Three-Dimensional Synthetic Niche Components to Control Germ Cell Proliferation

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Spermatogonial stem cells (SSCs) are increasingly studied for potential use in tissue regeneration due to their ability to dedifferentiate into embryonic stem cell-like cells. For their successful therapeutic use, these cells must first be expanded *in vitro* using an appropriate culture system. We hypothesized that a hydrogel with proper biochemical and biomechanical properties may mimic the composition and structure of the native basement membrane onto which SSCs reside, thus allowing us to control SSC proliferation. This hypothesis was examined in two-dimensional (2D) and three-dimensional (3D) cultures using hydrogels formed from calcium cross-linked alginate molecules conjugated with synthetic oligopeptides containing the Arg-Gly-Asp sequence (RGD peptides). The RGD peptide density (N_{RGD}) in gel matrices was controlled by mixing alginate molecules modified with RGD peptides and unmodified alginate molecules at varied ratios. The mechanical stiffness was controlled with the cross-linking density of gel matrices. Interestingly, the RGD peptide density modulated cell proliferation in both 2D and 3D cultures as well as the number and size of SSC colonies formed in 3D cultures. In contrast, cell proliferation was minimally influenced by mechanical stiffness in 2D cultures. Overall, the results of this study elucidate an important factor regulating SSC proliferation and also present a bioactive hydrogel that can be used as a 3D synthetic basement membrane. In addition, the results of this study will be broadly useful in controlling the proliferation of various stem cells.

Introduction

THE USE OF STEM CELLS in efforts to repair tissues and organs has become increasingly prevalent in the field of tissue engineering. Stem cells reside in specialized microenvironments, or niches, which regulate their self-renewal and overall maintenance.^{1,2} In order to best utilize the therapeutic potential of stem cells, it is crucial to understand the interactions between them and their native environment.^{3,4} Significant progress has been made in characterizing the niche microenvironment of a variety of stem cells; more specifically, recent studies have investigated the hematopoietic, epidermal, intestinal, and neural stem cell niches.^{5,6} Within each niche, supporting cells and associated blood vessels produce the necessary growth factors and stimuli that will modulate stem cell behavior. In addition, the extracellular matrix (ECM) is responsible for cell attachment and can also regulate cellular response to surrounding stimuli.^{7,8} Therefore, the composition of the ECM and the interactions between cells and their surrounding matrix are crucial in regulating stem cell fate.

Stem cells belonging to epithelial structures generally reside onto a specialized ECM called the basement membrane. Although composed of a small set of specific proteins, such as collagen IV, laminin, entactin, fibronectin, and perlecan, the basement membrane shows an impressive tissue- and site-specific variability in its composition.⁹ Maintenance of stemness, proliferation, and migration take place in the basal layer of epithelia, in contact with the basement membrane. These biological activities are regulated in part through interactions between individual components of the basement membrane and cell surface receptors called integrins.^{10,11} Integrins recognize and bind various ECM proteins, including those that contain the Arg-Gly-Asp (RGD) peptide sequence.^{12,13}

Biomaterials have been increasingly used to simulate and even modulate cell adhesion to the ECM in order to regulate signaling pathways that control cell function and tissue morphogenesis. For this purpose, biomaterials have been designed to mimic the biochemical and biomechanical properties of the natural ECM.^{14–16} Extensive chemical modifications have been used to incorporate synthetic cell adhesion

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oligopeptides into biomaterials, and the spatial organization of these cell adhesion cues at the nanometer and micrometer scale has been used to modulate the function of progenitor and precursor cells.^{17–20} In addition, the mechanical stiffness of biomaterials to which cells are adhered has been shown to significantly regulate the phenotypes of precursor cells.^{21,22}

Mammalian spermatogenesis is a tightly regulated and continuous process in which spermatogonial stem cells (SSCs) self-renew and differentiate to ultimately form spermatozoa. The SSC niche is found within the seminiferous epithelium where nursing Sertoli cells provide structural support and growth factors to the stem cells. Other stimuli are provided by the vascular network and interstitial cells between the seminiferous tubules.²³ Another important component of the testicular niche is the basement membrane, to which stem cells and Sertoli cells are attached through integrins. In 2004, the group of T. Shinohara demonstrated that mouse SSCs can dedifferentiate *in vitro*, reverting into cells that acquire an embryonic stem cell (ES) cell phenotype.²⁴ These ES-like cells can be differentiated into cells belonging to the three embryonic germ layers. More recently, the same potential for pluripotency has been demonstrated for adult SSCs.²⁵ Therefore, SSCs could potentially be used to regenerate and engineer specific tissues in an autologous manner. While progress has been made recently in expanding SSCs *in vitro* using cocktails of growth factors,^{26,27} growing and maintaining these cells *in vitro* can be further optimized. Therefore, other niche parameters that are important for SSC proliferation *in vitro*, specifically the composition of the ECM, need to be characterized.

In the present study, we hypothesize that a bioactive hydrogel designed to mimic some of the biochemical and biomechanical properties of the basement membrane can be used as part of a synthetic niche; by mimicking the *in vivo* niche, such a synthetic niche can be used to regulate SSC proliferation. This study presents an initial set of parameters that has been known to significantly regulate the phenotypes of precursor cells, namely cell adhesion ligand density and mechanical properties. Calcium cross-linked alginate hydrogels, which do not naturally facilitate protein adsorption and cell adhesion,^{28,29} were chemically coupled with synthetic oligopeptides containing the Arg-Gly-Asp (RGD) sequence (RGD peptides) in order to control the number of cell adhesion sites for an SSC line. As a model for SSCs, the C18-4 spermatogonial cell line was used.³⁰ This cell line exhibits many of the characteristics of normal SSCs, including the expression of the GFR α -1/Ret membrane receptor complex that binds the growth factor glial cell line-derived neurotrophic factor (GDNF). Upon stimulation with GDNF, the cells increase their rate of proliferation, which is mediated by Src- and Ras-dependent signaling pathways.^{31,32} The mechanical stiffness of the hydrogels was varied from 8 to 110 kPa, which is within the stiffness range of the natural basement membrane,^{33,34} to examine the effects of scaffold stiffness on SSC behavior. The hydrogel properties were first optimized through cell culture on 2D hydrogel surfaces; the cell adhesion and proliferation were then subsequently examined in three-dimensional (3D) hydrogel systems.

Materials and Methods

Material chemistry

Sodium alginate (FMC Biopolymer, Philadelphia, PA) was used as high-molecular-weight alginate ($M_w \sim 250,000$ g/mol).

Low-molecular-weight alginate ($M_w \sim 60,000$ g/mol) was obtained by irradiating the sodium alginate with a cobalt-60 source for 4 h at a dose of 5.0 Mrad.³⁵ Alginate molecules were covalently coupled with (Gly)₄-Arg-Gly-Asp-Ala-Ser-Ser-Lys (G₄RGDASSKY) oligopeptides (Peptides International, Louisville, KY) using a carbodiimide chemistry as previously described.²⁹ The mole ratio between RGD peptides and alginate was kept constant at 2:1. All alginate solutions were filtered, lyophilized, and reconstituted with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) to obtain a 2% w/v solution prior to gelation.

Cell line and cell culture

The C18-4 cell line was established by stably transfecting undifferentiated type A spermatogonia with the Large T antigen gene driven by the ecdysone promoter. These cells exhibit many features of SSCs, including the expression of Oct-4, Vasa, and the receptor complex for GDNF, a crucial stimulus for self-renewal.^{36,37} Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 100 units/mL penicillin-streptomycin (PS; Invitrogen) at 37°C in a 5% CO₂ incubator. The cell culture medium was changed every other day.

2D cell culture on hydrogels

High-molecular-weight alginate was used for 2D cell culture studies. Gels with three different densities of cell adhesion peptides were made by mixing unmodified high-molecular-weight alginate with G₄RGDASSKY-conjugated high-molecular-weight alginate in ratios of 9:1, 1:1, and 1:9. Alginate solutions were mixed with a 20% (w/w) calcium sulfate (CaSO₄) slurry at a ratio of 40 μ L CaSO₄ slurry to 1 mL alginate solution and allowed to gel between glass plates with a 1-mm spacer.

For experiments to examine the effects of hydrogel stiffness on cell proliferation, both high-molecular-weight and low-molecular-weight alginates were used. The ratio between CaSO₄ slurry and alginate was varied between 20 and 60 μ L CaSO₄ slurry to 1 mL high-molecular-weight alginate solution and 20–40 μ L CaSO₄ slurry to 1 mL low-molecular-weight alginate. Gel disks were punched using a 5-mm-diameter puncher and incubated in DMEM for 36 h, changing media every 12 h. The elastic modulus of the hydrogel was calculated from the slope of the compressive stress versus strain curve acquired using an MTS Insight Electromechanical Testing System. Cells were seeded on the disks at a density of 640 cells/mm² and incubated in complete growth media. Media were changed after 24 h and every 48 h thereafter.

3D cell culture in hydrogels

Low-molecular-weight alginate was used for 3D cell culture studies. Gels without RGD peptides were made from unmodified low-molecular-weight alginate. Gels with RGD peptides were made by mixing unmodified low-molecular-weight alginate with G₄RGDASSKY-conjugated low-molecular-weight alginate in ratios of 9:1, 1:1, and 1:9. Cells were encapsulated in alginate gels at a density of 6×10^5 cells/mL by mixing equal volumes of the cell suspension and an alginate solution. The cell–alginate mixture was cross-linked with CaSO₄ slurry at a ratio of 40 μ L CaSO₄ slurry to

FIG. 1. The overall density of RGD peptides immobilized on hydrogel surfaces (N_{RGD}) influenced cellular adhesion and colony formation. Intracellular actin stress fiber formation was visibly enhanced as N_{RGD} was increased. (A), (B), and (C) represent C18-4 cells attached to gels presenting N_{RGD} of 6.2, 31, and 56×10^7 RGD/mm², respectively. The number of cells per colony also increased as N_{RGD} was increased from 31×10^7 RGD/mm² (D) to 56×10^7 RGD/mm² (E). Green fluorescence in microphotographs represents

actin fibers stained with Oregon Green 514 phalloidin. Images were captured after 5 days of culture. Color images available online at www.liebertonline.com/ten.

1 mL cell–alginate mixture and allowed to gel between glass plates with a 1-mm spacer. Gel disks were punched using a 10-mm-diameter puncher and incubated in complete growth media. Media were changed after 24 h and every 48 h thereafter.

Analysis of stress fibers for 2D cultures

After culture of cells on the hydrogel surfaces for 5 days, cell–hydrogel constructs were fixed overnight in a solution of 4% formaldehyde, washed with phosphate-buffered saline (PBS), and permeabilized with a solution of 0.5% Triton X-100 and 5% dry milk. Intracellular actin stress fibers were stained using Oregon Green 514 phalloidin (Invitrogen). Oregon Green 514 phalloidin was diluted in PBS to 5 units/mL, and 1 mL was used to stain each gel for 30 min. Gels were rinsed with 0.5% Triton X-100 and 5% dry milk and then washed extensively in DMEM. The stained gels were visualized using a Leica TCS SP2 confocal system.

Cell proliferation assay for 2D cultures

Cell numbers on the two-dimensional (2D) gels were analyzed on days 1, 3, and 5. Four disks for each condition on each day were used for analysis. Four different micrographs were taken from random locations on each gel, and the total number of cells in each set of four micrographs was counted. The cell counts were normalized to those counted on day 1.

Cell proliferation and viability assays for 3D cultures

Cell proliferation in the 3D gels was analyzed with MTS tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (Promega, Madison, WI). Cells were treated with MTS, and viable cells were evaluated by the formation of a soluble formazan product. The amount of formazan produced was quantified with absorbance at 490 nm, and the quantity of formazan measured was directly proportional to the number of viable cells in culture.

To visualize viable cells after 5 days of culture, the 3D cell–gel constructs were treated with tetrazolium MTT [3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] (ATCC, Manassas, VA). Viable cells were evaluated by the formation of an insoluble dark purple formazan resulting from the reduction of the MTT reagent in mitochondria of viable cells. The treated cell–gel constructs were visualized using a Leica DMI 4000B microscope.

Immunohistochemistry analysis for 3D cultures

After 5 days of culture, the 3D cell–hydrogel constructs were fixed overnight in either 4% paraformaldehyde in PBS or 10% formaldehyde in PBS, both at pH 7.4. The hydrogels were then dehydrated in ascending percentages of ethanol, cleared in xylene, and embedded in low-temperature paraffin. Sections were cut at a thickness of 5 μ m and affixed to

pretreated glass slides. Slides were deparaffinized, hydrated, and boiled for 10 min in a microwave for antigen retrieval with 0.01 M citrate buffer, pH 6.0. Sections were incubated in the appropriate serum (10% normal goat or rabbit), followed by incubation with the primary antibody for 1 h at 37°C. Following a rinse with PBS, the sections were incubated with the appropriate biotin-conjugated secondary antibody at room temperature for 1 h, rinsed again, and incubated with ABC solution (Vector Laboratories, Burlingame, CA) for 30 min. Sections were colorized with diaminobenzidine, counterstained with Mayer hematoxylin (Sigma-Aldrich, St. Louis, MO), dehydrated, and coverslipped. Negative control sections were treated in an identical manner but were incubated with PBS instead of the primary antibody. Antibodies used were anti-rabbit DDX4/MVH Primordial Germ Cell Marker (VASA) and anti-rat c-Kit (Caltag Laboratories, Burlingame, CA). Secondary antibodies used were a biotinylated goat anti-rabbit (DAKO USA, Carpinteria, CA) and a biotinylated goat anti-rat (Abcam, Cambridge, MA).

Statistical analysis

Statistical significance between two data populations was evaluated using an unpaired, two-tailed Student's *t*-test in Microsoft Excel. Differences were considered statistically significant for $p < 0.05$. Statistical significance among more than two data populations was evaluated using a one-way analysis of variance (ANOVA) in Minitab. Data populations were considered statistically similar for $p > 0.1$.

Results

Effects of RGD peptide densities and hydrogel stiffness on cell proliferation in 2D cultures

Hydrogels presenting different RGD peptide densities (N_{RGD}) were prepared by altering the ratio between alginate molecules modified with RGD peptides and unmodified alginate molecules from 1:9 to 9:1 to vary N_{RGD} from 6.2 to 56×10^7 RGD/mm². The RGD concentration on the surface of the hydrogel was calculated from the degree of substitution of RGD peptides and the ratio between modified and un-

modified alginates assuming an alginate molecule occupies the same volume as a hard sphere with a radius equal to the radius of gyration of an alginate molecule. Changes in N_{RGD} had minimal effects on the hydrogel stiffness and swelling ratio (results not shown).

The effects of N_{RGD} on the morphology and cytoskeleton of cells adhered to the gel surface were investigated by evaluating the formation of actin stress fibers. Increasing N_{RGD} enhanced the formation of intracellular actin stress fibers, which were remodeled upon cellular contractility (Fig. 1A–C). SSC colony formation was enhanced with increasing N_{RGD} (Supplemental Fig. 1, available online at www.liebertonline.com/ten), while cell spreading was maximized at an intermediate N_{RGD} ; for single cells, the average projected cell area at 6.2 , 31 , and 56×10^7 RGD/mm² was 84 , 1500 , and $510 \mu\text{m}^2$, respectively.

Cell proliferation on 2D hydrogel surfaces was significantly dependent on N_{RGD} . Each SSC seeded formed distinct colonies of daughter cells, and the number of cells in each colony increased with N_{RGD} during 5 days of cell culture, qualitatively indicating an enhancement in cell proliferation with N_{RGD} (Fig. 1D, E). A quantitative analysis of cell number over time also confirmed that increasing cell adhesion sites promoted cell proliferation (Fig. 2A). The lowest N_{RGD} (6.2×10^7 RGD/mm²) showed a minimal increase in cell number, and the difference between normalized cell numbers for the lowest and highest N_{RGD} at day 5 was statistically significant. The cell proliferation rate (k) was further quantified from Eq. (1)

$$\frac{N}{N_0} = 2^{kt} \quad (1)$$

where N represents the number of cells measured at a given time, N_0 represents the initial number of cells measured at day 1, and t represents the cell culture period. The calculated k increased as N_{RGD} increased. The cell doubling time calculated from k decreased significantly as N_{RGD} was increased from 31 to 56×10^8 RGD/mm² (Fig. 2B).

In contrast, the stiffness of hydrogels played a minimal role in regulating cell proliferation. A quantitative analysis of

FIG. 3. The stiffness of hydrogels did not significantly influence C18-4 cell proliferation. Cell proliferation rate (k) on 2D hydrogel surfaces, calculated from the increase in cell number over time, was roughly independent of the elastic modulus of the hydrogel (E). N_{RGD} was kept constant at $6.2 \times 10^8 \text{ RGD/mm}^2$. The calculated k values were statistically similar on gels of different stiffness, as determined with ANOVA. Values represent the mean and standard deviation from four independent measurements.

cell number over time showed that at a constant N_{RGD} of $6.2 \times 10^8 \text{ RGD/mm}^2$, changes in the elastic modulus did not significantly alter the extent of cell spreading, the number of cells per colony, or the overall cell numbers (Supplemental Fig. 2, available online at www.liebertonline.com/ten). The k values quantified using Eq. (1) were statistically similar on hydrogels of different stiffness, indicating that k was roughly independent of the elastic modulus (Fig. 3).

Effects of RGD peptide densities on cell proliferation in 3D cultures

Based on the results from cell proliferation on 2D gel surfaces, SSCs were encapsulated within four hydrogel formulations: unmodified alginate and alginate modified with RGD peptides at an N_{RGD} of 1.5, 7.3, and $13 \times 10^{12} \text{ RGD/mm}^3$ in order to confirm the critical roles of cell adhesion peptides on cell proliferation in 3D cultures. Low-molecular-weight alginate molecules were used for cell encapsulation in order to circumvent a loss of cell viability during the encapsulation process.³⁸ The elastic moduli of the gels were kept constant at 15 kPa since cell proliferation was not dependent on hydrogel stiffness in 2D cell culture.

Cell viability was evaluated by identifying viable cells with an MTT assay. The MTT reagent was reduced to an insoluble dark purple formazan in the mitochondria of living cells, thus allowing viable cells to be identified with a dark stain. Increasing N_{RGD} from 0 to $1.3 \times 10^{13} \text{ RGD/mm}^3$ increased the number of viable colonies (Fig. 4A, B) and the

FIG. 4. The overall density of RGD peptides influenced C18-4 cell proliferation in 3D cultures. Increasing N_{RGD} from 0 (A) to $1.3 \times 10^{13} \text{ RGD/mm}^3$ (B) within a 3D hydrogel matrix roughly doubled the average diameter of cell colonies from 19 to 40 μm . The number of viable cells positively stained with MTT are represented with asterisk (*), while nonviable cells, unstained with MTT, are circled. The average diameter of viable cell colonies increased with increasing N_{RGD} (C). Differences between average cell colony diameters in gels without RGD as compared to gels presenting RGD were statistically significant ($*p < 0.05$). The cell proliferation rate (k) also increased with increasing N_{RGD} (D). Differences between calculated k values were statistically significant on gels with different N_{RGD} , as determined with ANOVA. The elastic modulus of the hydrogel was kept constant at 15 kPa.

FIG. 5. C18-4 cell colonies in 3D hydrogels were examined with immunocytochemistry. Cells cultured in 3D hydrogels expressed Vasa but did not express c-Kit. N_{RGD} was varied from 1.5×10^{12} RGD/mm³ (A–C) to 1.3×10^{13} RGD/mm³ (D–F). Cells expressed Vasa at all N_{RGD} , as indicated with arrows (A, D). Cells did not express the c-Kit protein at any N_{RGD} (B, E). (C) and (F) represent negative controls, colonies stained without primary antibodies.

diameter of viable colonies (Fig. 4C). Further, cells encapsulated within hydrogels modified with RGD were mostly viable after 5 days in culture as compared with cells encapsulated within unmodified hydrogels (Fig. 4A, B).

Cell proliferation was quantified with an MTS-based cell proliferation assay. Formazan produced by viable cells in the reduction of MTS was detected with absorbance at 490 nm. The measured absorbance, which was directly proportional to the number of viable cells, increased significantly with N_{RGD} (Supplemental Fig. 3, available online at www.liebertonline.com/ten). The cell proliferation rate (k) quantified using Eq. (1) also increased with N_{RGD} (Fig. 4D). Gels with no RGD peptides resulted in negative k values, likely due to significant cell death.

The integrity of the SSCs cultured within hydrogels modified with RGD peptides was further examined. The expression of Vasa, a marker for undifferentiated SSCs, and c-kit, a marker for differentiating SSCs, were probed via immunostaining. Cells cultured within hydrogels modified with RGD peptides were positively stained for Vasa (Fig. 5A, D) and negatively stained for c-kit (Fig. 5B, E), indicating that the cells retained their undifferentiated germ cell properties. In the unmodified hydrogels, which did not contain RGD, cells did not proliferate and died after 5 days in culture; therefore, their phenotype could not be analyzed (data not shown). At an N_{RGD} of 1.5 and 7.3×10^{12} RGD/mm³, cells proliferated but the size of the colonies remained small (Fig. 5A–C). In comparison, colony sizes doubled with cells cultured at an N_{RGD} of 1.3×10^{13} RGD/mm³ (Fig. 5D–F). While N_{RGD} influenced the size of cell colonies, it did not affect Vasa or c-kit expression.

Discussion

This study uses an advanced 3D cell culture system, which mimics some of the biochemical and biomechanical components of the basement membrane, to support SSC proliferation. The culture system used in this study consisted of a bioactive hydrogel presenting cell adhesion molecules (RGD peptides) and was tested with varying cell adhesion sites and stiffness. Interestingly, SSC proliferation could be regulated both in 2D and 3D cell cultures solely by altering hydrogel properties, without the addition of GDNF or other exogenous soluble factors.

The proliferation rates of SSCs and cell adhesion to the gel matrix were significantly dependent on the density of RGD peptides. In 2D cultures, the number of RGD peptides contributed to increasing the number of intracellular stress fibers but did not control the extent of SSC spreading. In 3D cultures, the number of RGD peptides contributed to the formation of viable cell colonies. It is likely that an increase in RGD peptide density led to an increase in the number of bonds between cellular integrins and RGD peptides, as previously examined. This increase in bond number promoted focal adhesion formation at the cell adhesion sites and subsequent intracellular stress fiber formation. It has been reported that this intracellular cytoskeletal reorganization influences the signaling pathways for cell division in both 2D and 3D cell culture.³⁹ SSCs prominently express $\alpha_6\beta_1$ integrins, which are known to bind to laminin in the basement membrane, and laminin is known to be important in the maintenance of stem cells in general.⁴⁰ The fact that RGD modulates SSC proliferation *in vitro* implies either that the

RGD on the α chain of laminin is important for SSC biology, or/and that other basement membrane proteins containing RGD play a role in SSC biology.⁴¹

In contrast, SSC proliferation was not influenced by mechanical stiffness of the hydrogels. Previous studies have demonstrated that the mechanical stiffness of hydrogels plays a critical role in regulating the proliferation of preosteoblast cells but a minimal role in regulating the proliferation of bone marrow-derived stem cells.⁴² Previous studies have also demonstrated that mechanical stiffness plays a role in regulating the differentiation of both stem and precursor cells.⁴³ The lack of dependency of SSC proliferation on hydrogel stiffness is thus similar to the response of bone marrow-derived stem cells. This result may be due to the lack of intrinsic factors in SSCs that respond to mechanical stress, in comparison to more differentiated cells or cells belonging to other lineages, such as preosteoblasts.

Overall, this study demonstrates that a bioactive hydrogel modified with synthetic oligopeptides can be highly useful in controlling SSC proliferation in both 2D and 3D cell culture by mimicking some of the biochemical properties of the basement membrane underlying the seminiferous epithelium. In addition, the results of this study identified a key ECM variable that might regulate the proliferation of primary SSCs in the natural stem cell niche. The differentiation of primary SSCs into specific lineages may be regulated with the appropriate soluble factors for cells cultured within the hydrogel or for cells isolated from the hydrogel. Thus, the hydrogel system used in this study presents strong potential for use as a primary SSC transplantation vehicle in various cell-based therapies. The crucial role of natural stem cell niches in regulating stem cell activities can also be further clarified using this synthetic hydrogel system. Therefore, the results of this study would be broadly applicable to understanding the interactions between various stem cells and their microenvironments and ultimately controlling the diverse activities of a broad array of stem cells.

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