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Adhesive Peptide Sequences Regulate Valve Interstitial Cell Adhesion, Phenotype and Extracellular Matrix Deposition

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Abstract

Knowledge of how extracellular matrix (ECM) binding impacts valve interstitial cells (VICs) is critical not only to better understanding the etiology of valvular diseases but also to constructing living valve substitutes that can grow and remodel. Use of ECM-mimicking adhesive peptides with specific affinity to different receptors provides insights into adhesion-mediated cell signaling and downstream outcomes. Expression of adhesion receptors by VICs was assessed by flow cytometry and used to guide the choice of peptides studied. The peptide RGDS with affinity to multiple integrin receptors, and specific receptor-targeting peptides DGEA (integrin $\alpha_2\beta_1$), YIGSR (67kDa laminin/elastic receptor; 67LR), and VAPG (67LR) were incorporated into hydrogels to investigate their effects on VICs. DGEA, YIGSR, and VAPG alone were insufficient to induce stable VIC adhesion. As a result, these peptides were studied in combination with 1 mM RGDS. For VICs cultured on two-dimensional hydrogel surfaces, YIGSR and VAPG down-regulated the expression of smooth muscle α -actin (myofibroblast activation marker); DGEA promoted VIC adhesion and VIC-mediated ECM deposition and inhibited the activity of alkaline phosphatase (osteogenic differentiation marker). Further, YIGSR and DGEA in combination promoted ECM deposition while inhibiting both myofibroblastic and osteogenic differentiation. However, VICs behaved differently to adhesive ligands when cultured within three-dimensional hydrogels, with most VICs assuming a healthy, quiescent phenotype under all peptide conditions tested. DGEA promoted ECM deposition by VICs within hydrogels. Overall, we demonstrate that the presentation of defined peptides targeting specific adhesion receptors can be used to regulate VIC adhesion, phenotype and ECM synthesis.

Keywords

VICs; ECM remodeling; tissue engineering; scaffolds; PEG hydrogels

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CONFLICTS OF INTEREST

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1 Introduction

Aortic valve disease causes approximately 60,000 hospitalizations every year in the United States⁴⁷. However, to date there are no medical agents that are FDA-approved to prevent valve disease progression, and current options for aortic valve replacements are non-viable and thus lack the ability to grow and remodel. Developing new treatments (medications or new living devices) for valve disease will require greater understanding of the biology and pathology of valvular cells. Valvular interstitial cells (VICs), the predominant cell population in valve leaflets, are responsible for active extracellular matrix (ECM) synthesis in the valve tissue⁴⁹. Complicating this, VICs are highly heterogeneous and dynamic in phenotype, comprising of progenitor endothelial/mesenchymal cells, quiescent fibroblasts, activated myofibroblasts, and osteoblast-like cells³⁴. In healthy adult valves, more than 95% of VICs are quiescent fibroblastic cells, characterized by the expression of mesenchymal marker vimentin but not smooth muscle α -actin (α SMA)^{6,44}. During valve injury or disease conditions, accompanying valve stiffening, VICs can undergo myofibroblast activation or osteogenic differentiation^{6,38,45}. Myofibroblast activation is characterized by increased contractility due to the expression of α SMA and increased secretory properties (ECM and matrix metalloproteinases (MMPs)), whose persistence may cause valve fibrosis⁶. Osteogenic differentiation is associated with alkaline phosphatase (ALP) upregulation followed by tissue calcification, which is the leading cause of valve diseases⁴⁶.

As an ECM regulator in the valve tissue, VIC phenotype and function are also influenced by ECM motifs, largely via adhesion-mediated cell signaling^{6,15}. For example, functional coupling of VICs to collagen via integrin $\alpha_2\beta_1$ was shown to be necessary for force generation in mitral leaflets⁵¹. VICs cultured on fibrin had greater α SMA expression and calcification than those cultured on collagen and fibronectin⁴⁵, involving mitogen-activated protein kinase signaling⁶. However, the usage of natural ECM to study the complex cell-matrix interplay within engineered tissue is limited by its inherent drawbacks, such as presence of multiple types of ligands, susceptibility to loss of bioactivity, batch-to-batch variation, and risk of inducing immune rejection. ECM-mimicking adhesive peptides (small, synthetic) serve as a better substitute to overcome these drawbacks and control cell-matrix interactions when conjugated with bioinert, synthetic polymers, such as poly(ethylene glycol)²⁵. Several adhesive peptides have been developed to control integrin and non-integrin mediated cell adhesion, such as RGDS^{2,12,17,18,23}, YIGSR^{12,19,59}, DGEA^{2,22,23} and VAPG^{16–18}. RGDS is the most often employed peptide sequence to promote cell attachment in tissue engineering based upon its widespread distribution and capability of binding to versatile adhesion receptors²⁶. However, since RGDS has affinity for approximately half of the 24 known integrins²⁶, it is impossible to specifically regulate adhesion-mediated cell signaling. Thus, adhesion ligands with specific affinity for each receptor are preferable to better control cell-matrix interactions. For example, previous research showed that VICs cultured on polystyrene plates coated with YIGSR and DGEA better resisted calcification than those on the standard RGDS-coated surface²². Additionally, blocking integrin 67 kDa receptor for laminin and elastin (67LR) precipitated the formation of calcified nodules²², suggesting the anti-calcification effect of 67LR binding.

Therefore, in the present study, the peptide RGDS and adhesive peptides with affinity to specific receptors of potential beneficial effects (DGEA, YIGSR and VAPG) (Table 1) were covalently bound to poly(ethylene glycol) diacrylate (PEGDA) hydrogels to investigate their effects on VICs. We demonstrated how the presence of these peptides alone or in combination influenced VIC adhesion, phenotype, and ECM deposition on two-dimensional (2D) hydrogel surfaces or within three-dimensional (3D) environments. Regulation of VIC adhesion, phenotype and ECM remodeling via adhesion ligands is crucial not only to understanding the etiology of valvular diseases but also to constructing living valve substitutes.

2 Materials and Methods

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise indicated.

2.1 Cell isolation and culture

As previously described⁵⁰, primary VICs were harvested from aortic valve leaflets dissected out of fresh porcine hearts from a commercial abattoir (Fisher Ham and Meats, Spring, TX, USA). Briefly, the leaflets were incubated with collagenase type II (550 U/ml, 30 min; Worthington Biochemical Corp, Lakewood, NJ) followed by manually scraping off endothelial cells and mincing the leaflets, which were then digested with collagenase type III (260 U/ml, 4 h; Worthington) in an incubated shaker. Isolations from six different donors were pooled together to compensate for biological variability. Cells were cultured on tissue culture polystyrene flasks in VIC growth media [1:1 DMEM (Corning, Tewksbury, MA, USA):F12 (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA) with 10% bovine growth serum (BGS; Hyclone), 1.6% 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Thermo Fisher Scientific), and 1% antibiotic-antimycotic solution (Lonza, Walkerville, MD)] before being seeded. VICs between passage 2 and 4 were used for study.

2.2 Flow cytometry

VICs (2×10^5) were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 10 min and permeabilized in 0.25% triton X-100 in phosphate buffered saline (PBS, Corning, pH=7.4) for 10 min. 1% bovine serum albumin (BSA, Fisher Bioreagents, Loughborough, UK) in PBS was used to block unspecific binding before 20 min incubation with 100 μ L primary antibody (anti-integrin α_v , abcam, 3 μ g/mL; anti-67LR, abcam, 3 μ g/mL; anti-integrin $\alpha_2\beta_1$, abcam, 6 μ g/mL). Samples were washed with 1% BSA/PBS to rinse off unbound primary antibodies followed by 100 μ L secondary antibody incubation (Alexa Fluor 488, 1:500, Life Technologies, Grand Island, NY, USA) for 20 min. Cells were then washed with 1% BSA/PBS to rinse off unbound secondary antibodies, and resuspended in PBS for flow cytometry test (BD FACSCanto II, BD Biosciences, San Jose, CA, USA). Samples with secondary antibody incubation but without primary antibody incubation were used as the negative controls. A minimum of 1×10^4 events (single cells) were counted for each sample and three parallel samples were used for each receptor testing.

2.3 Synthesis and purification of PEGDA, PEG-YIGSR, PEG-DGEA, PEG-VAPG, PEG-RGDS, and degradable PEG-PQ-PEG

PEGDA was synthesized and purified as previously described¹⁰. Briefly, PEG (MW 6kDa) was reacted with acryloyl chloride at a 1:4 molar ratio in anhydrous dichloromethane (DCM) with triethyl amine (TEA, PEG/TEA at a 1:2 molar ratio) overnight under argon at room temperature. PEGDA was purified via phase separation using 2 M K₂CO₃. The PEGDA-containing organic phase was dried using anhydrous MgSO₄ and filtered. PEGDA was precipitated with cold diethyl ether, filtered, and dried under vacuum. The resultant PEGDA was then characterized by ¹NMR (Avance 400 MHz; Bruker, Billerica, MA, USA) to verify acrylation and stored at -20°C until use.

PEG-peptide and PEG-peptide-PEG were synthesized and purified as previously described³⁹. Briefly, the adhesive peptides GGGYIGSRG, GGGDGEAGG, GGGVAPGGG and MMP-2- and -9-sensitive peptide GGGPQGIWGQK (PQ)^{39,57} were synthesized with a solid phase peptide synthesizer (Aapptec, Louisville, KY, USA) via standard Fmoc chemistry, and characterized with Maldi-MS (Applied Biosystems, Grand Island, NY, USA). The above three adhesive peptides and RGDS (American Peptide, Sunnyvale, CA, USA) were reacted with heterobifunctional acrylate-PEG-succinimidyl valerate (PEG-SVA; Laysan Bio, Arab, AL, USA) at a 1.2:1 molar ratio in HEPBS buffer (pH 8.3), yielding PEG-X monoacrylate (X=YIGSR, DGEA, VAPG or RGDS). The resultant PEG-X was then dialyzed, lyophilized and stored at -20°C until use. PEG-PQ-PEG was synthesized with a similar process by reacting PQ with PEG-SVA at a 1:2 molar ratio, adding a PEG-monoacrylate chain to the N-terminus and to the ε-amine group on the lysine at the C-terminus of the peptide. Conjugation of peptides onto PEG chains was confirmed by gel permeation chromatography equipped with UV and evaporative light-scattering detectors (Varian, Palo Alto, CA, USA).

2.4 PEGDA hydrogel polymerization and cell seeding

10 wt% PEGDA with specific concentrations of PEG-X was dissolved in 10 mM HEPES buffered saline (HBS, pH 8.3) with 1.5% v/v triethanolamine (TEOA). 10 μM eosin Y and 3.5 μL/mL N-vinylpyrrolidone (NVP) were added to the solution to form hydrogel precursor solution. A 18 μL droplet of the precursor solution was sandwiched between a Sigmacote-treated glass slide (Sigma, treated according to the supplier's instructions) and a methacrylate-modified glass cover slip (described below), and photocrosslinked by white light exposure for 35 sec (Fiber-Lite Series 180, 150 W halogen, Dolan Jenner, Dayton, Ohio, USA), forming 380 μm-thick, peptide-functionalized PEGDA hydrogel disks. VICs were seeded on hydrogel surfaces at 1×10⁴ cells/cm² (for cell adhesion assay, evaluated at day 2), or 4×10³ cells/cm² (for immunostaining, evaluated at day 5).

Methacrylate-modified glass cover slips were prepared by cleaning cover slips with 30% (v/v) hydrogen peroxide and 70% sulfuric acid, and exposing to 2% 3-(trimethoxysilyl) propyl methacrylate in 95% ethanol overnight. Modified cover slips were rinsed with 95% ethanol and baked at 90°C for 30 min before use.

2.5 Cell encapsulation in PEG-PQ-PEG hydrogels

VICs were encapsulated within MMP-sensitive, cell-adhesive PEG hydrogels at a seeding density of 1.5×10^7 cells/ml. Briefly, hydrogel precursor solution was prepared with 4 wt% PEG-PQ-PEG, PEG-RGDS (1 mM or 4 mM), PEG-DGEA (0 mM, 3 mM or 5 mM), 10 μ M eosin Y, 1.5% v/v TEOA, and 3.5 μ L/mL NVP in 10 mM HBS. VICs were mixed homogeneously with the precursor solution and a 10 μ L droplet was photocrosslinked in a similar manner as PEGDA gel preparation with an exposure time of 35 sec, forming 380 μ m-thick “PEG-PQ” hydrogels. Cell-laden hydrogels were cultured in VIC growth media and incubated at 37°C and 5% CO₂ for 28 days, with media change every three days, before being evaluated for phenotype marker expression and ECM production.

2.6 Cell adhesion and spreading quantification

To assess the percentage of cell adhesion, VICs adhered to hydrogel surfaces were rinsed with PBS and harvested using 0.25% trypsin-EDTA solution at day 2. The number of VICs in suspension was assessed via Quant-iT PicoGreen dsDNA Kit (Life Technologies) per manufacturer's protocol using a conversion factor of 5.6 pg DNA/cell^{20,40,58}. The percentage of cell adhesion was calculated as the measured cell density divided by the seeding density.

To assess cell spreading, bright field images of VICs on peptide-functionalized hydrogels were taken with a Zeiss Axiovert 135 inverted microscope at day 2. Cell spread areas and aspect ratios were analyzed from the manually traced cell outlines using Image J. Aspect ratio is defined as the ratio between the two principal (long/short) axes. A minimum of 140 cells were analyzed for each group.

2.7 Immunohistochemistry

VIC-seeded PEGDA hydrogels were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100, and blocked with 3.5% BSA in PBS. Samples were then incubated with primary antibodies against ki67 (1:100, abcam), vimentin (1:200, abcam), smooth muscle α -actin (α SMA, 1:200, abcam), and alkaline phosphatase (ALP, 1:200, abcam). After rinsing with 0.01% Tween in PBS, samples were incubated with secondary antibodies: anti-rabbit and anti-mouse antibodies conjugated with either Alexa Fluor 555 or Alexa Fluor 647 (Life Technologies). Lastly, to aid morphological analysis, actin and nuclei were counter stained with 1:150 dilution of rhodamine phalloidin (Life Technologies) in 2 μ M DAPI. For ECM staining, the same procedure was followed with the exception of the permeabilization step, which was eliminated to reduce intra-cellular staining. The primary antibodies used were anti-fibronectin (Fn, 1:400, abcam), anti-collagen I (Col I, 1:400, abcam) and anti-collagen III (Col III, 1:400, abcam). Cell-laden PEG-PQ hydrogels were processed in a similar manner except that the primary and secondary antibody concentrations were doubled. Fluorescent z-stacks were obtained on a confocal microscope (Zeiss LSM 510, Jena, Germany). Z-projections covering a depth of 6 μ m (2D) or 80 μ m (3D) were obtained via Image J for visualization and quantitative analysis. Relative fluorescence was calculated by thresholding all channels uniformly and normalizing the mean fluorescent intensities of a specific channel to the corresponding DAPI channel using Image J. Proliferation ratios were calculated by dividing proliferating cell numbers (ki67 positive cells) to the total cell

numbers (DAPI) using Image J “Adjust Threshold” and “Analyze Particles” function. Eighteen z-stacks from 6 hydrogels (2D) or twelve z-stacks from 4 hydrogels (3D) were taken for quantification.

2.8 Statistical analysis

Statistical analysis was performed using JMP 11 (SAS Institute Inc., Cary, NC, USA). The statistical significance of differences was determined by one- or two-way ANOVA followed by *post-hoc* Tukey HSD. P-values less than 0.05 were considered statistically significant. Labels that do not share a common letter are statistically different. Results are reported as the mean $\pm$ standard deviation. For fluorescence quantification, sample numbers were expressed as the numbers of hydrogels (n=6 for 2D study and n=4 for 3D study) whereas images taken from the same hydrogels were considered as replicates of one sample.

3 Results and discussion

3.1 Expression of target adhesion receptors by VICs

RGDS has affinity for approximately half of the 24 known integrins, including the integrin α_v family ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$)³⁵. Hence, the expression of integrin α_v on VICs was analyzed via flow cytometry. Results showed that $98.4 \pm 0.2\%$ of VICs expressed integrin α_v (Figure 1A), supporting the use of RGDS to induce VIC attachment on otherwise cell non-adherent PEG^{5,9}. Next, the expression of 67LR, which has affinity to YIGSR and VAPG, was evaluated, and $99.7 \pm 0.0\%$ of VICs expressed 67LR (Figure 1B). To our knowledge, this was the first time that the presence of 67LR on VICs was characterized and confirmed. Lastly, integrin $\alpha_2\beta_1$, the target receptor of DGEA, was characterized, and $75.0 \pm 1.2\%$ of VICs expressed integrin $\alpha_2\beta_1$ (Figure 1C). The expression of target adhesion receptors by VICs supports the study of these peptides.

3.2 VIC adhesion and proliferation on hydrogels via RGDS, or 67LR- and integrin $\alpha_2\beta_1$ -specific peptides (2D)

PEG hydrogels were used as scaffold materials to study VIC adhesion, proliferation, phenotype, and ECM deposition both on 2D and within 3D. The mechanical properties of PEG hydrogels are highly tunable, and in this work were designed for physiological relevance to valve tissue. The tensile (Young's) moduli of the valve tissue are on the order of MPa, i.e., ~ 1.6 -7.5 MPa in the radial direction, and ~ 5.9 -14.6 MPa in the circumferential direction from different investigators³⁶. To our best knowledge, the compressive moduli of valve leaflets have not yet been reported largely due to their thin and region-dependent thickness, typically with a thickness < 0.4 mm at the cusp coaptation region, and ~ 0.8 mm in cusp basal region^{37,53,56}. The compressive moduli of valve sinus, which is thicker, with a thickness of ~ 1.3 -1.9 mm, were reported to be ~ 17 kPa²⁹. While these values are good indicators of the mechanical properties of the valve tissue materials, they may not represent the stiffness that cells actually sense. VICs have been shown to activate from a quiescent fibroblast phenotype to a myofibroblast phenotype on substrates above a threshold modulus in the range of 4-10 kPa^{11,30,43,55}, much lower than any of the values reported for the valve tissue. In this work, PEG-PQ hydrogels for 3D cultures were designed with a compressive modulus below the threshold (~ 2.1 -4.7 kPa, Supplementary Fig. S1B) in order to preserve

the quiescent, *in vivo*-like phenotype of VICs. Stiffer PEGDA hydrogels (~77-109 kPa, Supplementary Fig. S1A) were used as the cell culture substrates on 2D because they share similar chemical properties (except for the lack of proteolytically degradable sites) with PEG-PQ hydrogels but are easier to handle due to enhanced compliance.

Cell adhesion was quantified by determining the number of adherent cells on the hydrogel surfaces using a DNA assay and comparing this to the total number of cells seeded on the surface. Results showed that RGDS induced high percentages of VIC adhesion (2 mM, $66\pm 27\%$; 5 mM, $94\pm 26\%$) whereas DGEA led to moderate adhesion (5 mM, $39\pm 10\%$), and YIGSR and VAPG induced low adhesion ($< 20\%$) (Figure 2A). While VICs that adhered to RGDS proliferated rapidly and became confluent within a few days, those that adhered to the other three peptides alone generally detached after a few days. Previous work by the Anseth group used a basal level of 0.8 mM RGDS in conjunction with other small peptides to promote VIC adhesion¹⁸, but cell adhesion on these peptides alone without RGDS supplementation was not reported. Their inability to support stable cell adhesion might have had been the reason. Similar phenomena were also reported for human mesenchymal stem cells (hMSCs)¹². hMSCs attachment and viability upon binding to peptides RRETAWA, IKVAV or YIGSR could only be supported for less than 48h, so a minimum level of RGD had to be added together with these peptides¹². Hence, supplementation of RGDS seems necessary to maintain cell adhesion for long-term culture of these cell types. VIC adhesion on various concentrations of RGDS showed that 1 mM RGDS was able to induce $70\pm 13\%$ cell adhesion and increasing RGDS concentration led to enhanced adhesion to different extents (Figure 2B). As a result, 1 mM RGDS was supplemented with the other three peptides for the remaining studies to induce basal adhesion and to evaluate the effects of adding the other three peptides on VIC adhesion, phenotype, and ECM deposition. Although the addition of the other three peptides slightly reduced the compressive moduli of hydrogels compared to RGDS only group, the compressive moduli of hydrogels with any of these three peptides were not significantly different from each other (Supplementary Fig. S1). Therefore, the stiffness should not be the factor influencing VIC behaviors discussed below.

When 1 mM RGDS was supplemented, the addition of 5 mM DGEA enhanced VIC adhesion by 33% whereas YIGSR and VAPG did not significantly increase adhesion (Figure 2C). VICs in all groups showed mixed morphologies of well-spread cells and more round cells at day 2 (Figure 2D). We measured the cell spread areas and aspect ratios of over 140 cells/group using Image J. Compared to the RGDS only group, the addition of 5 mM DGEA increased the average cell spread area by 25% (Figure 2E). DGEA group also had higher aspect ratios (long/short axis) than YIGSR and VAPG group although statistical significance was not achieved comparing to the RGDS only group (Figure 2E). Hence, VICs tended to spread over a larger area and toward a more elongated morphology via DGEA binding, suggesting a strengthened adhesion considering the positive correlation between adhesion strength and spreading⁷. On the contrary, VAPG seemed to decrease the cell spread area, which was not statistically significant at day 2 but became visually more obvious at day 5 (Figure 3 and Figure 4, not quantified due to intensive cell overlap at day 5). This may correlate with its effects on interrupting actin stress fiber distribution (Figure 4) and cell proliferation. The addition of VAPG significantly reduced VIC proliferation ($\sim 25\%$ of cells ki67+, $p<0.05$) whereas YIGSR and DGEA did not significantly influence VIC proliferation

ratio (~40% of cells ki67+) as shown by ki67 staining compared to DAPI (Supplementary Fig. S2). Previous work from Anseth group showed that the addition of peptides VGVAPG and P15 significantly reduced the elongation factor, metabolic activity, and MMP activity of VICs¹⁷, which may indicate negative effects of VAPG on VIC spreading and growth. However, there were no significant differences in the proliferation of hMSCs that adhered to RGDS or DGEA². The cell densities from different peptide groups were again quantified at day 5. Indicative of active cell proliferation, there was a 2- to 11-fold increase in cell number (relative to the seeding density) during the five days of VIC culture on different peptide-functionalized hydrogels, with the highest to lowest rank as DGEA+ (11.1 fold) > RGDS only (7.7 fold) $\approx$ YIGSR+ (7.1 fold) > VAPG+ (2.6 fold) (Figure 2F). Together with the results from VIC adhesion and proliferation study, the finding of the highest cell density from DGEA+ group is likely due to the enhanced initial cell adhesion by DGEA; the finding of the lowest cell density from VAPG+ is likely due to the inhibitory effect of VAPG on cell proliferation; and YIGSR did not significantly influence either cell adhesion or proliferation. Therefore, VIC number (impacted by both adhesion and proliferation) can be regulated via the use of adhesive peptides. The use of specific targeting adhesive peptides also allows the investigation and regulation of VIC phenotype and functions, which is limited when using RGDS alone.

3.3 Adhesive peptides regulate the expression of phenotype markers of VICs cultured on hydrogels (2D)

The expression of VIC phenotype markers including vimentin (mesenchymal), α SMA (myofibroblast) and ALP (osteogenic) were characterized after VICs were cultured on the surfaces of non-degradable PEGDA hydrogels for 5 days.

Vimentin, an intermediate filament expressed by mesenchymal cells, is commonly employed as a marker for fibroblasts, but not expressed exclusively by fibroblasts. VICs on hydrogel surfaces stained positively for vimentin and the expression level of vimentin was not significantly different among groups at day 5 (Figure 3A, D). Consistent with our findings here, previous studies have shown that almost all VICs stained positively for vimentin both in native valves and during culture^{31,42,48}, and vimentin expression was maintained at a similar level for VICs of different phenotypes (fibroblasts versus myofibroblasts)⁵⁵.

VIC differentiation into pathological myofibroblasts or osteoblast-like cells plays crucial roles in valve disease progression. As a result, inhibiting myofibroblast activation and osteogenic differentiation is critical for the success of developing new treatments for valve diseases. On surfaces of PEGDA hydrogels covalently modified with different peptides, VICs stained positively for α SMA at day 5, indicating an activated myofibroblastic phenotype (Figure 3B). Compared to RGDS only, the addition of YIGSR and VAPG reduced α SMA expression by 22% and 33% respectively whereas DGEA showed no significant effect (Figure 3E). Interestingly, the aforementioned work from Anseth group showed different effects of a peptide similar to VAPG on myofibroblast activation: the addition of 1.2 mM VGVAPG increased myofibroblast activation^{17,18}. This is probably due to the lack of cell spreading and growth in that work (with low concentrations of adhesion peptides, low serum (1%) medium) which are associated with pathological cell growth and

differentiation^{1,11}. Overall, our results suggest that 67LR binding reduces myofibroblast activation since both YIGSR and VAPG target 67LR.

ALP upregulation is an important osteogenic differentiation marker and precedes the onset of calcification^{33,45}, which is the leading cause of aortic valve dysfunction⁴⁶. As a result, ALP expression was analyzed next. Fluorescence quantification of ALP staining of VICs on hydrogel surfaces at 5 days showed that DGEA down-regulated ALP expression level by 23% whereas VAPG and YIGSR showed moderate or no effect (Figure 3C, F). Consistent with this work, previous literature investigating the effects of RGDS and DGEA on osteogenic differentiation of hMSCs showed that DGEA induced a lower level of ALP gene expression³ and ALP activity² than RGDS, indicating the inhibitory effect of DGEA on osteogenic differentiation compared to RGDS. Thus, DGEA is potentially beneficial to inhibit pathological osteogenic differentiation which may lead to valve calcification. Moreover, the inhibition of myofibroblast activation or osteogenic differentiation based only on the presented ECM-mimicking ligands holds immense potential for *in vivo* application by incorporating peptides into the valve replacements.

3.4 Adhesive peptides regulate ECM deposition by VICs cultured on hydrogels (2D)

A significant challenge of heart valve tissue engineering is to recapitulate the ECM components of the native valve tissue. VICs are largely responsible for the ECM synthesis and maintenance in the valve tissue⁵². Hence, strategies to promote VIC-mediated ECM remodeling hold great promise for future living valve replacement therapies. The ECM components of native valves consist of collagens, elastin, glycoproteins and glycosaminoglycans (GAGs). Bundles of collagen fibers (mainly collagen I and III) associate closely with VIC filopodia⁴. These collagen fibers are circumferentially aligned to provide tensile strength to withstand high-pressure loads⁵⁴. Radially aligned elastin fibers provide flexibility that allows stretch and recoil during valve opening and closure⁵⁴. Elastin was not evaluated in this work because appreciable elastin deposition by cultured VICs was not observed in our previous work or in most published literature^{13,58}. The glycoprotein fibronectin (Fn) serves as an intermediate protein, linking cells to other extracellular components, such as collagen and GAGs³². Hence, deposition of collagen (Col) I, Col III, and Fn were investigated to evaluate VIC-mediated ECM remodeling in this work.

VICs cultured on PEGDA hydrogel surfaces for 5 days deposited Fn, Col I and Col III, which were mainly pericellular (Figure 4A-C and Supplementary Fig. S3A-B). Compared to the RGDS only group, the addition of DGEA enhanced VIC-mediated Fn, Col I and Col III deposition by 48%, 20% and 19% respectively whereas YIGSR and VAPG had no significant effect (Figure 4D-E and Supplementary Fig. S3C). These results suggest that integrin $\alpha_2\beta_1$ binding via DGEA promotes VIC-mediated ECM remodeling. The up-regulation of collagen synthesis and MMP secretion via integrin $\alpha_2\beta_1$ binding has also been reported for human osteosarcoma cells, acting through the activation of p38 pathway^{24,28}.

3.5 DGEA promotes ECM deposition and preserves fibroblastic phenotype of VICs within 3D biomimetic hydrogels (3D)

3D culture guides cells to produce *in vitro* responses that are more physiologically relevant than does planar 2D culture via affecting integrin ligation, cell contraction and associated intracellular signaling²¹. Therefore, the effects of DGEA on VIC phenotype and ECM production were further characterized in 3D, proteolytically degradable PEG-PQ hydrogels. In contrast to the 2D study, most VICs lost their α SMA expression upon encapsulation in 3D (Figure 5A), as reported in our previous work⁵⁸. The addition of DGEA did not significantly influence the expression of phenotype markers α SMA or ALP for VICs in 3D hydrogels at 28 days (Figure 5A-D), presumably because expression of the myofibroblast and osteogenic markers were essentially nil in 3D cultures. This may result from several factors. 3D matrix adhesions may differ in composition, localization and function from 2D focal adhesions⁸. Relative to 2D focal adhesions, 3D matrix interactions have been shown to display narrowed integrin usage and enhanced cell biological activities, such as enhanced cell attachment and accelerated migration rates⁸. In addition, the PEG-PQ hydrogels (~2.1-4.7 kPa) were ~30 times softer than PEGDA hydrogels (~77-109 kPa), leading to altered mechanotransduction. VICs cultured on the stiffer 2D hydrogels assumed activated myofibroblast phenotype, resembling that in the diseased condition, whereas those that cultured within the softer 3D hydrogels assumed quiescent fibroblast phenotype, resembling that in the healthy condition. Previous work investigating the effects of substrate stiffness on hMSCs showed that ALP activity of hMSCs on stiff substrates (>22 kPa) increased with time during 15 days of culture whereas activity on soft substrates (~3 kPa) was maintained at a baseline level¹⁴, indicating that the upregulation of ALP activity is supported by substrate stiffness. The different responses of VICs within 3D versus on 2D hydrogels in this work may reflect their changes in baseline phenotype.

However, similar to the effects of DGEA on ECM deposition in the 2D study, the addition of 3 mM DGEA significantly enhanced the deposition of Fn, Col I and Col III within hydrogels by 119%, 49% and 47% respectively at 28 days when compared to the 1 mM RGDS only group (Figure 6 and Supplementary Fig. S4). On the contrary, the addition of 3 mM RGDS (for a total of 4 mM RGDS, immobilized on hydrogel surfaces in the same manner as 3 mM DGEA) did not significantly influence the deposition of these ECM proteins (Figure 6 and Supplementary Fig. S4), indicating that it was not the availability of extra binding sites but rather the targeted binding to integrin $\alpha_2\beta_1$ via DGEA that promoted the VIC-mediated ECM deposition. This is the first time reporting enhanced ECM deposition by VICs via targeted binding of adhesive receptors, not only on 2D but also within more physiologically relevant 3D environments. This finding holds great promise to apply specific targeting cues in the design of valve scaffolds and in the treatment of valve disease.

Furthermore, two dosages of DGEA were tested in this study, i.e., 3 mM and 5 mM. These dosages were chosen based on the results of 2D cultures that 5 mM DGEA significantly increased cell adhesion whereas 2 mM was not enough (Figure 2A). This choice was also based on the previous publication that a concentration of 2.8-5 mM VAPG induced full spreading of ~50-90% of smooth muscle cells whereas a lower concentration dramatically reduced cell adhesion and spreading¹⁶. However, our results showed that these two dosages

were not significantly different from each other in terms of influencing ECM deposition and phenotype marker expression of VICs (Figure 5, Figure 6 and Supplementary Fig. S4).

3.6 Combination of YIGSR and DGEA inhibits the expression of pathological differentiation markers of VICs while enhancing their ECM deposition on hydrogels (2D)

As discussed in sections 3.2 through 3.4, YIGSR and VAPG inhibited VIC myofibroblast activation whereas DGEA inhibited VIC osteogenic differentiation and promoted VIC-mediated ECM deposition on PEGDA hydrogels. As an effort to inhibit both myofibroblast activation and osteogenic differentiation at the same time and to promote ECM deposition, the combination of YIGSR and DGEA (together with 1 mM RGDS) was evaluated. VAPG was not chosen because it severely compromised cell proliferation whereas sufficient cell proliferation is generally desired in tissue engineering. As shown in Figure 7, the combination of 3 mM YIGSR and 3 mM DGEA [3 mM (Y+D)+] significantly reduced the expression of phenotype markers α SMA and ALP by 46% and 24% respectively on PEGDA hydrogel surfaces at 5 days. The combination also significantly enhanced the deposition of Fn, Col I and Col III by 38%, 25% and 37% respectively on hydrogel surfaces (Figure 8 and Supplementary Fig. S5). The higher dosage of 5 mM of each peptide was not significantly different from the 3 mM combination for each of the ECM protein tested (Figure 7, Figure 8 and Supplementary Fig. S5).

3.7 Combination of YIGSR and DGEA [(Y+D)+] promotes ECM deposition and preserves fibroblastic phenotype of VICs within 3D biomimetic hydrogels (3D)

The combined effects of YIGSR and DGEA [(Y+D)+] were also tested within 3D PEG-PQ hydrogels. The combination of YIGSR and DGEA significantly enhanced the deposition of Fn, Col I and Col III by 21%, 29% and 40% respectively within hydrogels at 28 days (Figure 9 and Supplementary Fig. S6). Similar to the effects of DGEA in 3D hydrogels, the combination of YIGSR and DGEA did not significantly influence the expression of phenotype markers α SMA and ALP, with the majority of VICs negative for α SMA (Supplementary Fig. S7), indicating a quiescent, fibroblastic phenotype.

4 Conclusions

The ECM-mimicking, adhesive peptides circumvent limitations associated with animal-derived, full-length ECM proteins when considering materials for tissue culture substrates and scaffolds. This work aimed to regulate VIC adhesion, phenotypes and ECM synthesis via adhesive peptides with affinity to specific adhesion receptors. We demonstrated that in combination with a basal level of RGDS, adhesive peptides YIGSR and VAPG (with affinity to 67LR) down-regulated myofibroblastic differentiation whereas DGEA (with affinity to integrin $\alpha_2\beta_1$) promoted VIC adhesion and VIC-mediated ECM deposition and reduced osteogenic differentiation on 2D PEGDA hydrogel surfaces. The combination of YIGSR and DGEA [(Y+D)+] promoted ECM deposition and reduced both myofibroblastic and osteogenic differentiation on PEGDA hydrogels. However, within 3D PEG-PQ hydrogels, DGEA alone or the combination of YIGSR and DGEA promoted ECM deposition without significantly influencing VIC phenotype. This work not only shows potential in regulating VIC behavior via adhesion-mediated cell signaling, but also demonstrates different VIC

responses to adhesion regulation on 2D surfaces and within 3D scaffolds, giving insights into VIC regulation in heart valve tissue engineering. Moreover, the knowledge of cell-matrix interactions via adhesion provides vital information for the future development of specific targeting drug carriers for valve disease therapy. Yet, the use of these peptides with specific affinity is limited by the fact that they alone do not support stable cell adhesion that is critical for VIC survival and function, requiring supplementation of a basal level of RGDS with affinity for multiple receptors, which renders the complete control over VIC adhesion impossible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

VICs	valve interstitial cells
ECM	extracellular matrix
67LR	67kDa laminin/elastic receptor
αSMA	smooth muscle α -actin
ALP	alkaline phosphatase
2D	two-dimensional
3D	three-dimensional
PEG	poly(ethylene glycol)
PEGDA	poly(ethylene glycol) diacrylate
MMP	matrix metalloproteinase
hMSCs	human mesenchymal stem cells
PQ	GGGPQGIWGQGK

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Figure 1.

Representative profiles of adhesion receptor expression of VICs tested via flow cytometry.

(A) Integrin α_v , with affinity to RGDS; (B) 67LR, with affinity to YIGSR and VAPG; (C)

Integrin $\alpha_2\beta_1$, with affinity to DGEA. n=3 runs with each run $> 1 \times 10^4$ events (single cells).

Figure 2.

VIC adhesion and proliferation on the surfaces of peptide-functionalized hydrogels. (A) VIC adhesion on hydrogels covalently modified with 2 mM or 5 mM PEG-X (X= YIGSR, DGEA, VAPG or RGDS) at day 2. Two-way ANOVA identified both “peptides” and “concentrations” as significant factors affecting VIC adhesion, $p < 0.05$. Then, each pair of group was compared using *post-hoc* Tukey HSD. (B) VIC adhesion on hydrogels covalently modified with various concentrations of RGDS at day 2. (C-E) VIC adhesion and spreading on hydrogels covalently modified with 1 mM PEG-RGDS and 5 mM PEG-X’ (X’=YIGSR, DGEA, VAPG or nothing) at day 2. Average cell spread areas and aspect ratios (E) of >140 cells of each group. Scale bar=100 μm. (F) Significantly increased VIC cell density on hydrogels covalently modified with 1 mM PEG-RGDS and 5 mM PEG-X’ at day 5 compared to the seeding density (dashed line). Cell adhesion percent was calculated as the cell number quantified by PicoGreen dsDNA assay at day 2 divided by the seeding density. $p < 0.05$ for comparisons with different letters, $n > 5$ hydrogels per group.

Figure 3.

Phenotype marker expression of VICs cultured on the surfaces of peptide-functionalized hydrogels at day 5. Representative images and fluorescence intensity quantification of vimentin (A, D), α SMA (B, E) and ALP (C, F) of VICs on hydrogels covalently modified with 1 mM PEG-RGDS and 5 mM PEG-X'. $p < 0.05$ for comparisons with different letters, $n = 6$ hydrogels per group. No statistical significance was achieved for vimentin expression (D). Scale bar = 50 μ m.

Figure 4.

Extracellular matrix deposition by VICs cultured on the surfaces of peptide-functionalized hydrogels at day 5. Representative images and fluorescence intensity quantification of Fn (B, D) and Col I (C, E) by VICs on hydrogels covalently modified with 1 mM PEG-RGDS and 5 mM PEG-X'. $p < 0.05$ for comparisons with different letters, $n = 6$ hydrogels per group. Scale bar = 50 μm .

Figure 5.

DGEA did not significantly change the expression of phenotype markers αSMA and ALP of VICs within 3D PEG-PQ hydrogels at day 28. Representative images and fluorescence intensity quantification of αSMA (A, C) and ALP (B, D) of VICs within PEG-PQ hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM DGEA, or total 4 mM RGDS. No groups achieved statistical significance, n=4 hydrogels per group. Scale bar=50 μm.

Figure 6.

DGEA promoted ECM deposition by VICs within 3D PEG-PQ hydrogels at day 28. Representative images and fluorescence intensity quantification of Fn (B, D) and Col I (C, E) by VICs within PEG-PQ hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM DGEA, or total 4 mM RGDS. $p < 0.05$ for comparisons with different letters, $n = 4$ hydrogels per group. Scale bar = 50 μm .

Figure 7.

Combination of YIGSR and DGEA significantly reduced the expression of phenotype markers α SMA and ALP of VICs cultured on the surfaces of hydrogels at day 5. Representative images and fluorescence intensity quantification of α SMA (A, C) and ALP (B, D) of VICs on the surfaces of hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM YIGSR and DGEA. $p < 0.05$ for comparisons with different letters, $n = 6$ hydrogels per group. Scale bar = 50 μ m.

Figure 8.

Combination of YIGSR and DGEA significantly enhanced ECM deposition by VICs cultured on hydrogel surfaces at day 5. Representative images and fluorescence intensity quantification of F α (B, D) and Col I (C, E) by VICs on the surfaces of hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM YIGSR and DGEA. $p < 0.05$ for comparisons with different letters, $n = 6$ hydrogels per group. Scale bar = 50 μ m.

Figure 9.

Combination of YIGSR and DGEA significantly enhanced ECM deposition by VICs within 3D PEG-PQ hydrogels at day 28. Representative images and fluorescence intensity quantification of Fn (B, D) and Col I (C, E) by VICs within PEG-PQ hydrogels covalently modified with 1 mM RGDS only, or 1 mM RGDS supplemented with 3 mM or 5 mM YIGSR and DGEA. $p < 0.05$ for comparisons with different letters, $n = 4$ hydrogels per group. Scale bar = 50 μm .

Table 1Source and affinity of adhesion ligands^{26,27,41}.

Ligand	Source	Affinity
RGDS	Fibronectin	$\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_8\beta_1$, $\alpha_{11b}\beta_3$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, $\alpha_v\beta_8$, and to some extent $\alpha_2\beta_1$, $\alpha_4\beta_1$
DGEA	Collagen I	Integrin $\alpha_2\beta_1$
YIGSR	Laminin	67LR (via residues 205-229)
VAPG	Elastin	67LR (via three separate sites)