collagen types I and IV are up-regulated in cells on IRGD substrates, but not in cells on cRGD substrates [58]. The observed increases in integrin receptor transcripts and the corresponding increases in collagen I and collagen IV suggests that the poor strength of ligand–receptor interactions on the low-affinity RGD substrates causes a significant up-regulation of major ECM proteins as well as their associated binding receptors, whereas cells that are adherent to the cRGD-terminated monolayers are less actively remodeling their matrices.

While the low-density RGD substrates used in this study provide a sufficient density of ligand for adhesion, the strength of the ligand–receptor interactions significantly affects signaling that leads to downstream changes in gene expression [70–72]. Cells adherent to IRGD substrates display decreased expression of the cyclin family of proteins that regulate the cell cycle and adhesion compared to fibronectin-coated substrates. Cyclin A, a cell adhesion-dependent regulator of proliferation in fibroblasts, is down-regulated in cells on IRGD substrates but not in cells on cRGD substrates [73]. This difference suggests that cells on IRGD substrates are not able to obtain the required adhesive strength leading to cell proliferation and division, further supported by the trend of down-regulated expression for focal adhesion and cytoskeletal proteins.

The strength and quality of adhesion to a substrate significantly influences cell survival and proliferation. Our work shows a general increase in the expression of Rab GTPases in cells cultured on the peptide-terminated monolayers relative to fibronectin. This family of proteins is implicated in cellular transport processes that are key to cell proliferation. Compared to cells cultured on fibronectin, cells cultured on IRGD show significant changes in many of the Rab transcripts. Most notably, Rab13, Rab26, and Rab31 gene transcripts, which are involved in regulating cell–matrix interactions, show significant increases in expression in cells adherent to IRGD substrates compared to fibronectin-coated substrates [74,75]. This

trend suggest that cells on IRGD substrates are less stable, turning over membrane and ECM proteins like integrin receptors and ECM proteins at a higher rate than cells on fibronectin or cRGD.

Some of the greatest changes in expression were observed in the ferritin family of proteins. These proteins have been implicated in focal adhesion stability as well as promoting cell adhesion and survival [76]. There is a general trend of increased expression for these genes in cells on both RGD substrates compared to cells on fibronectin-coated substrates. The ferritin proteins also play a role restoring key survival and adhesion signaling pathways, suggesting the RGD substrates are both able to support cell survival. However, the larger increases in expression of these proteins in cells on IRGD substrates imply that cells on cRGD substrates induce a lower degree of cell stress. This further supports our conclusion that cRGD should be preferentially used over IRGD and that it may be suitable for long-term culture.

4.4. Monolayers provide value to the design of ECM mimics

Many studies that use materials modified with the RGD peptide do not employ surface chemistries that are intrinsically inert to the adsorption of protein [77]. These substrates can give inconsistent results stemming from, for example, adsorption of unknown proteins to the surface prior to cell attachment, or likewise, a non-specific attachment of cells stemming from adsorption of various cell surface components to the substrate [26,78]. In both cases, the presence of the RGD peptides do not serve as functional mimics of ECM, since they may not interact with their cognate cell-surface receptors, and may not directly control cell adhesion to the surface [79,80]. For the peptide modified substrates, the monolayers we used present a background of tri(ethylene glycol), which is very effective at preventing the non-specific adsorption of protein [19]. Hence, it is possible to control entirely the ligand–receptor interactions that mediate cell adhesion. Finally, we also note that the monolayer substrates provide additional benefits as mimics of ECM, including the ability to pattern the shapes, sizes and positions of individual cells, to immobilize ligands in gradient patterns, to prepare substrates having multiple ligands, and to prepare substrates that are dynamic in that the activities of immobilized ligands can be switched on and off [20,21,39,81–83]. These benefits have made monolayers on gold particularly well-suited as model substrates for understanding and controlling the adhesion of cells.

5. Conclusions

This study demonstrates that gene expression can be used as a measure to assess the extent to which RGD biomimetic peptides serve as an effective substitute for purified ECM proteins when incorporated into biomaterials. Gene expression of cells adhered to RGD substrates revealed that a cyclic RGD peptide alone was an effective mimic of the fibronectin protein in a short-term study. In comparison, IRGD was a less effective mimic of fibronectin, though it was superior to the use of substrates lacking a bioactive motif. The properties of cRGD that make it a more effective mimic of fibronectin, compared to IRGD, likely relate to the higher affinity of integrins for the former. Most significantly, this work suggests that cyclic RGD peptides should always be used in place of the more common linear RGD peptide. The use of global analysis tools is an important method for assessing the performance of ECM, in conjunction with the conventional methods of assessing cell adhesion and spreading.

Acknowledgments

This research was supported by the Center of Cancer Nanotechnology Excellence (CCNE) initiative of the National Institutes of Health (NIH) under Award number U54 CA151880 and the Zell Family Foundation Faculty Recruitment Gift. Microarray expression profiling was performed by the Northwestern University Genomics Core Facility. Expression analysis was performed by the Northwestern University Bioinformatics Core.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at <http://dx.doi.org/10.1016/j.biomaterials.2015.02.045>.

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