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Influence of Discrete and Continuous Culture Conditions on Human Mesenchymal Stem Cell Lineage Choice in RGD Concentration Gradient Hydrogels

Laura A. Smith Callahan^{†,§}, Gina M. Policastro[†], Sharon L. Bernard[†], Erin P. Childers[†], Ronna Boettcher[†], and Matthew L. Becker^{*,†,‡}

[†]Department of Polymer Science, The University of Akron, Akron, Ohio, United States

[‡]Center for Biomaterials in Medicine, Austen Bioinnovation Institute in Akron, Akron, Ohio, United States

Abstract

Stem cells have shown lineage-specific differentiation when cultured on substrates possessing signaling groups derived from the native tissue. A distinct determinant in this process is the concentration of the signaling motif. While several groups have been working actively to determine the specific factors, concentrations, and mechanisms governing the differentiation process, many have been turning to combinatorial and gradient approaches in attempts to optimize the multiple chemical and physical parameters needed for the next advance. However, there has not been a direct comparison between the cellular behavior and differentiation of human mesenchymal stem cells cultured in gradient and discrete substrates, which quantitates the effect of differences caused by cell-produced, soluble factors due to design differences between the culture systems. In this study, the differentiation of human mesenchymal stem cells in continuous and discrete polyethylene glycol dimethacrylate (PEGDM) hydrogels containing an RGD concentration gradient from 0 to 14 mM were examined to study the effects of the different culture conditions on stem-cell behavior. Culture condition was found to affect every osteogenic (alkaline phosphatase, Runx 2, type 1 collagen, bone sialoprotein, and calcium content) and adipogenic marker (oil red and peroxisome proliferator-activated receptor gamma) examined regardless of RGD concentration. Only in the continuous gradient culture did RGD concentration affect human mesenchymal stem-cell lineage commitment with low RGD concentrations expressing higher osteogenic differentiation than high RGD concentrations. Conversely, high RGD concentrations expressed higher adipogenic differentiation than low RGD concentrations. Cytoskeletal actin organization was only affected by culture condition at low RGD concentrations, indicating that it played a limited role in the differences in lineage commitment observed. Therefore, the role of discrete versus gradient strategies in high-throughput experimentation needs to be considered

*Corresponding Author: Phone: 330-972-2834 Fax: 330-972-5290. becker@uakron.edu.

§Present Address: Laura A. Smith Callahan: Department of Neurosurgery & Center for Stem Cell and Regenerative Medicine, University of Texas Health Science Center at Houston, 1825 Pressler Street, Suite 630F, Houston, Texas 77030, United States. Laura.A.SmithCallahan@uth.tmc.edu. Tel: (713) 500-3431.

Notes

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when designing experiments as we show that the respective strategies alter cellular outcomes even though base scaffolds have similar material and chemical properties.

Graphical abstract

INTRODUCTION

Regenerative medicine promises to provide solutions for many unmet or undeserved clinical needs.¹ However, each clinical need requires a carefully tailored material to support the cellular component. Complicating the multidimensional engineering problem is the fact that no single material or strategy will serve every need. However, an aversion to risk on the part of researchers and a lack of diversity in commercially available degradable polymers is the limiting development of novel tailored materials. Another significant limitation on regenerative medicine is sourcing the cellular component.² Human mesenchymal stem cells (hMSCs) are one of the promising cell sources for clinical regenerative medicine due to their multilineage potential and ability to modulate immune response.^{3–6} However, few hMSCs are recovered during each isolation, making expansion necessary before use in regenerative medicine applications.⁷ Extrinsic factors significantly affect hMSC behavior,^{8–10} and crosstalk between different cell types or exposure to different extracellular matrix (ECM) conditions have been shown to alter hMSC differentiation and proliferation.^{11–14} For instance, increased adipogenesis leads to decreased osteogenesis in the bone marrow,^{15,16} demonstrating the significant effect small environmental changes can have on hMSC behavior and differentiation.

Systematic approaches are necessary to identify and optimize the chemical and mechanical properties of scaffolds for ex vivo expansion and directed differentiation of stem cells. Many have turned to combinatorial methods for solutions to the multicomponent optimization challenges, and examples using continuous gradient technologies^{17–19} and array formats^{20–22} have been used. Continuous gradients combine two or more components in continuously changing ratios to produce a sample containing virtually every possible combination of the components, while array formats present a limited number of discrete defined combinations of the components. Design differences between these two systems can have a significant effect on the cellular response to the material, even though substrates with identical mechanical, chemical, and bioactive properties can be generated in both continuous gradient and discrete array formats, as demonstrated by a recent study of cellular proliferation, which found decreased mouse preosteoblast proliferation on continuous samples compared with discrete gradient samples.²³ However, studies examining differentiation or utilizing less committed stem cells could not be found in the literature.

Polyethylene glycol dimethacrylate (PEGDM) hydrogels containing an RGD concentration profile were either fabricated and cultured as a continuous gradient or cut into discrete samples to form an array and isolated for the duration of culture to examine the effect of continuous gradient and discrete array culture on hMSC differentiation. The concentration of RGD, a common, integrin-binding adhesion sequence in a number of proteins, is known to affect the behavior of numerous cell types.^{24–27} In 3D culture, RGD has been shown to improve survival of hMSC;^{28,29} however, more advanced studies examining lineage commitment in 3D could not be found in the literature. In 2D culture, RGD has been shown to alter osteogenic and adipogenic differentiation of hMSC.^{26,27,30} In this report, the effects of the continuous and discrete culture conditions will be examined on hMSC differentiation toward the osteogenic and adipogenic lineages through analysis of ECM expression and nuclear markers in low cell density culture.

EXPERIMENTAL METHODS

Hydrogel Fabrication

PEGDM 11.5% (~8000 g/mol) (Monomer-Polymer & Dajac Laboratories, Trevose, PA) solutions in alpha minimal essential medium (α -MEM) with or without 15 mM RGD-derivatized acrylate (American Peptide, Sunnyvale, CA) were prepared containing 0.1% Irgacure 2959 (Ciba Specialty Chemicals, Basel, Switzerland). Solutions were loaded into 1 mL syringes and placed in a computer driven syringe pump system to create gradient hydrogels, as previously described.¹⁹ Computer-controlled syringe pumps were used to dispense 11.5% PEGDM solutions with and without 15 mM RGD monomer in inverse ramping profiles ranging from 53 to 0 mL/h over 90 s into a custom mold while 11.5% PEGDM solution without RGD was dispensed at a constant rate of 10 mL/h (Figure 1D). The mold possessed a depth profile (1 mm) to minimize diffusional mixing during gradient formation. Unless otherwise noted, all samples for analysis were 5 mm $\times$ 10 mm $\times$ 1 mm. Hydrogels were photopolymerized using ~2.3 mJ/cm² UVA light for 5 min and then placed in α -MEM media for storage. For cellular experiments, 11.5% PEGDM solution without RGD was dispensed at a constant rate contained 3.85×10^6 cells/mL, leading to a final cell content of ~7800 cells per analysis sample for both discrete or continuous culture conditions.

Hydrogel Characterization

RGD peptide concentration in the gradient hydrogels was measured using a Lowry assay.³¹ In brief, gradient substrates were placed in ultrapure water for 3 days on an orbital shaker at 50 rpm. The water was changed daily. The gradient substrates were then placed in Reagent B (Dc Protein assay, Biorad, Hercules, CA) on the orbital shaker. After 30 min, the substrates were placed in Reagent A for 30 min. The gradient substrates were washed in ultrapure water, and a circular sample was taken every 10 mm down the length of the gradient. The absorbance at 750 nm was read from each gradient sample and compared with the absorbance of hydrogel samples of known RGD content for normalization.

The bioavailability of RGD was determined in a manner similar to one previously described using a peptide designed to mimic the natural integrin receptor (CWDDGWLC-biotin)

(American Peptide, Sunnyvale, CA) and Alexaflour 488 streptavidin colloidal gold (Invitrogen).^{19,32} In brief, test samples were blocked for 1 h with bovine serum albumin in RGD blocking buffer, washed for 5 min in RGD wash buffer five times, and incubated overnight at ambient temperature on an orbital shaker at ~75 rpm in 0.1 mg/mL integrin mimicking peptide in RGD wash buffer. Samples were washed five more times for 5 min each in RGD wash buffer to remove unbound peptide and incubated in 3 ng/mL Alexaflour 488 streptavidin colloidal gold overnight at ambient temperature on an orbital shaker at ~75 rpm. Samples were washed five times for 5 min each in RGD wash buffer to remove unbound Alexaflour 488 streptavidin colloidal gold and then viewed on a IX81 microscope (Olympus, Center Valley, PA). Gradient samples were placed in polypropylene culture test tubes (Fisher Brand) filled with 0.5 mL of PBS to keep the samples moist and loaded in a Skyscan 1172 Micro CT (100 keV, Micro Photonics) to detect the colloidal gold RGD in the RGD gradient hydrogels and hydrogels of known RGD concentration (0, 1, 5, 10, 15 mmol). Each sample was scanned at 6.7 μ m pixels, medium camera size, eight average frames, 88% partial width, 0.42° rotation step, a voltage of 70 kV, and a current of 114 μ A. The hydrogel 2D scans were reconstructed in NRecon and analyzed in CTan. The average grayscale unit value was reported for 10 random locations within each standard sample to generate an average RGD concentration (mmol) to construct the standard curve. At each 10 mm position in the full length gels, the average of 12 grayscale unit values was reported. Grayscale values at each gradient position were converted to concentration of RGD using the formulated standard curve.

Cross-link density was calculated using the rubber elasticity theory phantom network model using the following equation.^{33,34}

$$G = (1 - 2/\Phi) V_e RT V_2^{2/3}$$

where G , the storage modulus, was measured using 8 mm diameter samples punched from the gradients every 10 mm with a gasket punch on a ARES-G2 rheometer (TA Instruments, Newcastle, DE) with 8 mm serrated parallel plates using a strain amplitude of 1% and 30N constant normal force to prevent slippage over an angular frequency sweep from 100 to 1 rad/s with 10 points per decade. Modulus data are reported at an angular frequency of 1 rad/s because gels did not show to be frequency-dependent. Φ , the functionality of cross-link junctions, is assumed to be 4. R is the gas constant and T is the absolute temperature. V_2 , the volume fraction of cross-linked polymer, was determined by the following equation.³⁵

$$V_2 = (1 + (q_f - 1)\rho/d)^{-1}$$

where q_f is the mass of the swollen hydrogel divided by the mass of the freeze-dried hydrogel. The density of the solvent (alpha minimum essential media), d , is assumed to be 0.997 g/mL. The density of the polymer, ρ , is 1.113 g/mL.

Human Mesenchymal Stem Cell Culture

hMSCs (Tulane Center for Gene Therapy, New Orleans, LA or Lonza, Walkersville, MD) were expanded according to the manufacturer's protocol. For differentiation experiments, hMSCs (passage 5) were encapsulated in RGD gradient hydrogels and cultured in α -MEM (Invitrogen, Grand Island, NY) supplemented with 16.5 vol % fetal bovine serum, 100 U/mL penicillin–streptomycin, 10^{-7} M dexamethasone $10 \mu\text{g/mL}$ insulin and 0.5 mM 3-isobutyl-1-methylxanthine, and 200 μM indomethacin, 50 $\mu\text{g/mL}$ ascorbic acid, and 10 mM β -glycerol phosphate in a 37 °C incubators with 5% CO₂ for up to 3 weeks. Discrete samples were cut into 5 mm sections and cultured in individual wells of a 24-well plate, while continuous samples were left whole and cultured in a 100 mm dish. Media was changed every other day for the duration of culture.

Histological Staining and Immunohistochemistry

All samples were fixed overnight in 4% paraformaldehyde (Sigma). Whole mount histological staining samples were transferred to PBS and stained with alkaline phosphatase (AP, Sigma) for 3 h and oil red (OR) for 30 min at room temperature. Samples were then washed with PBS and water and imaged on a CKX41 microscope (Olympus). Image J (downloaded from the National Institute of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij/>) was used to determine the fraction of cells expressing AP and OR from at least 50 cells in four separate gradients at each position. Whole mount cytoskeletal staining samples were transferred to 0.5% Triton X-100 in cytoskeleton stabilization (CS) buffer (0.1 M PIPES, 1 mM EGTA, and 4% (w/v) 8000 MW polyethylene glycol) at 37 °C for 10 min, rinsed thrice for 5 min each in CS buffer, and incubated in 0.05% sodium borohydride in PBS at ambient temperature for 10 min. Whole mount samples were then blocked in 5% donkey serum for 20 min at 37 °C and incubated overnight at 4 °C with vinculin antibody (V4505, Sigma, 1:100) and rhodamine phalloidin (1:200). Samples were then washed thrice with 1% donkey serum for 5 min, followed by appropriate secondary antibodies conjugated to FITC. DAPI was used to stain the cell nuclei. Image J was used to determine cellular area and number of focal adhesions from at least 20 cells from three separate gradients per position. Samples for histological sectioning were transferred to 70% ETOH for at least 1 h, 80% ETOH for 1.5 h, 95% ETOH for 12 h, ETOH for 1.5 h twice, and xylene for 1 h. Samples were then placed in a 60 °C paraffin bath for 12 h and embedded in a paraffin block for sectioning.³⁶ Blocks were removed from a –20 °C freezer and cut into 7 μm sections. After 2 days of drying in a 37 °C oven, xylene was used to remove the paraffin and the samples were rehydrated through an ethanol gradient. Samples were incubated in 0.5% pepsin for 10 min at 37 °C for antigen retrieval. Nonspecific antibody binding was blocked by incubating in 10% goat serum; then, samples were exposed to collagen type 1 (1:100) and bone sialoprotein (BSP) (1:200), Runx 2 (1:100) and peroxisome proliferator-activated receptor gamma (PPAR- γ) (1:100), or collagen type IV (col IV) (1:200) and osteocalcin (OC) (1:100) antibodies, followed by appropriate secondary antibodies conjugated to Alexafluor 488 (BSP, PPAR- γ , Col IV) or Alexafluor 546 (col 1, Runx 2. os). DAPI was used to stain the cell nuclei. The BSP antibody (WVID1(9C5)) by Michael Solursh and Ahnders Franzen was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Col 1A (sc-25974), runx 2 (sc-10758), osteocalcin (sc-18322), and PPAR- γ (sc-1984)

antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), Col IV (ab6586) was obtained from Abcam; and DAPI was obtained from Sigma. Image J was used to determine the mean grayscale intensity from 20 cells in three separate gradients in each position for each antibody.

Biochemistry

Collagen content was quantified using dimethylaminobenzaldehyde (DAB) to observe chloramines T-oxidized hydroxyproline, as previously described.^{19,37,38} In brief, samples were homogenized with a Tissue-Tearor (BioSpec Products, Bartlesville, Oklahoma) and then digested with proteinase K overnight at 60 °C. Samples for hydroxyproline detection were dehydrated, autoclaved at 120 °C with 2N NaOH for 20 min, oxidized with chloramines T solution for 25 min at room temperature on an orbital shaker at 100 rpm, and then incubated with DAB for 20 min at 65 °C. The absorbance was then read at 550 nm. Calcium samples were processed according to the manufacturer's instructions (Calcium LiquiColor, Stanbio Laboratory, Boerne, Texas). For normalization, total protein was measured in samples with a Dc Protein assay (Biorad, Hercules, CA) according to manufacture protocol. Quantification of DNA was determined with a fluorescence DNA assay kit (Sigma, St. Louis, MO) according to the manufacturer's protocol. OR content was quantified as previously described.³⁹ In brief, OR was extracted from stained whole mount samples with isopropyl alcohol for 5 min on an orbital shaker at 75 rpm. Solution containing dye was centrifuged for 1 min at 4000 rpm; then, the absorbance of the supernatant was read at 540 nm.

Statistics

All experiments were conducted at least three times ($n = 3$). All quantitative data are presented as the average $\pm$ standard deviation. One-way analysis of variance (ANOVA) with Tukey post hoc analysis was performed where applicable. Significance was set at a p -value of <0.05 .

RESULTS AND DISCUSSION

Combinatorial methods are being utilized to accelerate the optimization of scaffolds for regenerative medicine.⁴⁰ However, differences in cellular environment created by continuous and discrete gradient culture have been shown to affect cellular behavior, illustrating the importance of gradient construction in substrate optimization strategies.²³ For instance, discrete arrays that do not isolate the soluble factor crosstalk from other sample positions^{41,42} provide a cellular environment similar to a continuous gradient instead of the discrete sample strategy they emulate. To examine the effects of culture condition on hMSC differentiation, we fabricated PEGDM hydrogels containing a RGD concentration gradient. The RGD concentration gradient was characterized for both total peptide content using a modified Lowry Assay (Figure 1A) and bioavailability using a peptide designed to mimic the natural integrin receptor conjugated to colloidal gold (Figure 1B), demonstrating that an RGD gradient from approximately 0 to 14 mM was formed. The controlled variation of RGD concentration did not affect the mechanical properties of the hydrogel, which maintained a cross-link density of $25.5 \pm 1.6 \text{ mol/m}^3$ throughout the gradient.

hMSCs were encapsulated at low density (at least 1/3 less than previously reported cellular densities^{28,29}) to reduce the effects of crosstalk between culture conditions and then cultured up to 3 weeks. Markers of osteogenic (AP, Runx2, Col 1, BSP, and Ca content; Figures 2, 4, and 5) and adipogenic (OR, PPAR- γ and Col IV; Figures 2–4) differentiation were examined and quantified. After 2 weeks of culture, a significantly higher fraction of hMSCs encapsulated in discrete samples with low RGD concentrations (2.7 and 4 mmol RGD gradient positions) expressed alkaline phosphatase, an early osteogenic differentiation marker, compared with hMSC encapsulated in the continuous samples at the same concentration, whereas, after 3 weeks of culture, a significant difference in AP expression occurred between culture conditions in hMSC encapsulated in samples with higher RGD concentration (9.2, 10.4, and 11.8 mmol RGD, respectively, Figure 2A), indicating that culture conditions affect osteogenic differentiation. Significant differences in AP expression were observed in hMSC encapsulated at the 2.7 mmol concentration position in both the discrete and continuous samples after 2 weeks of culture relative to higher RGD concentrations regions of the same gradient (Figure 2A), indicating that RGD concentration also has an effect on osteogenic differentiation.

As AP expression increased with higher RGD concentrations in hMSCs encapsulated in continuous samples after 2 weeks of culture, OR expression decreased in hMSC encapsulated within continuous samples with higher RGD concentrations (Figure 2B). After 3 weeks of culture, significantly more hMSCs encapsulated in higher RGD concentration regions (9.2, 10.4, and 11.8 mmol RGD concentration positions) of continuous samples exhibited oil red staining than hMSC in discrete samples at the same position.

Runx 2, a key regulator of osteogenic differentiation,⁴³ was expressed strongly in hMSCs encapsulated in continuous samples with low RGD concentration, and the level of expression decreased as the concentration of RGD increased after 3 weeks of culture (Figure 2C) in a time-dependent manner similar to AP expression. hMSCs encapsulated in discrete samples had low Runx 2 expression regardless of the RGD concentration (Figure 2D). A previous 2D study found RGD concentration to have no effect on Runx 2 expression in hMSC,⁴⁴ similar to the discrete sample results. The differences in Runx 2 expression could be due to the modulation of Runx 2 by PPAR- γ , which can inhibit osteogenic differentiation.^{45–47}

PPAR- γ , a key regulator of adipocyte differentiation, is essential for maintenance of adipocyte phenotype and function.⁴⁸ hMSC encapsulated in continuous samples with high RGD concentration was found to have the highest expression of PPAR- γ (Figure 2E). As the RGD concentration decreased, so did the expression of PPAR- γ in hMSC encapsulated in continuous samples, which is the opposite expression pattern observed in the continuous samples for Runx 2. hMSC encapsulated in discrete samples had a similar expression of PPAR- γ regardless of RGD concentration (Figure 2F). However, quantification of OR staining showed increased intensity of staining in discrete samples compared with continuous samples after 2 weeks of culture (Figure 3). Both continuous and discrete samples have similar DNA content over the time course regardless of RGD concentration (data not shown). Therefore, differences in cell number are unlikely to contribute to the difference in OR quantification.

Collagen is an important ECM component in many tissues. After 2 weeks of culture, discrete samples contain significantly more collagen than continuous samples at 2.7 mmol RGD concentration (Figure 4A). After 3 weeks of culture, discrete samples in general have a higher collagen output than the continuous samples. However, hMSC encapsulated in discrete samples is surrounded by lower intensity staining for type I collagen, the predominant collagen during osteogenic differentiation,⁴⁹ expression compared with hMSC encapsulated in the continuous samples (Figure 4B,D,E). Type IV collagen, which is predominant during adipogenic differentiation,²⁶ was found to be expressed at a higher intensity around hMSC encapsulated in discrete samples containing the highest RGD concentration compared with hMSC encapsulated in continuous samples with the same RGD concentration after 3 weeks of culture (Figure 4C,F,G). These variations in type I and type IV collagen around encapsulated hMSC further illustrated variations in hMSC differentiation due to changes in culture condition.

Later ECM markers of osteogenic differentiation, such as bone sialoprotein (BSP) and calcium content, were also examined over 3 weeks (Figure 5). A previous 2D study found that changes in RGD concentration affected BSP gene expression in hMSC over time.⁴⁴ After 3 weeks of culture, hMSC encapsulated in continuous samples with lower RGD concentrations was surrounded by more BSP than hMSC encapsulated in continuous sample with higher RGD concentrations or hMSC encapsulated in discrete samples (Figure 5A–C) similar to the trend observed with other osteogenic differentiation markers. Calcium content also varied relative to culture condition and RGD concentration over the time course (Figure 5D).

hMSC lineage commitment is influenced by cytoskeletal shape.^{50–52} The difference between gradient culture conditions could be modulating cytoskeletal interactions to produce the lineage differences observed. Whereas we are not capturing the genomic signaling in this experiment, after 3 weeks of culture, the cellular area of hMSC encapsulated in the lowest RGD concentration region of the gradient (~ 1.4 to 4 mmol) is significantly larger ($p = 0.03$) for hMSCs encapsulated in discrete samples with an average area of $281 \pm 68 \mu\text{m}^2$ (avg $\pm$ std dev) compared with hMSC encapsulated in continuous samples (Figure 6B), which have an average area of $158 \pm 56 \mu\text{m}^2$ after 3 weeks of culture (Figure 6A). hMSC encapsulated in other gradient positions had a similar cellular area regardless of whether they were encapsulated in discrete or continuous gradients, indicating that in higher RGD concentration regions cytoskeletal shape is not playing a significant role in the observed differences in lineage choice. The number of focal adhesion assemblies on hMSCs was not affected by culture condition or RGD concentration. Although the number of focal adhesions was not different between positions at the observed time points, RGD spacing has been previously shown to affect hMSC adipogenic and osteogenic lineage choice,²⁶ and differences in the spacing of RGD due to concentration change may be contributing to the observed effects on lineage choice.

Overall, the results of this study indicate that the factors governing hMSC lineage choice are complex and can be significantly affected by the construction of the gradient. For combinatorial methods to be effective in accelerating scaffold optimization for clinical tissue engineering applications, further study is needed to elucidate the mechanisms and the factors

affecting cellular environment and determine which construction, continuous or discrete, is a better indicator of successful in vivo tissue formation and integration.

CONCLUSIONS

Differences in cellular environment created by continuous and discrete gradient culture have significant effect on hMSC lineage choice. Whereas an individual cell is unlikely to sense a nano- or picomolar change over the length of the cell, adjacent cells over very few increments could have significant influence, and the diffusional length scales are small enough that the paracrine effects would be sensed by cell populations in close proximity. We are currently pursuing these studies and are looking at additional combinations of factors on the lineage commitment choices. The observed osteogenic (AP, Runx 2, col 1, BSP, and calcium content) and adipogenic (OR, PPAR- γ , and col IV) differentiation markers were all affected by culture condition. RGD concentration played a significant role in the lineage choice of hMSC in continuous gradients but had little effect on the differentiation of hMSC in discrete gradient samples. The literature is awash with multiple reports of researchers using continuous gradient profiles, isolated culture discrete samples, or array technologies, and mountains of results have been reported, often in direct contrast with each other. This report is the first to offer a direct comparison and was executed using conditions used by many others over concentration ranges deemed relevant by the majority of the literature. The primary findings show the length (concentration) and temporal regimes where the significance occurs and call attention to the role of significant paracrine effects on the commitment choices. Further studies including Western blots and spatially defined gene expression analyses must be done to isolate the crosstalk factors contributing to these differences in differentiation to ensure that 3D combinatorial methods are fabricated in ways to provide reliable results for cellular response to truly expedite the optimization of tissue engineering scaffolds.

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Figure 1.

Summary of RGD concentration within the hydrogel gradient by (A) quantification of RGD by (Lowry) and gold nanoparticle-labeled receptor mimic measured via microcomputed tomography (uCT) within gradient that corresponds to RGD bioavailability. (B) Bright-field microscopy images every 10 mm (with the corresponding RGD concentration) of gold-particle-tagged receptor mimic peptide bound to the bioavailable RGD. Scale bar = 50 μ m. (C) Rheological properties and mesh size calculations of the hydrogel properties.

Figure 2.

Effect of gradient culture condition on human mesenchymal stem cell lineage selection. (A) Fraction of cells expressing alkaline phosphatase. (B) Fraction of cells with adipogenic vacuole staining. Images were taken every 10 mm of human mesenchymal stem cells culture in: (C) continuous and (D) discrete for immunofluorescence of Runx2 expression after 3 weeks: (E) continuous and (F) discrete for PPAR- γ expression after 3 weeks. Scale bar = 5 μ m. The corresponding RGD concentrations are shown below the respective images. * indicates p-value <0.05 between discrete and continuous values at gradient position for the time point. # indicates p-value <0.05 between gradient position and 2w C 35 mm (12.2 mM RGD). \$ indicates p-value <0.05 between gradient position and 2w C 5 mm (4.4 mM RGD). ** indicates p-value <0.05 between gradient position and 2w D 5 mm (4.4 mM RGD). ^ indicates p-value <0.05 between gradient position and 2w D 10 mm (5.7 mM RGD). & indicates p-value <0.05 between gradient position and 3w C 40 mm (14 mM RGD).

Figure 3.

Quantification of extracted oil red dye from different culture conditions over 3 weeks of culture. * indicates p -value <0.05 between discrete and continuous value at gradient position for the time point. & indicates p -value <0.05 between gradient position and 2w C 40 mm (14 mM RGD). @ indicates p -value <0.05 between gradient position and 2w C 45 mm (16.1 mM RGD).

Figure 4.

Effect of culture condition on collagen content within the respective samples over 3 weeks of culture. (A) Hydroxyproline quantification, (B) mean intensity of collagen type I expression per cell in immunofluorescence, and (C) mean intensity of collagen type IV expression per cell in immunofluorescence. Images from (D) continuous and (E) discrete samples stained for collagen type I A and from (F) continuous and (G) discrete samples stained for collagen type IV expression every 10 mm. The corresponding RGD concentrations are shown below the respective images. Scale bar = 25 μ m. * indicates p-value <0.05 between discrete and continuous values at gradient position for the time point. # indicates p-value <0.05 between the 2 and 3 week time points for the gradient position and RGD concentration. ^ indicates p-value <0.05 between gradient position and 2w C 20 mm (7.2 mM RGD).

Figure 5.

Markers of mature osteoblast differentiation Images of (A) continuous and (B) discrete samples expressing bone sialoprotein expression after 3 weeks. Images were taken every 10 mm. The corresponding RGD concentrations are shown below the respective images. Scale bar = 5 μ m. (C) Mean intensity of bone sialoprotein expression per cell (D) Calcium quantification. * indicates p-value < 0.05 between discrete and continuous value at gradient position for the time point. # indicates p-value < 0.05 between the 2 and 3 week time points for the gradient position and RGD concentration. ^ indicates p-value < 0.05 between gradient position and 2w C 20 mm (7.2 mM RGD).

Figure 6. Fluorescence microscopy images showing actin (red), vinculin (green), and nuclear (blue) staining after 3 weeks of culture in (A) continuous and (B) discrete gradients. Images were taken every 10 mm down the length of the gradient. The corresponding RGD concentrations are shown below the respective images. Scale bar = 5 μm .