# Positively and Negatively Modulating Cell Adhesion to Type I Collagen Via Peptide Grafting

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The biophysical interactions between cells and type I collagen are controlled by the level of cell adhesion, which is dictated primarily by the density of ligands on collagen and the density of integrin receptors on cells. The native adhesivity of collagen was modulated by covalently grafting glycine–arginine–glycine–aspartic acid–serine (GRGDS), which includes the bioactive RGD sequence, or glycine–arginine–aspartic acid–glycine–serine (GRDGS), which includes the scrambled RDG sequence, to collagen with the hetero-bifunctional coupling agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The peptide-grafted collagen self-assembled into a fibrillar gel with negligible changes in gel structure and rheology. Rat dermal fibroblasts (RDFs) and human smooth muscle cells demonstrated increased levels of adhesion on gels prepared from RGD-grafted collagen, and decreased levels of adhesion on RDG-grafted collagen. Both cell types demonstrated an increased ability to compact free-floating RGD-grafted collagen gels, and an impaired ability to compact RDG-grafted gels. RDF migration on and within collagen was increased with RDG-grafted collagen and decreased with RGD-grafted collagen, and dose–response experiments indicated a biphasic response of RDF migration to adhesion. Smooth muscle cells demonstrated similar, though not statistically significant, trends. The ability to both positively and negatively modulate cell adhesion to collagen increases the versatility of this natural biomaterial for regenerative therapies.

## Introduction

CELL MIGRATION IS A UBIQUITOUS PROCESS that is of fundamental importance in tissue morphogenesis, wound healing, and tissue engineering. Different tissue cells can demonstrate distinct morphologies and mechanisms during migration. Fibroblasts, which exhibit relatively slow migration, explore the direction of migration by extending a leading edge, attaching to the matrix, strengthening attachments by the formation of focal adhesion sites, contracting actin bundles to advance the cell body, and finally releasing rear attachments to propel forward.[1–3] In contrast, keratinocytes, which can migrate more quickly, deploy a treadmilling mechanism of myosin contraction of the actin cytoskeleton–integrin–matrix links to give the appearance of a gliding motion.[4] For each mechanism, however, effective migration requires the successful transfer of internal cytoskeletal forces to external tractional forces against an extracellular substratum. Traction is exerted on the substratum via specific cell surface receptors and complimentary adhesion ligands present in the substratum, and is balanced by translocation of the cell and/or reorganization of the matrix.

The strength of cell adhesion to the substratum contributes critically to the balance of external and internal forces that governs cell migration.[5,6] In two-dimensional (2D) in vitro systems, cells exhibit a biphasic response of migration with respect to adhesion strength, with the highest migration coefficients occurring at intermediate levels of available ligands and/or receptors.[5–7] If adhesion strength is low or too few adhesions are made, the cell is unable to develop sufficient traction to propel. If adhesion strength is too great or adhesions are too plentiful, ligand–receptor dissociation is impeded and cells cannot remove themselves from the substrate to effectively locomote. A similar biphasic relation between adhesion and cell migration has been demonstrated and modeled in three-dimensional (3D) matrices.[1,8–11]

Biopolymeric gel–based in vitro assays—particularly, type I collagen systems—provide an especially valuable platform to examine cell migration. Collagen is a ubiquitous ECM protein that forms the structural framework for many soft tissues. Solutions of collagen can be used to coat 2D substrates, or can self-assemble into fibrillar 3D hydrogels that offer a better representation of the microenvironment of living tissues when compared to traditional 2D systems. When tissue cells are entrapped in an entanglement of collagen fibers to form the so-called tissue equivalents, cells can attach to and exert traction on the fibers, which can both compact the matrix and/or propel the cells.[12,13] As a natural

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ECM substrate, collagen provides a significant basal level of adhesivity via several peptide sequences, including RGD and GFOGER,[14,15] that can vary among cell types depending on the nature of integrin expression, and therefore places different cells at different locations on the biphasic curve relating cell migration to cell adhesion.

Physiologically, the strength and specificity of adhesion can be modulated by environmental factors to enhance cell migration, such as during wound healing, when resident fibroblasts are stimulated by cytokines and growth factors released at the wound site to migrate into the provisional matrix.[16] In vitro, fibroblasts in collagen gels exhibit minimal migration under standard culture conditions, but increase in motility significantly when exposed to growth factors such as the BB isoform of platelet-derived growth factor,[17] basic fibroblast growth factor,[18] or epidermal growth factor.[19] Clinically, the ability to increase cell migration has broad applications in regenerative therapies, where repopulating damaged or engineered tissues with host cells can accelerate healing and recovery, including wound healing[20] and peripheral nerve and spinal cord regeneration.[21] Although cell migration can generally be enhanced via exogenous soluble factors,[22,23] genetic reprogramming,[24,25] or by blocking adhesion with soluble, competitive ligands,[26] from a tissue engineering perspective, the ability to enhance cell migration through collagen scaffolds via intrinsic properties of the biomaterial without the inclusion of technology for the controlled release of soluble factors or the genetic manipulation of cells would have great clinical potential. The specificity and strength of collagen adhesivity have been increased by covalently grafting bioactive peptide sequences associated with integrin-mediated binding to the collagen backbone.[8,27,28] However, to increase the speed or motility of many cell types on or within collagen, a decrease in the adhesivity is required.[7,29] Herein, we show that grafting glycine–arginine–aspartic acid–glycine–serine (GRDGS), nominally a scrambled, nonadhesive peptide control for glycine–arginine–glycine–aspartic acid–serine (GRGDS),[26] decreases fibroblast and smooth muscle cell (SMC) adhesion to collagen gels. The decrease in cell adhesion decreased the ability for these cells to compact free-floating collagen gels, and, at low-to-moderate concentrations of grafted peptide, increased cell migration.

## Materials and Methods

### Cell culture

Experiments were performed with rat dermal fibroblasts (RDFs) and human aortic SMCs. RDFs were obtained using a primary explant technique from rat pups that constitutively express green fluorescent protein via an actin promoter (tissue generously provided by the W.M. Keck Center for Collaborative Neuroscience). Cell lines were initiated for culture by thawing an aliquot of cells and centrifuging at 2000 rpm for 2 min at 4°C. The pellet of cells was resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin–streptomycin (pen–strep) (1% v/v) and L-glutamine (1% v/v) (Sigma, St. Louis, MO). Cells were plated in T25 flasks using 4 mL of DMEM with 10% fetal bovine serum (FBS), 1% pen–strep, and 1% L-glutamine, and kept in a humidified $CO_2$ incubator at 37°C. Trypsin/EDTA (Sigma) was used to passage the cells once a week at a 1:8 dilution.

Flasks (75–90% confluent) were harvested with 0.5% Trypsin/EDTA and washed twice with DMEM supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA), 1% pen–strep, and 1% L-glutamine. All experiments were conducted before the 12th passage, at which point a new culture was initiated from frozen cells. SMCs (a gift from Dr. Gary Nackman at the University of Medicine and Dentistry of New Jersey [UMDNJ]) were cultured in MCDB-131 medium with SmGM-2 SingleQuot (Cambrex–Clonetics, Walkersville, MD). SMCs were stored and cultured in a manner similar to RDFs. All experiments with SMCs were conducted before passage 8.

### Conjugation of peptides to collagen backbone

Two peptide sequences, GRGDS (RGD) and a scrambled version, GRDGS (RDG), were custom synthesized (Genscript, Piscataway, NJ) and were conjugated to the backbone of collagen in suspension. A third peptide, TVFHFRLL (a non-RGD–related peptide used in the laboratory as a scrambled control for peptide mimics of carbohydrates involved in neural cell adhesion[30]), was similarly acquired and tested in adhesion assays to evaluate the specificity of the observed responses on the RDG scrambled peptide. A hetero-bifunctional coupling agent, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC; Sigma), was used to activate the carboxylic group of the peptide by mixing 1 mL of a 1 M solution of EDC in MES buffer (pH 2–4; Sigma) with 2 mg of peptide for 10 min at 37°C. The peptide–EDC mixture was added to 5 mL of a 3 mg/mL suspension of type I collagen (Cat #C857, acid extracted from calf skin with >95% purity; Elastin Products, Owensville, MO) in 0.02 N acetic acid. The activated peptide covalently bound to free amines on residues on the collagen backbone via nucleophilic attack. A low pH buffer was used while coupling peptides to the collagen backbone to avoid self-assembly of collagen fibers. Peptide–EDC–collagen mixtures were incubated on a shaker overnight at 4°C and then dialyzed against 0.02 N acetic acid for 12 h using snakeskin dialysis tubing with a 10 kDa molecular weight cutoff (Pierce Biotechnology, Rockford, IL) to remove unconjugated peptide. Dialyzed peptide-grafted collagen was lyophilized at −150°C and 50 mtorr for 12 h to remove all water. Lyophilized product was resuspended in 0.02 N acetic acid to make a 3 mg/mL solution of grafted collagen. The efficiency of peptide grafting was measured indirectly by grafting peptides that included a FITC tag during synthesis (Genscript) and comparing the fluorescence intensity of grafted collagen after reconstitution to a standard curve created by admixing fluorescent peptide into collagen solution. From these comparisons of fluorescence intensities, the efficiency of peptide grafting ranged between 50% and 60% of the original mass of added peptide, resulting in 67–80 μg peptide/mg collagen or ∼10–12 peptides per collagen chain, based on the molar ratios of the peptides to monomeric type I bovine collagen fibers, although we note that the collagen used in this study is not monomeric but rather a suspension of short oligomers. To evaluate the effects of the EDC treatment on adhesion, separate solutions of control collagen were prepared as above without the inclusion of peptides.

### Collagen gel preparation

Type I collagen gels were prepared by mixing 20 μL 1 M HEPES buffer (Sigma), 140 μL 0.1 N NaOH (Sigma), 100 μL

$10\times$ minimum essential medium (Sigma), 62 µL medium 199 (Invitrogen, Carlsbad, CA), 1 µL pen–strep from a stock solution of 5000 units of penicillin and 5000 mg streptomycin/ mL in 0.85% saline (Sigma), 10 µL L-glutamine from a stock solution of 29.2 mg/mL in 0.85% saline (Sigma), and 667 µL of 3.0 mg/mL collagen (grafted or native) to make a 2.0 mg/mL collagen solution. To prepare gels with different concentrations of grafted collagen, peptide-grafted collagen solutions were diluted with native collagen solutions at varying ratios. The native and grafted collagen solutions self-assemble into a gel upon incubation at 37°C. For assays with RDFs, three concentrations of grafted RGD, RDG, or TVFHFRLL peptides were investigated: a high (H) concentration of 67 µg peptide/mg collagen, a medium (M) concentration of 33.5 µg/mg, and a low (L) concentration of 16.75 µg/mg, each in a 2 mg/mL collagen gel. For the RDG and RGD peptides, these concentrations result in $\sim 0.25$ mM, 0.125 mM, and 0.0625 mM for the high, medium, and low cases. For experiments with SMCs, only the medium concentrations of grafted RDG and RGD were assayed.

### Fibril size and density

High-magnification confocal microscopy was used to estimate the effects of the peptide grafting on collagen fiber size and density. Straight, 1-cm-long, 500-µm-wide, and 100-µm-deep channels were generate in poly(dimethyl siloxane) (PDMS) (Fisher Scientific, Pittsburgh, PA) using standard soft lithography and were bonded to glass coverslips. The channels were filled with grafted or native collagen solution spiked with FITC-labeled collagen (10% v/v; Elastin Products), to allow for visualization of fibers. Devices were transferred to a Leica TCS SP2/MP confocal microscope (Leica Microsystems, Exton, PA). Images were taken at $63\times$ with a $2\times$ digital zoom at 488 nm excitation with a 500–535 nm emission bandpass filter. All image frames underwent two line and frame averaging. Three images were taken at random in each device. Each image was divided into nine equal squares. The average number and diameter of fibers were determined in three of the nine squares with image analysis software. The analysis was repeated for three gels in each condition, and results were compared with ANOVA (significance set at $p < 0.05$).

### Mechanical testing of scaffolds

Potential effects of the grafting procedure on the mechanical properties of the resulting self-assembled gels were assessed via parallel plate rheometry using a Rheometrics SR-2000 rheometer (TA Instruments, New Castel, DE), as previously described.[31] Briefly, a 25-mm-diameter hole was punched in a 4-mm-thick layer of PDMS. Collagen solution (800 µL) was pipetted into the punched well and transferred to a 37°C incubator to induce self-assembly. The gels were carefully removed with a spatula and transferred to the bottom plate of the rheometer. The top plate was lowered to a height of 0.8 mm. The dynamic storage and loss moduli of the gel were evaluated at 1% shear strain amplitude at frequencies ranging from 0.1 to 5 Hz for three samples prepared from separate batches of native collagen and RGD-grafted collagen. The storage moduli were compared with ANOVA at discrete frequencies. Significance levels were set at $p < 0.05$.

### Adhesion assay

RGD-, RDG-, and TVFHFRLL-grafted collagen solutions at low, medium, and high concentrations of grafted peptide, as well as native collagen solutions and EDC-treated collagen solutions (200 µL), were pipetted into separate wells of a 24-well plate in triplicate and allowed to self-assemble at 37°C and 100% humidity. RDFs (100 µL of a 50,000 cells/mL suspension) were seeded on the gels and allowed to settle and attach for 1 h. Wells were rinsed three times with 1 mL PBS in 5-min intervals. The remaining cells were stained with calcein AM (Invitrogen) and imaged using an Olympus IX81 inverted epifluorescent microscope (Olympus, Melville, NY) with a $4\times$ objective and an FITC filter. Images were captured digitally (Hamamatsu ORCA, Hamamatsu City, Japan) using Olympus Microsuite software. The number of cells in each field of view was counted using Image Pro Plus (version 5.1 for Windows; Media Cybernetics, Bethesda, MD). Three randomly selected fields were imaged and quantified for each well. The data were analyzed statistically with ANOVA. *Post hoc* pairwise comparisons were performed with Tukey's test. Significance levels were set at $p < 0.05$. The adhesion assay was repeated with SMCs for the RGD-M, RDG-M, and the control collagen conditions. Images and data were collected and analyzed in a similar manner.

### Gel compaction assay

The effects of peptide grafting on the ability of cells to exert traction on the fibrillar collagen network were indirectly evaluated by measuring cell-mediated compaction of free-floating collagen disks. RDFs (50,000 cells/mL) were suspended in type I collagen solutions with a specified volume fraction of RDG- or RGD-grafted collagen. A PDMS annulus (4.75 mm ID and 4 mm height) was placed in a well of a 24-well plate and was filled with 35 µL of the cell/collagen suspension. The plate was transferred to a humidified incubator operating at 37°C and 5% $CO_2$ to induce self-assembly of the collagen and entrap the cells within the forming fibrillar network.[32] The collagen did not adhere to the PDMS, and after self-assembly, addition of 500 µL of medium to the well caused the gelled disc to float out of the ring. The plate was then returned to the incubator. Without a mechanical constraint to compaction, traction is exerted isotropically,[12,33] and as such, the cell-mediated compaction resulted in a uniform reduction in the size of the disk. An image of the compacting collagen disc was taken at day 0 and then every 24 h for 6 days using a dissection microscope (Carl Zeiss, Thornwood, NY) equipped with a digital camera (MatrixVision, Oppenweiler, Germany). The area of the collagen disc was measured from the images using ImageJ (NIH, Bethesda, MD). Each condition was done in (at least) triplicate on a particular day, and the design was repeated four times. The amount of compaction was determined by comparing the area of the disk to its initial area at day 0. Statistical significance was evaluated with a one-way ANOVA. Significance levels were set at $p < 0.05$. The compaction assay was repeated with SMCs for the RGD-M, RDG-M, and control collagen conditions. Images and data were collected and analyzed in a similar manner.

### Migration assay

The effects of peptide grafting on cell migration both on collagen gels (2D migration) and within collagen gels (3D

migration) were assayed using methods similar to those of Shreiber *et al.*[13] A 200 µL aliquot of collagen solution was pipetted into wells of a 24-well plate and allowed to self-assemble at 37°C and 100% humidity. RDFs or SMCs (5000/well) were seeded on the gels. SMCs were pre-stained with Lysotracker Red (Invitrogen) to visualize the cells using TRITC optics during time-lapse epifluorescent microscopy. A custom-built on-stage incubator was placed on the computer-controlled stage of the Olympus IX81 to maintain temperature and humidity during the time-lapse experiment. RDFs were visualized using FITC optics via the constitutively expressed GFP. For experiments in collagen gels (which were not free floating, but rather were adherent to the tissue culture plastic at the bottom and around the perimeter of the gel), 1000 cells/well were introduced into the collagen before casting the gel, similar to the compaction studies described above. Fewer cells were used in gels than on gels to limit cell-mediated compaction of the gel, which introduces a significant convective component to cell position that complicates quantifying cell migration. FITC-fluorescent beads were included in two experiments to assess convective movement of collagen gels, which was judged to be minimal (data not shown). The plates were returned to the incubator, and the cells were allowed to attach and spread for 3 h before initiating time-lapse microscopy.

Time-lapse microscopy of cell migration was performed via a computer-controlled stage of an Olympus IX81 inverted microscope with a temperature-controlled incubation chamber set to maintain 37°C (TA Instruments). Air-buffered media (medium 199 [Invitrogen, Carlsbad, CA] with Hanks' salts supplemented with 10% FBS, 1% pen–strep, and 1% L-glutamine) was used for the duration of the time-lapse experiment. Images for the migration assay were captured digitally using Olympus Microsuite Imaging Software. Three fields of view were imaged in each well with a 4×objective at 10–15 min for 12–14 h, which captured the X–Y position of cells within the field. The Z-position of cells, even within gels, was not recorded.

Images were processed using Matlab's image processing toolbox (Mathworks, Natick, MA); original images were filtered and binarized to subtract background fluorescence, when required. Processed images were imported into Image Pro Plus (Media Cybernetics) to determine 2D spatial cell tracks for each cell in view, which were then verified manually. At least 50 cells in each well were tracked. The average mean squared displacement ($\langle d^2(t)\rangle$) for each well was cal-

culated over all time intervals using overlapping intervals.[34] The resulting $\langle d^2(t)\rangle$ versus time interval ($t$) data were fit to a persistent random walk model assuming 2D migration,

$$\langle d^2(t)\rangle = 4\mu\left[t - P\left(1 - e^{-t/P}\right)\right]$$

to determine the cell motility coefficient $\mu$ and the persistence time, $P$.[35]

For RDFs within collagen and SMCs on collagen, three conditions (RDG-M, RGD-M, and collagen) were assayed in each experiment in triplicate, and the design repeated three times. For the dose–response RDF experiments on collagen, separate experiments were performed with RDG (RDG-L, RDG-M, RDG-H, and collagen) and with RGD (RGD-L, RGD-M, RGD-H, and collagen), with each condition performed in triplicate and each design repeated three times. The cell motility coefficients and persistence times were analyzed for statistical significance using a one-way ANOVA. Significance levels were set at $p < 0.05$.

## Results

### Physical characteristics of collagen gels

The effects of peptide grafting on the collagen fiber microstructure were estimated from high-magnification confocal micrographs (Fig. 1). No differences in the average number of collagen fibers (ANOVA, $p = 0.753$) or the average thickness of fibers ($p = 0.420$) were detected between peptide-grafted and native collagen. The effects of peptide grafting on the mechanical properties of the collagen post-self-assembly were evaluated with parallel plate rheometry (Fig. 2). Profiles of storage and loss moduli versus shear strain frequency for peptide-grafted and untreated collagen gels were consistent with our previous reports.[31] No significant differences were detected in storage or loss moduli at 0.1, 1, 2.5, or 5 Hz (ANOVA, min. $p = 0.70$). Together, these results indicate that grafting peptides to collagen oligomers does not interfere significantly with self-assembly into a fibrillar hydrogel, which is consistent with previous studies employing similar techniques.[27]

### Adhesion

Grafting bioactive and scrambled peptides significantly affected cell adhesion to collagen gels (Fig. 3). RDF adhesion (Fig. 3A) increased above controls on collagen grafted with
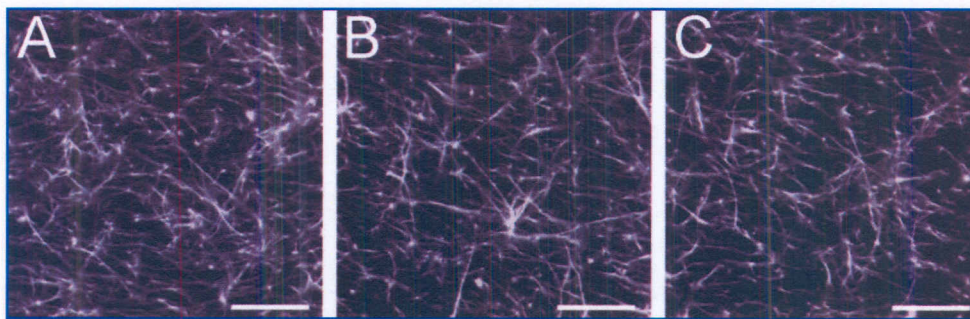


**FIG. 1.**   Confocal micrographs of (**A**) native, (**B**) RGD-grafted, and (**C**) RDG-grafted collagen. No overt differences were apparent in collagen gels formed following peptide grafting, and no significant differences were identified in estimates of fiber number (ANOVA, $p = 0.753$) or size (ANOVA, $p = 0.420$). Scale bars = 25 µm.
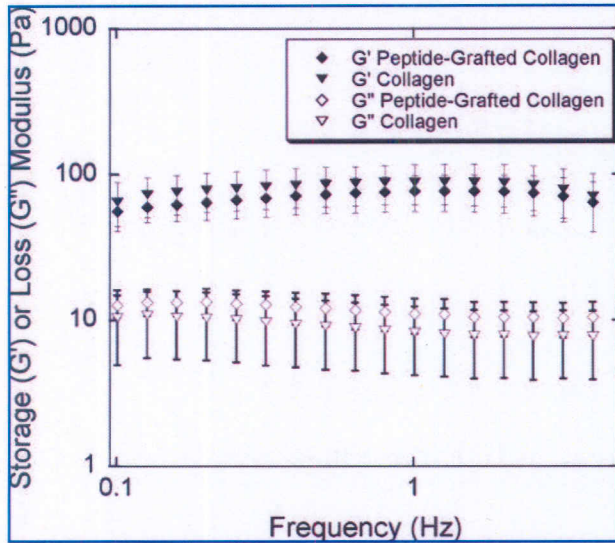
**FIG. 2.** Average storage and loss moduli (±standard error) for native and peptide-grafted collagen. The procedure for grafting peptides on to collagen had little effect on the rheological properties of the resulting fibrillar gel, as measured with parallel plate rheology. No significant differences in storage or loss moduli were detected across the frequency sweep (max. $p = 0.703$).

RGD, and decreased below controls on collagen grafted with RDG (ANOVA, $p < 0.001$). A similar decrease in RDF adhesion was observed with collagen grafted with TVFHFRLL ($p < 0.001$) (Fig. 3B). RDF adhesion was tested at varying concentrations of grafted RGD and RDG, which together modulated cell adhesion in a roughly sigmoidal, dose-dependent manner (ANOVA, $p < 0.001$). *Post hoc* analysis with Tukey's test demonstrated that all pairwise comparisons were significantly different (max. $p = 0.009$) except the following pairs: RDG-H and RDG-M ($p = 0.995$), RDG-M and RDG-L ($p = 0.061$), and RGD-L and RGD-M ($p = 0.530$). Similar *post hoc* analysis with the TVFHFRLL-grafted collagen results demonstrated that all pairwise comparisons were significantly different (max. $p = 0.013$) except TVFHFRLL-L and TVFHFRLL-M ($p = 0.079$) and TVFHFRLL-M and TVFHFRLL-H ($p = 0.407$). SMC adhesion (Fig. 3C) also increased above controls on collagen grafted with RGD, and decreased below controls on collagen grafted with RDG (ANOVA, $p < 0.001$). Treatment of collagen solutions with EDC without addition of peptides did not affect adhesion ($p = 0.838$).

## Compaction

The effects of modulating cell adhesion to collagen via peptide grafting on cell-mediated compaction of collagen gels were evaluated in cell-populated, free-floating collagen gels over a 6-day period (Fig. 4). Increasing cell adhesion via grafting of RGD peptides increased the degree of cell-mediated compaction by both RDFs and SMCs. Conversely, decreasing cell adhesion via grafting of RDG peptides decreased the ability of these cells to compact collagen gels. Significant differences were found in compaction among conditions at each of the 6 days following cell seeding for
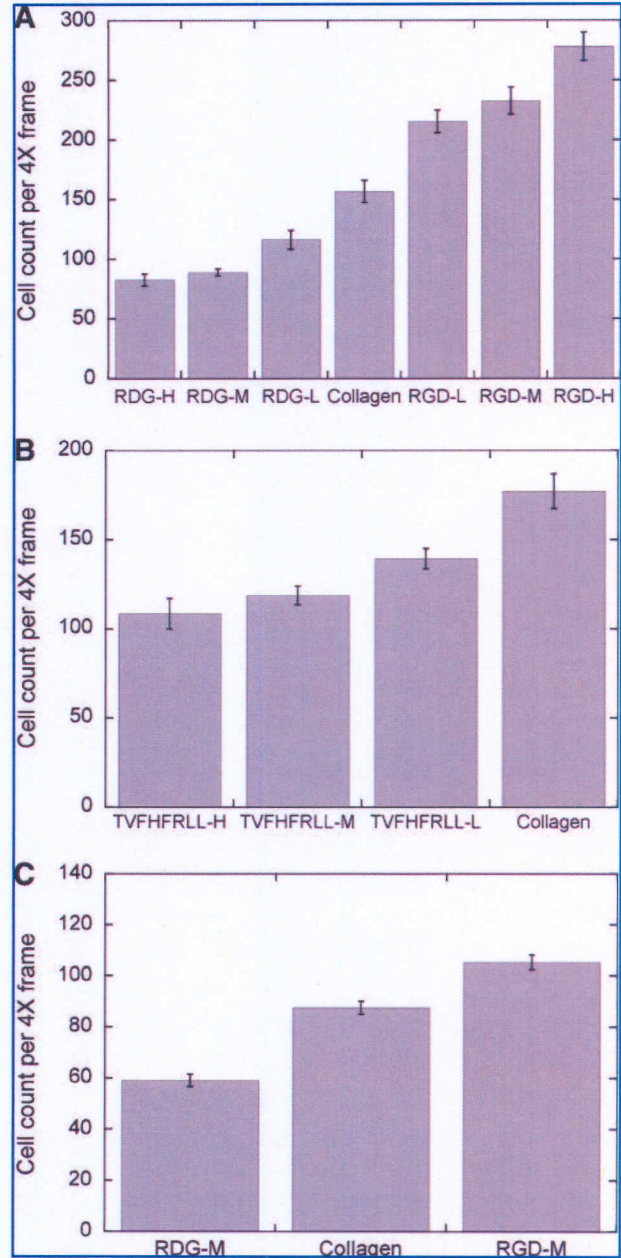


**FIG. 3.** Effects of peptide grafting on RDF and SMC adhesion. (**A**) RDF attachment (average ± standard error) was increased on RGD-grafted gels and decreased on RDG-grafted gels. RDF adhesion was assayed at three concentrations of RDG and RGD, and demonstrated a dose–response relationship (ANOVA, $p < 0.001$). (**B**) RDF attachment was also decreased in a dose–response fashion when a separate, non-RGD–related control peptide (TVFHFRLL) was grafted to the collagen (ANOVA, $p < 0.001$). (**C**) SMC adhesion was assayed at the medium concentration of grafted peptides, and also demonstrated a significant dependence on peptide grafting (ANOVA, $p < 0.001$).
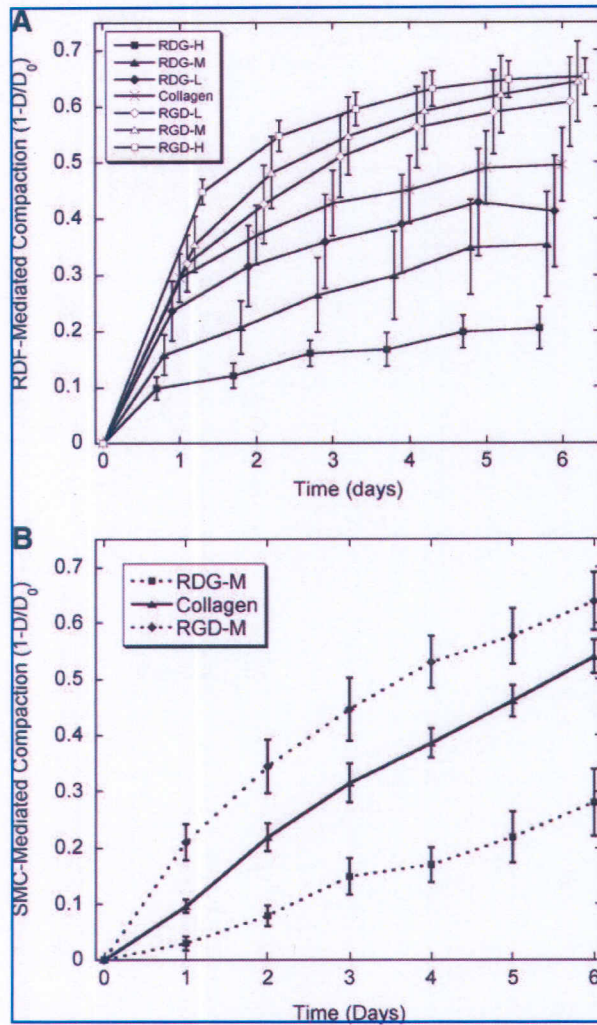
**FIG. 4.** Effects of peptide grafting on (**A**) RDF-mediated and (**B**) SMC-mediated compaction of free-floating collagen gels (average degree of compaction ± standard error). RDF-mediated compaction demonstrated a dose–response dependence on the concentration of grafted RGD or RDG peptide (ANOVA, $p < 0.001$.) Compaction was increased in gels prepared with RGD-grafted collagen and decreased in gels prepared with RDG-grafted collagen. Similar significant trends were identified with SMCs at the medium concentration of peptide grafting (ANOVA, $p = 0.007$).

SMCs (ANOVA, max. $p = 0.009$) and RDFs (ANOVA, max. $p < 0.001$). In general, decreasing adhesion (via RDG grafting) had a greater negative effect on compaction than the positive effect induced by increasing adhesion (via RGD grafting).

*Migration*

The influence of modulating adhesion to collagen gels via peptide grafting on cell migration was assayed in 2D and 3D systems with RDFs, and in a 2D system with SMCs (Figs. 5 and 6). Separate sets of RDF dose–response experiments were performed with RGD-grafted collagen and with RDG-grafted collagen, each with native collagen controls. Cell

motility was significantly decreased below controls for RGD-grafted collagen ($p = 0.025$). No significant differences were detected among the RGD conditions (pairwise comparisons with Tukey's test, min. $p = 0.99$) (Fig. 5A). In contrast, cell motility demonstrated a statistically significant (ANOVA, $p = 0.003$) bimodal dependence on the concentration of RDG that was grafted to collagen, first increasing at low and medium concentrations of grafted RDG, and then decreasing to near control levels when adhesion was further decreased via a high concentration of grafted RDG (Fig. 5B). Tukey's test revealed significant differences between RDG-L and collagen ($p = 0.005$) and RDG-L and RGD-H ($p = 0.024$). No other comparisons were significantly different (min. $p = 0.077$).

Cell migration assays were also performed with the RDG-M and RGD-M conditions for RDFs migrating within a collagen gel (Fig. 6A) and for SMCs migrating on a collagen gel (Fig. 6B). RDF migration within collagen gels significantly depended on peptide grafting ($p = 0.005$). Migration was increased in RDG-grafted gels and decreased in RGD-grafted gels compared to collagen controls. Similar trends were observed with SMCs migrating on collagen gels, but these differences were not statistically significant ($p = 0.157$). No significant differences were observed in the persistence time of each cell population on or in the various scaffolds (min. $p = 0.256$).

*Adhesion–migration–compaction relationships*

To examine the relationships among cell adhesion, cell migration, and cell traction via cell-mediated compaction, data from RDF dose–response adhesion, compaction (after 24 h), and migration experiments were plotted on the same sets of axes. First, data sets from individual experiments were normalized to the average control condition for that day, and the normalized values were averaged across all experiments. These data were then grouped according to the grafting condition (RGD-L, -M, and -H or RDG-L, -M, and -H) and plotted as (adhesion, migration) and (adhesion, compaction) X–Y pairs. Figure 7 demonstrates the observed biphasic response of migration to cell adhesion and the monotonic response of gel compaction.

**Discussion**

It is generally accepted that a biphasic relationship exists between cell adhesion and cell migration, where an optimum density of integrin-ligand binding creates the most favorable force balance for cell migration.[7] We demonstrated that this could be accomplished for naturally adhesive biomaterials by covalently grafting nonadhesive peptide sequences to mask native adhesion. Addition of nonadhesive RDG decreased adhesion and cell-mediated compaction, and increased cell migration, whereas addition of bioactive RGD to collagen increased adhesion and cell-mediated compaction, but had little effect on cell migration. Addition of a separate, scrambled, nonadhesive peptide (TVFHFRLL) also decreased cell adhesion, which suggests that the blocking of adhesion was not due to specific inhibitory bioactivity of the RDG peptide.

*In vitro*, bioactive peptides are commonly applied to functionalize a synthetic, nonadhesive surface[36] or are added in a soluble form to inhibit adhesions.[37–39] These
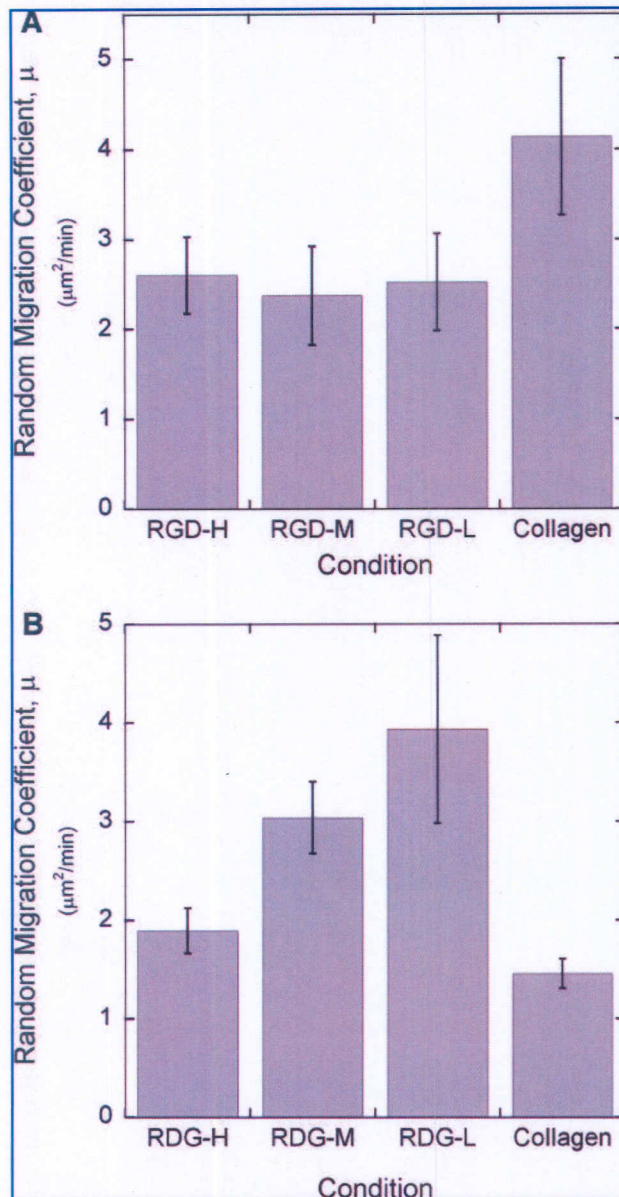
**FIG. 5.** Dose–response effects of peptide grafting on the random migration coefficient (average ± standard error) for RDFs migrating on collagen gels. (**A**) RGD-grafted collagen; (**B**) RDG-grafted collagen. Cell migration was decreased on RGD-grafted gels compared to collagen (ANOVA, $p = 0.025$), but no dose dependence was identified in *post hoc* tests (Tukey's test, min. $p = 0.99$). Cell migration was significantly affected by RDG grafting (ANOVA, $p = 0.003$). *Post hoc* tests revealed that migration on the RDG-L condition was significantly greater than RDG-H ($p = 0.024$) and collagen ($p = 0.005$).

peptides can elicit cell behavior through synergistic adhesion-mediated phenomena[37] and binding-mediated intracellular pathways.[40] Scrambled sequences or sequences with a substituted peptide are often used as controls for these experiments, and cells are typically blind to these scrambled versions.[41–44] Using hetero-bifunctional coupling systems,
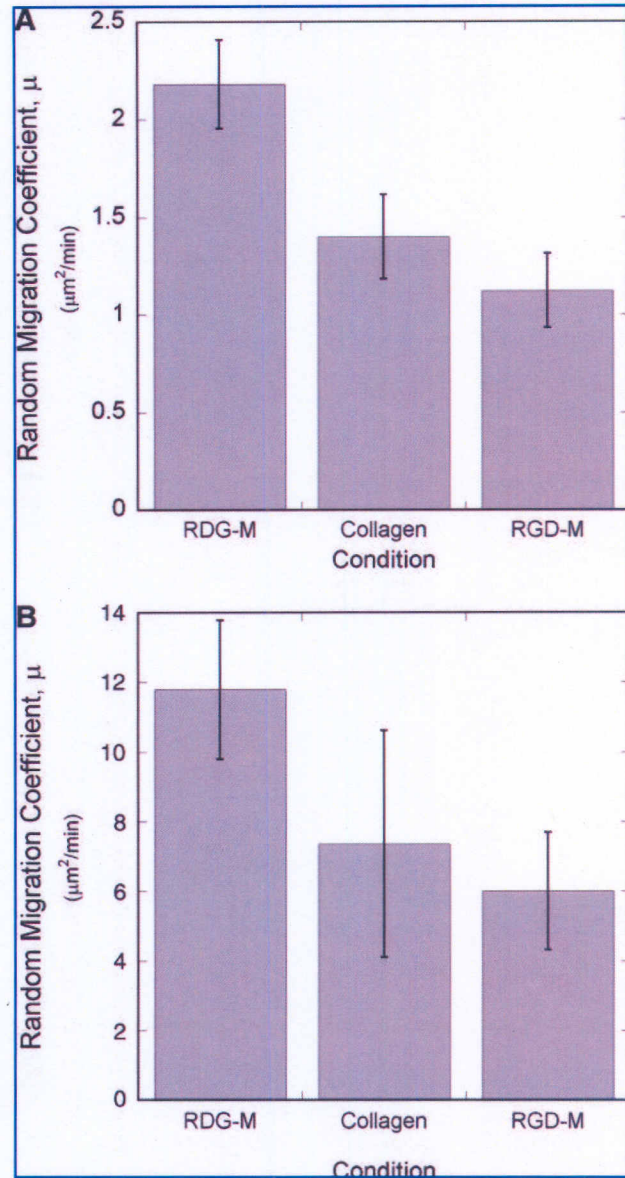


**FIG. 6.** Effects of peptide grafting on (**A**) RDF migration in collagen gels and (**B**) SMC migration on collagen gels. Both cells demonstrated increased migration with RDG-M collagen and decreased migration with RGD-M collagen. The differences were statistically significant (ANOVA, $p = 0.005$) for RDFs migrating in collagen gels, but not significant ($p = 0.157$) for SMCs migrating on collagen gels.

covalent grafting of the bioactive peptides to naturally bioactive biomaterials, including collagen, has been employed to increase the level and specificity of adhesion without significantly altering the physical structure of the assembled network of collagen fibers or the mechanical properties.[27] In general, increasing adhesion will result in increased cell traction,[11,45] which can be inferred from measurements of gel compaction. Our compaction results with RGD-grafted collagen are consistent with this notion. However, rarely are the results of grafting control peptides reported. Notable exceptions are reports from Burgess *et al.*[8] and Myles *et al.*,[27] who
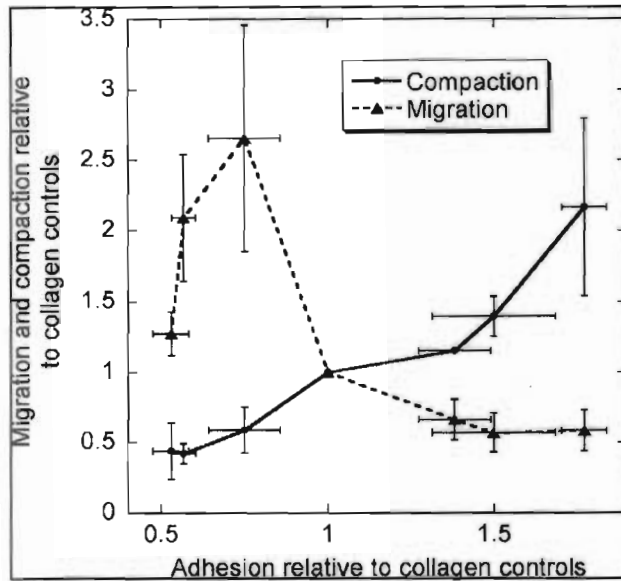
**FIG. 7.** Inferred dependence of cell migration and cell-mediated compaction on the level of cell adhesion. For the dose–response RDF experiments, the average adhesion, migration, and compaction (after 24 h) from each experimental set was normalized by the average response in the control conditions for that set. These normalized values were then averaged across all experiments and plotted as (adhesion, migration) and (adhesion, compaction) X–Y pairs (±standard error). At low levels of adhesion, both cell migration and cell-mediated gel compaction (via cell traction) are inhibited. As adhesion increases, an optimal level is reached for cell migration, after which the elevated adhesive forces limit the ability for cells to detach from the substrate, which is observed as a decrease in migration. Conversely, the elevated adhesive forces increase the ability for the cells to exert traction and compact the fibrillar collagen network.

demonstrated a monotonic increase in adhesion of K1735 melanoma cells to increasing concentrations of GRGDSPC grafted to type I collagen, but a biphasic response in cell migration within a 3D collagen gel. No changes were noted in adhesion or migration with the scrambled peptide, GRGESPC.

The contradictory results from Myles et al. and Burgess et al. compared to those presented herein are likely explained by important differences in the respective cell type, control peptides, and/or coupling agents. Although melanoma cells express $\alpha1\beta1$ and $\alpha2\beta1$ integrins, which have a high affinity for collagen and are generally accepted to recognize the peptide sequence GFOGER,[14] the motility of the highly metastatic K1735 cells is mediated by expression of $\alpha v\beta3$ integrins,[46,47] which primarily recognizes RGD sequences in vitronectin and fibronectin. As such, increasing adhesion via addition of RGD to collagen shifts the position of the cells on the curve toward the right, first toward the peak of the curve and an optimal adhesion level for migration, and then to a level of adhesion too great for effective migration.

Surprisingly, Burgess et al. found that, while the K1735 cells demonstrated a biphasic relationship between cell migration and cell adhesion, the relationship was not due to a similar response in cell speed, but rather a biphasic response in persistence time. Instead, cell speed was found to decrease monotonically with increasing adhesion. This result may be indicative of the colonizing nature of the metastatic K1735 cells. Burgess et al. also note potential distinctions between assaying migration on a 2D surface and within a 3D network. In a 3D system, increased cellular interactions with and reorganization of the matrix are involved, and the adhesivity, stiffness, and porosity can work in concert to affect migration.[45,48] However, we observed an inverse correlation between adhesion and migration for cells both in and on gels.

Interestingly, RGE demonstrates bioactivity in certain cell types, including epidermal cells[49] and, importantly, human[50] and mouse[51] melanoma cell lines. Humphries et al. showed that the soluble peptides GRGDS and GRGES had very similar inhibitory effects on mouse melanoma cell spreading, but only GRGDS similarly inhibited spreading of fibroblastic baby hamster kidney cells.[51] The peptide GRDGS had no effects in either cell type. Smith and Giachelli found that human melanoma cell adhesion to osteopontin was primarily dictated by $\alpha v\beta3$-mediated binding to RGD domains. When the RGD domains were mutated to RGE, adhesion dropped by 50%, but when it was mutated to RAA (or removed completely), adhesion was completely blocked. The residual adhesion offered by RGE was eliminated by antibodies to $\alpha9\beta1$ integrins.[50] These integrins, for which adhesive ligands include osteopontin and tenascin-C,[52,53] are expressed by a variety of epithelial cells as well as the melanoma cells, and interactions of $\alpha9\beta1$ with RGE peptides have been shown to influence migration.[49,50]

In our experiments, we used RDG peptides, which are not, to our knowledge, bioactive, and normal RDFs and SMCs. Both of these cells express $\alpha1\beta1$ and $\alpha2\beta1$ integrins and employ these integrins in regulating the collagen network and cellular and tissue functions.[54–57] Moreover, neither cell is naturally motile unless stimulated by a change in environmental cues, as occurs, for instance during wound healing,[20] and both the RDFs and SMCs would reside naturally on the right end of the biphasic relationship between adhesion and migration. Accordingly, further increasing adhesion via addition of RGD has a negative to minimal effect on migration, and a disruption in adhesion is required to increase migration. A decrease in adhesion would also be reflected in gel compaction, where the ability to exert traction sufficient to compact the collagen gel is diminished.

The other chief difference between the studies was the reagent(s) used to couple the peptides to collagen. In Myles et al. and Burgess et al., sulfosuccinimidyl 6-[30-2-(pyridyldithio)-propionamido] hexanoate (sulfo-LC-SPDP) was used to conjugate RGD peptides to collagen, whereas we used EDC.[8,27] Neither technique appears to overly influence the mechanical or structural properties of the fibrillar gels, and treatment of collagen with EDC without including peptides did not alter the adhesivity of the collagen. We note that the confocal technique, with a resolution limit of ~200 nm, offers a less precise estimate of structure than the scanning electron micrography used by Myles, but when combined with the rheological data and dose–response adhesion data, provides reasonable assurance that the observed differential response of the cells to the gels was due to adhesive binding (or lack thereof).

The sulfo-LC-SPDP reacts with the short collagen oligomers to form an amine bond, and then the 2-pyridyl disulfide

group is reacted with the sulfhydryl group of the cysteine residue on GRGDS or GRGES to form a disulfide bond. As a result, a 15Å spacer is introduced between the conjugated peptides and the collagen,[58] and the nonadhesive, scrambled peptide may not interfere with binding to nearby ligands or with integrin clustering. The spacer may also influence the traction exerted on the collagen fibers via the grafted RGD by providing a potentially compliant structure between the peptide and fiber. Conversely, covalent coupling via EDC leaves no spacer,[59] and the grafted scrambled peptide may mask natural adhesion sites more efficiently, and allow more direct transfer of traction for RGD-grafted collagen. Hern and Hubbell have reported that fibroblast spreading on RGD-modified poly(ethylene glycol) diacrylate networks, which were naturally nonadhesive, was significantly affected by including a PEG spacer between the network and grafted peptide,[36] though that spacer was ~10× the molecular weight of the sulfo-LC-SPDP spacer. Similar results were demonstrated by Park *et al.* with a nonadhesive, thermoreversible N-isopropylacrylamide hydrogel.[60]

EDC covalently binds the peptides to collagen via nucleophilic attack of primary amines on the highly reactive lysine residues on collagen. Though the lysine residues are generally not included in the ligands for integrin receptors found on fibroblasts or SMCs, their cationic charge is particularly conducive for promoting nonspecific cell adhesion, as is exploited, for example, by coating surfaces with poly-L-lysine. Decreasing the cationic charge by adding anions to collagen via hydrolysis of carboxyamide side chains of asparagine and glutamine has been shown to decrease adhesion of a human erythroleukemia cell line,[61] but no differences were observed in similar experiments with mouse fibroblast and endothelial cell lines, where the net negative charge of collagen was increased via acetylation, succinylation, or treatment with glutaraldehyde.[62] The observed decrease in adhesion following grafting of RDG peptides to lysine may be due to the replacement of the charged amine on lysine with RDG. Conversely, grafting of RGD peptides to lysine may be increasing adhesion by replacing the charge on lysine with a stronger, ligand-specific mediator of adhesion.

To a lesser extent, grafting may occur on the amine on arginine residues, though it has significantly lower reactivity than lysine. Because many of the arginine residues are involved in integrin binding, primarily through GER sequences, grafting to these residues may replace an existing bioactive site with another ligand (RGD) that binds to a distinct integrin receptor, or with an inactive sequence (RDG) that effectively removes the existing adhesion site. For RGD, the switch from one integrin ($\alpha 2\beta 1$ or $\alpha 1\beta 1$) to another ($\alpha v\beta 3$ or $\alpha 5\beta 1$, for instance) may differentially modulate the level of adhesion (and, in this case, likely increase adhesion). Moreover, adding RGD sequences without significant removal or masking of GER sequences may enhance overall adhesion and integrin-mediated signaling. Reyes *et al.* showed that an HT1080 human fibrosarcoma cell line demonstrated enhanced adhesion and activation of focal adhesion kinase on substrates with mixed presentation of GFOGER and a recombinant fragment of fibronectin that spans the seventh to tenth type III repeats and contains adhesion motifs including RGD versus either ligand alone.[63]

Type I collagen has been extensively researched as a biomaterial for a variety of tissue-engineered regenerative therapies, including skin, blood vessels, ligaments and tendons, and peripheral nerves and spinal cords. The ability to manipulate the intrinsic adhesivity of collagen, and thereby modulate both cell migration and cell traction, can provide valuable flexibility to these approaches, particularly if spatial control of differentially grafted collagen can be achieved to provide distinct regions that promote adhesion or promote contraction. For example, engineered, bioartificial skin grafts can be designed to enhance the repopulation of the graft by host fibroblasts, or adhesion can be sufficiently masked to limit attachment and infiltration of any type of cell. It should be recognized that the cells in these different applications may require different degrees of peptide grafting (scrambled and/or bioactive) to achieve the desired properties. Additionally, the relationships among migration, adhesion, and traction become more complex when considering cells within a 3D system capable of cell-mediated reorganization, such as a collagen or fibrin gel, where the stiffness of the gel and the resultant change in porosity and cell density can also contribute to the observed behavior.[11] Our technique allows the adhesivity of these systems to be altered without affecting significantly the initial structure or stiffness, and may therefore provide a valuable platform to further elucidate these complex relationships.

## Disclosure Statement

No competing financial interests exist.

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