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8 **Regulating dynamic signaling between hematopoietic stem cells and niche**
9 **cells via a hydrogel matrix**

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ABSTRACT

Hematopoietic stem cells (HSC) reside in unique bone marrow niches and are influenced by signals from surrounding cells, the extracellular matrix (ECM), ECM-bound or diffusible biomolecules. Here we describe the use of a three-dimensional hydrogel to alter the balance of HSC-generated autocrine feedback and paracrine signals generated by co-cultured niche-associated cells. We report shifts in HSC proliferation rate and fate specification in the presence of lineage positive (Lin^+) niche cells. Hydrogels promoting autocrine feedback enhanced expansion of early hematopoietic progenitors while paracrine signals from Lin^+ cells increased myeloid differentiation. We report thresholds where autocrine vs. paracrine cues alter HSC fate transitions, and were able to selectively abrogate the effects of matrix diffusivity and niche cell co-culture via the use of inhibitory cocktails of autocrine or paracrine signals. Together, these results suggest diffusive biotransport in three-dimensional biomaterials are a critical design element for the development of a synthetic stem cell niche.

Keywords: hematopoietic, hydrogel diffusivity, autocrine, paracrine, signaling

1. Introduction

Hematopoietic stem cells (HSCs) are responsible for mediating the process of hematopoiesis, the continuous generation of the body's full complement of blood and immune cells. These events take place in unique regions of the bone marrow termed niches [1-6]. While the genetic information required to direct HSC fate specification events such as quiescence, self-renewal, or differentiation is contained within its DNA, signals from the niche – surrounding cells, the extracellular matrix (ECM), ECM-bound or diffusible biomolecules – trigger these events [7-9]. An artificial bone marrow would have significant clinical value, both for therapeutic expansion of HSCs to improve hematopoietic stem cell transplants and to facilitate studies of the etiology and treatment of hematologic diseases.

Recent efforts have focused on the development of biomaterials that regulate the presentation of matrix-based signals (*e.g.*, stiffness, composition) or exogenously-provided (soluble or matrix bound) growth factors [10-13] to direct HSC fate. However, exploring the role played by interactions between HSCs and supportive niche cells within the matrix provides a new avenue for investigation. Direct cell-cell interactions (juxtacrine signals) rely on the activation of membrane-bound cell adhesion molecules; these signals have been exploited in a biomaterial context to alter HSC survival and differentiation [14, 15]. Indirect cell-cell interactions between HSCs and niche cells (paracrine signals) or autocatalytic effects from the HSC itself (autocrine signals) are mediated by diffusible biomolecules [16-20]. Recently, experimental and modeling efforts have begun to examine niche-inspired paracrine and autocrine signals, primarily using liquid culture systems [19, 21]. While demonstrating the potential to alter HSC lineage

specification profiles, translation of such systems into fully three-dimensional biomaterial platforms offers an opportunity for innovation but remains poorly explored.

Within the niche, HSCs and supportive niche cells secrete and subsequently interact with a large number of cytokines and growth regulators [22]. Mesenchymal stem cells (MSCs) are believed to stimulate proliferation of HSCs through paracrine-mediated interactions via several molecules such as CXCL12, IL-6 and TPO [20]. Csaszar *et al.* demonstrated that paracrine-mediated IL-6 signaling between CD34⁺ human progenitor cells can be regulated by activation of the delta-1 ligand and is responsible for HSC expansion [19]. Similarly, the transforming growth factor β (TGF- β) pathway has been shown to be activated via both niche generated paracrine signals and HSC-generated autocrine feedback to inhibit HSC self-renewal and proliferation [16, 23]. *In vitro* autocrine feedback activated via vascular endothelial growth factor (VEGF) have also been shown to maintain HSC survival [17] while platelet derived growth factor (PDGF) mediated autocrine feedback can enhance proliferation [23, 24]. From an engineering context, Zandstra *et al.* demonstrated selective inhibition of paracrine and autocrine feedback signal transduction pathways in liquid culture to alter HSC fate [21, 23, 25]. Recently, Müller *et al.* described the use of arrays of microcavities to culture single or small groups of hematopoietic stem and progenitor cells (HSPCs), and reported that while autocrine feedback within the microcavity culture may play a role in HSC quiescence, paracrine signaling provided both stimulatory and inhibitory effects [26]. While these studies highlight the importance of autocrine and paracrine signaling mechanisms for HSC biomanufacturing, significant opportunity exists to develop a framework to examine the balance of these signals within a fully-3D biomaterial platform where matrix diffusive transport plays a critical role.

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2 Here we study the coordinated effects of paracrine signals produced by co-encapsulated Lin⁺
3 niche cells and HSC-generated autocrine feedback on HSC lineage specification. We
4 hypothesize that the mode of cell communication in a diffusion-restricted environment is a
5 function of niche cell density, responsible for establishing the concentration of paracrine signals,
6 and the diffusivity of the hydrogel encapsulating the cells. We further hypothesize that altering
7 the delivery of paracrine signals from niche cells or the balance of diffusive loss vs. retention of
8 HSC-generated autocrine signals alters HSC fate specification. We explored this concept using
9 the well-described murine hematopoietic stem cell system, choosing primary bone marrow
10 derived Lin⁻Sca1⁺cKit⁺ (LSK) sub-fraction as HSCs and Lin⁺ marrow cells as supportive niche
11 cells known to secrete biomolecular cues that stimulate HSC proliferation and lineage
12 specification [23]. HSCs were encapsulated with Lin⁺ niche cells in collagen hydrogels, varying
13 both hydrogel density (1, 3 mg/mL) and HSC:Lin⁺ ratio (1:0, 1:10, 1:100), with liquid culture
14 used as a control (**Fig. 1**). Cultures were limited to 2 days in order to explicitly consider early
15 HSC responses to mixed cultures and to limit the effects of dynamic feedback responses. We
16 traced HSC response via established metrics of HSC apoptosis, proliferation, surface antigen
17 expression, and colony forming unit capacity, and used selective inhibitors of paracrine and
18 autocrine signaling pathways to validate our findings. We report HSC fate specification as a
19 function of diffusion-restricted (autocrine feedback dominated) vs. diffusion unlimited (paracrine
20 dominated) biomaterial environments, and in so doing define a biomaterial-based approach to
21 regulate the balance of autocrine vs. paracrine signals to tune HSC proliferation vs.
22 differentiation.

Figure 1. Hematopoietic stem cell – Lin⁺ niche cell interactions within a hydrogel niche.

(A) A schematic depicting biomolecular interactions between HSCs and niche cells encapsulated within a hydrogel matrix. P: paracrine signals generated by the niche cell population. A₁: Fraction of HSC-generated autocrine signals that contribute to feedback loop. A₂: Fraction of HSC-generated autocrine signals that diffuse away. (B) Schematic of expected effects of changes in niche cell density versus matrix diffusivity on the magnitude of Paracrine vs. Autocrine feedback reaching the HSC population. Magnitude of signal depicted by the arrow width and font size. (C) Representative image of the HSC differentiation hierarchy depicting the starting Lin⁺Sca1⁺cKit⁺ (LSK) fraction along with the early vs. late hematopoietic progenitor cell populations. LT-HSC: Long-term repopulating HSC (LSK·CD34⁺·Flk2⁻). ST-HSC: Short-term repopulating HSC. MPP: Multipotent progenitor (LSK·CD34⁺·Flk2⁻). CMP: common myeloid progenitor. MEP: megakaryocyte-erythrocyte progenitor. GMP: granulocyte-macrophage progenitor.

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2. Methods and Materials

2.1 HSPC and Lin⁺ cell isolation

All work involving primary cells was conducted under approached animal welfare protocols

(Institutional Animal Care and Use Committee, University of Illinois at Urbana-Champaign).

Primary HSPCs were isolated from the bone marrow of the femur and tibia of female C57BL/6

mice (Jackson Labs; Ages 1 – 3 months) as described previously[27]. HSPCs were identified as the Lin⁻ Sca-1⁺c-kit⁺ (LSK) fraction by incubating the remaining bone marrow cells with a cocktail of antibodies (eBioscience San Diego, CA): PE-conjugated Sca-1 (1:100 dilution), APC-Cy7 conjugated c-kit (1:100 dilution), and a 1:100 dilution of a FITC-conjugated Lineage (Lin) cocktail (CD5, B220, Mac-1, CD8a, Gr-1, Ter-119). Both the LSK and Lin⁺ fraction was sorted using a BD FACS Aria II flow cytometer (BD FACS Diva software) and collected in PBS/FBS on ice for immediate use.

2.2 Collagen hydrogel preparation

Rat tail type I collagen (BD Biosciences, Bedford MA) was used to make the cell-laden collagen hydrogels. Collagen solutions of defined densities (1, 3 mg/mL) were prepared from the stock collagen solution of approximately 9 mg/mL (lot specific). The final concentrations were as follows: 1x M199 buffer (supplemented with 0.75% NaHCO₂ and 0.01 M HEPES) in a ratio of 1:4 with collagen stock; 1mM Dithiothreitol (DTT, Sigma); 5mM CaCl₂; 0.1mM Glyoxal; 0.4M NaOH added to adjust pH between 7.3–7.4 [28]. Final gel volume was achieved by adding the cell-media suspension to create a 60μL solution that was pipetted into a 96 well plate. Gelation was achieved within 1 hr of placing the gels in a humidified incubator at 37 °C and 5% CO₂.

2.3 Hydrogel biophysical characterization

Bulk rheological properties of the hydrogels were tested using a torque-controlled rotational rheometer (Discovery Series Hybrid Rheometer (DHR), model HR-3, TA Instruments) using a cone and plate geometry (60 mm cone diameter; 0.5° cone angle). The base plate was maintained at 37°C via a Peltier system, while a solvent trap was used to maintain hydrogel

1 samples in a humid environment. The collagen network was allowed to evolve in the confines of
2 the cone-plate rheometer setup for 3600 s and small oscillations in shear (3 rad/s, 1% strain
3 amplitude) in order to monitor gelation kinetics and to determine final viscoelastic properties
4 (**Supplementary Fig. 2**). Following gelation, hydrogels were subjected to an oscillation
5 frequency sweep at 1% strain amplitude (corresponding to the linear viscoelastic regime as
6 determined via previous oscillatory strain sweep; data not shown). Additionally, step stress tests
7 (to measure creep compliance) were used to characterize longer time behavior (up to 100 s) to
8 validate that the hydrogels were soft viscoelastic solids.

9
10 Hydrogel diffusivity was measured via fluorescence recovery after photobleaching (FRAP)
11 experiments using FITC-conjugated Dextran (40 kDa and 2000 kDa, Sigma Aldrich). Collagen
12 solutions (1 and 3 mg/mL) were prepared as described above and incubated for 1 hr. The gels
13 were incubated with FITC-conjugated Dextran solution overnight. Prior to analysis, the gels
14 were gently washed with PBS. FRAP measurements were performed using the Zeiss 710
15 Multiphoton confocal microscope as described previously [29]. Briefly, a 50 μm diameter spot
16 was bleached (Laser intensity: 0.05 mW) at the center of the gel. The sample was bleached for 5
17 seconds and recovery due to diffusive transport of fluorescent molecules from the surrounding
18 hydrogel was tracked for up to 15 minutes via fluorescent imaging. The half time ($t_{1/2}$) of the
19 recovery was calculated using the FRAP function available in the Zeiss software. The hydrogel
20 diffusion coefficient (D) was subsequently calculated as:

$$21 \quad D = \frac{w^2}{4t_{1/2}} \quad (1)$$

where D is the diffusion coefficient, w the photobleaching beam radius, and $t_{1/2}$ the recovery half time [30]. A maximum of 3 spots were bleached per sample, spaced away from each other to minimize effects of the previous photobleaching.

2.4 Mesh size estimation

To estimate the ratio of the average network mesh size ξ for varying collagen concentrations with fiber diameter d , we use the relation $G_0 \sim \xi^{-22/5} d^{28/5}$ from an existing theory for semi-flexible polymer networks by *Mackintosh et al.* [31], and scaling relations for structural elements of collagen proposed by *Yang et al.* [32]. Using the measured value of $G_0 \approx 5$ Pa for 1 mg/mL collagen, and $G_0 \approx 56$ Pa for 3 mg/mL collagen, and assuming no significant change in the fiber diameter with change in collagen concentration, we estimate $\xi_{3\text{mg/mL}} \sim 0.58 \cdot \xi_{1\text{mg/mL}}$. The accuracy of these estimates can be verified with mesh size estimates reported by *Arevalo et al.* via confocal reflectance imaging, predicting $\xi_{3\text{mg/mL}} \sim 0.7 \xi_{1\text{mg/mL}}$ [33]. This is close to our estimate using the network elastic moduli for these concentrations. It is worth noting that the pore size estimates from theories using diffusion in collagen gels are more accurate and representative of the true mesh size, and are much smaller than that reported using CRM imaging. These studies however do not report mesh sizes for 1 mg/mL collagen. Determining the actual mesh sizes require additional structural information such as fiber diameter, which is unavailable. Yet, this 40% decrease is consistent with existing reports from confocal reflectance imaging for the same range of concentrations [33].

2.5 Dextran hydrodynamic radius estimation

The *Mark-Houwink* equation relates the average molecular weight of the polymer M_w to its intrinsic viscosity $[\eta]$:

$$[\eta] = KM_w^\alpha,$$

where K and α are polymer constants and have been determined for dextran, $K = 49.3 \times 10^{-3}$ mL/g and $\alpha = 0.6$ [34]. For a polymer in solution, the radius of gyration R_g is given by [35]:

$$R_g = \left(\frac{[\eta]M_w}{6.2N_a} \right)^{\frac{1}{3}}$$

where N_a is the Avagadro number. The diffusion coefficient D is related to the polymer hydrodynamic radius R_h and the radius of gyration R_g as [36]:

$$D = \frac{kT}{6\pi\eta_s R_h} = \frac{0.203kT}{\sqrt{6}\eta_s R_g},$$

where η_s is the solvent viscosity. For the 40 kDa Dextran ($M_w = 40000$ g/mol), we find $D_{37^\circ\text{C}} = 5.37 \times 10^{-7}$ cm²/s and $R_h = 4.2$ nm, and for the 2000 kDa Dextran ($M_w = 2,000,000$ g/mol), we find $D_{37^\circ\text{C}} = 0.657 \times 10^{-7}$ cm²/s and $R_h = 35$ nm. These numbers are consistent with experimental reports from other studies [37].

2.6 Determination of characteristic diffusion time (t_D)

The cell-cell diffusion time for cell-secreted biomolecules for each co-culture condition was estimated as follows. For 3D culture, a homogeneous distribution of cells within the 60 μ L hydrogel and a simple cubic lattice structure arrangement of cells within the culture volume was assume. For a cell radius R , surface-surface cell spacing a , total number of cells N , and total hydrogel volume of 60 μ L:

$$N * (2R + a)^3 = 60\mu L$$

Therefore, we can solve for the cell spacing for each HSC:niche cell condition (1:0, 1:10, and 1:100). Further, biomolecular diffusion time (t_D ; **Supplementary Table 1**) for a given hydrogel diffusivity (D) is given by:

$$t_D(s) = \frac{a^2(\mu m^2)}{D(\mu m^2/s)}$$

2.7 Experimental culture conditions

LSKs were seeded in monoculture and co-culture with Lin⁺ bone marrow cells in 2D or 3D culture environments under the following conditions: LSK:Lin⁺ cells at 1:0, 1:10, 1:100, 1:500 in 2D (liquid), 1 mg/mL, 3 mg/mL collagen gels. 5000 LSK cells were used for each condition and cultured for 2 days prior to analysis. The cells were cultured in StemSpan SFEM media (Stemcell Technologies Inc., Vancouver, Canada) supplemented with 100 ng/mL Stem cell factor (SCF) (PeproTech, Rocky Hill, NJ).

2.8 Surface antigen expression analysis to determine cell phenotype

Cell phenotype was determined via surface antigen expression using flow cytometry on a BD LSRII flow cytometer (BD Bioscience). Prior to analysis, the collagen gels were digested using a 50 U/mL solution of type IV collagenase (Worthington Biochemical) for roughly 30 mins in the incubator. The cells were then washed, filtered and resuspended in 100 μ L PBS with 5% FBS (PBS/FBS) and 0.1% sodium azide. The cell suspension was then stained with a cocktail of the following antibodies (eBioscience): APC anti-CD34 (1:20 dilution, 60 mins), AmCyan anti-IL7R α (1:20, 60 mins BD Bioscience), APC-Cy7 anti-c-kit (1:100, 30 mins), PE anti-Sca-1 (1:100, 30 mins), PE-Cy7 anti-CD16/32 (1:100, 30 mins), PE-Cy5 anti-Flk2 (1:20, 30 mins), FITC-anti lineage cocktail (CD5, B220, Mac-1, CD8a, Gr-1, Ter-119, 1:100, 30 mins).

The surface antigen-based identification of the cells for all the progenitor sub-fractions is given in **Supplementary Table 2**.

2.9 LSK progenitor identification

The LSK progenitors were identified post culture by staining the initial LSK population with the CellTrace Violet Cell Proliferation dye (Invitrogen, Carlsbad, CA) as per manufacturer instructions. Briefly, cells were washed and resuspended in PBS. CellTrace Violet tracking dye was added at a final dye concentration of 1 μ M for 20 mins at 37 °C. Cells were quenched with cold PBS/FBS, incubated on ice for 5 mins followed by a wash with PBS. LSK progenitors were identified along with other surface markers on the flow cytometer. Progenitor sub-fractions were then calculated as a fraction of these LSK progenitors.

2.10 Apoptosis assay

Cells undergoing apoptosis were detected via staining with APC-labeled Annexin V as per manufacturer protocols [38]. Briefly, cells were washed and resuspended in 100 μ L of 1X Binding Buffer with 1% FBS and 5 μ L of the dye. The cells were stored in the dark at room temperature for 30 mins and analyzed within 1 hr. As a positive control, cells were treated with 1 μ M of Staurosporine. To further distinguish between live and dead cells, co-staining with propidium iodide (PI) was performed. A high percentage of cells that stained positive for Annexin V also stained positive for PI. To avoid spectral interference of PI with the surface antigen dyes PE-Cy7 and Cy5, only the Annexin V dye was added to distinguish between live and apoptotic cells. All cell samples were analyzed on a BD LSRFortessa flow cytometer (BD Bioscience) and using the FCS Express 4.0 software.

2.11 Colony-forming unit (CFU) assay

Colony forming potential was assessed via Methocult GF M3434 methylcellulose-based medium (StemCell Technologies, Vancouver, Canada). For this purpose, cells were isolated from collagen hydrogels after culture via collagen digestion mentioned above, filtered, and then resuspended in StemSpan SFEM media. Aliquots were made at one-third and one-fifth of the total sample volume, brought to a volume of 0.1 mL and mixed with the Methocult media for a total volume of 1.1 mL per sample. The samples were plated on a gridded 35 mm culture dishes (StemCell Technologies) and incubated for 12 days. Following incubation, the GEMM, GM, G, M, Mk, and E colonies were counted using an inverted microscope. The results are reported as the number of colonies counted per 1000 of the original LSK cells seeded for each condition.

2.12 Inhibitor molecules

Small molecule inhibitors for paracrine signaling were added to the 1 mg/mL collagen condition while inhibitors for autocrine signaling were added to the 3 mg/mL collagen condition as listed in **Supplementary Table 3**.

2.13 Statistical analysis

Statistical analysis was performed via one-way analysis of variance (ANOVA) tests after which a Tukey-HSD post-hoc test was used. Independent factors included niche cell density and hydrogel density. Analysis of LSK cell bioactivity (viability, proliferation index, surface antigen expression, CFU assay) used $n = 5$ constructs per group. Significance was set at $p < 0.05$. Error bars are reported as standard error of the mean unless otherwise noted.

3. Results

3.1 The presence of Lin^+ niche cells alters HSC progenitor fractions in liquid culture

Liquid culture was first used to determine the magnitude of the effect of Lin^+ niche cell co-culture on HSC response in a diffusion-unrestricted environment. Here, $\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^+$ (LSK) HSCs were seeded with Lin^+ niche cells over a range of HSC: Lin^+ densities (1:0, 1:10, 1:100). All experimental conditions contained an identical number of HSCs (5000). Overall viability of the HSC: Lin^+ cell co-cultures (1:0 to 1:100) was high (>90%) for all conditions (**Fig. 2A**). Flow cytometry analysis revealed that after two days of co-culture, the total number of LSK cells expanded (**Fig. 2B**) with increasing niche cell density. Further, early hematopoietic progenitors, long-term HSCs (LT-HSCs; $\text{LSK} \cdot \text{CD34}^{\text{lo}} \cdot \text{Flk2}^{\text{lo/-}}$), short-term HSCs (ST-HSCs; $\text{LSK} \cdot \text{CD34}^{\text{hi}} \cdot \text{Flk2}^{\text{lo/-}}$), and multipotent progenitors (MPPs; $\text{LSK} \cdot \text{CD34}^{\text{hi}} \cdot \text{Flk2}^{\text{hi}}$) showed significant increases with increasing Lin^+ niche cell density (**Fig. 2C-E**). Effects of Lin^+ co-culture on myeloid differentiation was also seen, with common myeloid progenitors (CMP; $\text{Lin}^- \cdot \text{IL7R}\alpha^- \cdot \text{c-kit}^+ \cdot \text{Sca-1}^- \cdot \text{CD34}^+ \cdot \text{CD16/32}^-$) showing a non-significant decrease with Lin^+ density and myeloid erythroid progenitors (MEP; $\text{Lin}^- \cdot \text{IL7R}\alpha^- \cdot \text{c-kit}^+ \cdot \text{Sca-1}^- \cdot \text{CD34}^- \cdot \text{CD16/32}^-$) showing a significant increase with Lin^+ density. No effect was observed for granulocyte macrophage progenitors (GMP; $\text{Lin}^- \cdot \text{IL7R}\alpha^- \cdot \text{c-kit}^+ \cdot \text{Sca-1}^- \cdot \text{CD34}^+ \cdot \text{CD16/32}^+$) (**Fig. 2F-H**). Colony forming unit (CFU) assays revealed minor functional changes as well. Notably, a decrease in the most primitive GEMM (granulocyte, erythrocyte, macrophage,) colonies, a significant ($p < 0.01$) increase in CFU-M (macrophage) colonies, and increasing trends in granulocyte macrophage (GM) and granulocyte (G) colonies (**Supplementary Fig. 1**). These results suggest that co-

Figure 2. Co-culture of LSK cells with Lin⁺ niche cells in liquid (2D) culture.

(A) Viability of Lin⁺Sca1⁺cKit⁺ (LSK) HSC as a function of niche cell density. (B) Total number of LSK cells remaining as a function of niche cell density. (C) – (H) Fraction of the early and late hematopoietic progenitors as a function of LSK:niche cell co-culture density. LT-HSC: Long-term repopulating HSC (LSK·CD34⁺·Flk2⁺). ST-HSC: Short-term repopulating HSC. MPP: Multipotent progenitor (LSK·CD34⁺·Flk2⁺). CMP: common myeloid progenitor. MEP: megakaryocyte-erythrocyte progenitor. GMP: granulocyte-macrophage progenitor. *: p < 0.05. **: p < 0.01. ***: p < 0.005. NS: No significance.

1 culture with Lin⁺ niche cells altered LSK proliferation and lineage specification, with
 2 particularly significant effects on early progenitor fractions.

3.2 Biophysical characterization of collagen hydrogels

5 The storage modulus ($G'(\omega)$) for both collagen hydrogel concentrations (1mg/ml, 3mg/ml) was
 6 significantly larger than the viscous modulus ($G''(\omega)$), suggesting the materials are effectively
 7 solid over the timescales (frequencies: $10^{-1} - 10^2$ rad/sec) probed. We report the plateau modulus
 8 G_o of the evolved state of the collagen network at 37°C. The 1 mg/ml hydrogel has a plateau

modulus of 5 Pa, while the 3 mg/ml collagen hydrogel exhibited viscoelastic behavior and a plateau modulus of 56 Pa. These rheological properties were also used to estimate changes in network mesh size ξ with collagen concentration. With fiber diameter d , we use the relation $G_0 \sim \xi^{-22/5} d^{28/5}$ from an existing theory for semi-flexible polymer networks [31, 32], suggesting a 40% decrease in mesh size for 3 vs. 1 mg/ml gels. Data at high frequency is affected by instrument rotational inertia, and cannot be used for analysis (dashed line) (**Supplementary Fig. 2A**) [39]. Longer timescales were probed with step-stress (creep) tests at an input stress of 0.03 Pa (Supplementary Fig. 2b). Up to timescales of 100s, both materials show solid-like behavior (plateaus of the creep compliance, $J(t)$), with time. Upon close inspection (Supplementary Fig. 2b inset), the 1 mg/ml collagen gel exhibits slight power law creep compliance

$$J(t) \approx \left[0.15 \frac{1}{\text{Pa s}^{0.1}} \right] t^{0.1}, \text{ suggesting soft solid behavior close to a critical gel [40].}$$

Gel diffusivity indicates collagen concentration and molecular size dependent effects on diffusivity. The diffusivity of 40 kDa Dextran decreased significantly with increasing hydrogel density (1 mg/mL: $15.9 \pm 0.67 \mu\text{m}^2/\text{sec}$; 3 mg/mL: $10.7 \pm 0.67 \mu\text{m}^2/\text{sec}$) (**Supplementary Fig. 2C**). Diffusivity of 2000 kDa Dextran was significantly lower for both hydrogel formulations, but was insensitive to collagen concentration, likely due to a larger hydrodynamic diameter ($d_h \sim 70 \text{ nm}$) on order of the collagen mesh size. For 40 kDa molecules, the hydrodynamic diameter $d_h \sim 8 \text{ nm}$ is much smaller than the collagen mesh size [33, 41]. As many hematopoietic associated cytokines (*e.g.*, SCF [10], CXCL12, VEGF, TGF β) range in size between 20 – 60 kDa, these two hydrogel variants present significantly different biotransport environments for paracrine or autocrine-mediated signals.

Cell spacing for each niche cell condition was calculated assuming a simple cubic lattice structure of cell arrangement in the 3D hydrogels. The characteristic diffusion time between cells (t_D) was calculated based on these distances and the experimentally determined diffusion coefficient (D) according to (**Supplementary Table 1**):

$$t_D(s) = \frac{\text{Cell spacing}^2(\mu\text{m}^2)}{D(\mu\text{m}^2/s)}$$

3.3 HSC progenitor response to Lin⁺ niche cells is altered by the surrounding hydrogel network

Early progenitor fractions (LT-HSC, ST-HSC, MPP) showed significant ($p < 0.05$) increases with increasing Lin⁺ co-culture in 1 mg/mL gels. While the trend still existed in 3 mg/mL hydrogels, the differences were no longer significant (**Fig. 3A**). Looking at differentiating hematopoietic populations, the number of common myeloid progenitors (CMPs) held constant for increasing Lin⁺ niche cells, while the further differentiated myeloid progenitors (MEP, GMP) showed significant increases ($p < 0.01$ for 1 mg/ml; $p < 0.05$ for 3 mg/ml) with Lin⁺ co-culture (**Supplementary Fig. 3B**). Analysis of the functional capacity of the HSCs via CFU assays revealed similar conclusions, notably an increase in downstream myeloid progenitors (GM, G, M and E colonies) in both 1 mg/ml and 3 mg/ml collagen gels (**Supplementary Fig. 4, 5**). Although not statistically significant, while numbers of the most primitive GEMM colonies decreased with increasing Lin⁺ co-culture in liquid media, GEMM colonies remained relatively constant or increased in hydrogel cultures.

Heat maps normalizing cell response of HSC:niche co-culture in the hydrogel network versus liquid co-cultures were used to compare the magnitude and effect of the presence of a hydrogel

Figure 3. Comparative analysis of hematopoietic progenitor populations via flow cytometry as a function of hydrogel matrix conditions and niche cell co-culture density.

(A) Early hematopoietic progenitor populations (LT-HSC, ST-HAS, MPP) increase with increasing niche cell density. (B) Representative schematic of the HSC differentiation hierarchy and heat map legend depicting changes in HSC:niche cell density ('*paracrine signal*') as well as hydrogel density ('*diffusivity*'). (C) Heat map depicting relative changes in progenitor cell populations in 1 and 3 mg/mL collagen hydrogel conditions normalized against observed changes in 2D liquid (diffusion-unlimited) culture. Separate heat map scales are used for Early vs. Late hematopoietic progenitors to better demonstrate effects of niche cell and hydrogel density. *: $p < 0.05$ w.r.t. 2D culture. **: $p < 0.01$ w.r.t. 2D culture. ***: $p < 0.001$ w.r.t. 2D culture. #: $p < 0.05$ w.r.t. 1 mg/mL culture.

- 1 network on HSC differentiation. For each cell state (e.g., CD34⁺Flk2⁺LSK LT-HSCs), a 3×2
- 2 heat map compares 6 microenvironmental conditions (Fig. 3B). Rows correspond to discrete
- 3 HSC:Lin⁺ cell densities (1:0, bottom; 1:10; 1:100 top) while columns correspond to hydrogel
- 4 density (1 mg/ml left; 3 mg/ml right). Therefore, the bottom right of each matrix corresponds to a
- 5 niche environment with no niche cells and increased restriction to biomolecule diffusion
- 6 (maximum autocrine feedback). The top left corner (high niche cell:HSC density; high

diffusivity) corresponds to the highest potential paracrine signal environment. Relative increases in early progenitor fractions (LT-HSC, ST-HSC) were observed for autocrine feedback dominated (3 mg/ml, 1:0 HSC:Lin⁺ ratio). However the greatest increase in late-progenitor cell populations (CMP, GMP) was observed in paracrine-dominated regimes (1 mg/mL hydrogel; 1:100 HSC:Lin⁺ ratio) (**Fig. 3B**). Heat maps regarding functional CFU formation were consistent with FACS results, suggesting that except for GEMM colonies, lower diffusivity (3 mg/mL) hydrogels resulted in lower colony numbers than higher diffusivity (1 mg/mL) hydrogels (**Supplementary Fig. 6**). Taken together, these results suggest that reduced gel diffusivity promotes expansion of early HSC progenitors at low niche cell densities, whereas conditions of high niche cell density and increased gel diffusivity promote expansion of differentiated hematopoietic progenitors.

3.4 Inhibition of paracrine signals affects hematopoietic progenitors at all levels

To better describe the effect of paracrine signals from Lin⁺ niche cells on HSC response, cells were again co-cultured in the 1 mg/ml collagen hydrogel (high diffusivity) in the presence of a cocktail of inhibitors for TGF- β , MIP-1 α and TNF α (**Fig. 4A**), paracrine signals known to inhibit the self-renewal capacity of primitive HSC progenitor populations [23]. Here, heat maps comparing HSC response (relative to liquid culture) contain rows for increasing Lin⁺ density as before, but columns now compare uninhibited (*left*) vs. paracrine signal inhibited (*right*) culture (**Fig. 4B**). While dependence of early HSC progenitors on niche cell density was qualitatively similar for the paracrine signal inhibited conditions, we observed a reduction in differentiated myeloid populations. The greatest effect was seen for late myeloid progenitors (CMP, GMP) at high niche cell densities. Compared to uninhibited cultures, early progenitor (e.g., CMP)

Figure 4. Effect of the addition of paracrine-inhibitory cocktail on HSC response to Lin⁺ niche cells within the hydrogel environment.

(A) A schematic depicting the effect of paracrine inhibition on HSCs in 1 mg/mL collagen co-cultures. HSC response was quantified via surface antigen expression using a modified heat map depiction of the effects of paracrine inhibition (right column) vs. culture in 1 mg/ml collagen (left column). Functional changes in HSC response were also profiled via CFU-assays (CFU-GEMM, CFU-GM, along with more mature Mk, E, G, M colonies). (B) Heat map-based analysis of effect of addition of paracrine inhibitor cocktails to HSC:Lin⁺ niche cell co-culture in paracrine-dominated (1 mg/mL) hydrogel environments showing fold change in cell fractions both without (left column) and with (right column) paracrine inhibitors, with values normalized against 2D co-culture. (C) CFU assay showing effect of paracrine inhibition on progressively mature colonies, GEMM (most primitive) to E (most mature). *: p < 0.05 w.r.t. 2D culture. **: p < 0.01 w.r.t. 2D culture. ***: p < 0.001 w.r.t. 2D culture. NS: Not significant.

fractions increased whereas the more mature GMP fractions decreased. Results from CFU assays exhibited a similar trend. While no significant changes were seen in the primitive GEMM colonies due to loss of paracrine signaling, more mature colonies such as GM and E no longer showed a significant increase in number with Lin⁺ niche cell density (Fig. 4C, Supplementary Fig. 8).

3.5 Inhibition of autocrine signaling primarily affects early hematopoietic progenitors

1 We subsequently co-cultured HSCs in 3 mg/mL collagen (low diffusivity) hydrogels in the
2 presence of a cocktail of inhibitors for VEGF, PDGF and FGF receptors (**Fig. 5A**). Autocrine
3 mediated feedback via these factors has been shown to promote primitive HSC expansion [17,
4 23, 42], suggesting their inhibition in hydrogel culture may reduce the numbers of early
5 hematopoietic progenitors in conditions where autocrine feedback is significant (low niche cell
6 densities; low diffusivity environments). Heat maps comparing HSC response (relative to liquid
7 culture) contain rows for increasing Lin⁺ density as before, but columns now compare

Figure 5. Effect of the addition of autocrine-inhibitors on HSC response within the hydrogel environment.

(A) A schematic hypothesizing the effects of autocrine inhibition in 3 mg/mL collagen co-cultures. HSC response was quantified via surface antigen expression using a modified heat map depiction of the effects of autocrine inhibition (right column) vs. culture in 3 mg/ml collagen (left column). Functional changes in HSC response were also profiled via CFU-assays (CFU-GEMM, CFU-GM, along with more mature Mk, E, G, M colonies). (B) Heat map-based analysis of effect of addition of autocrine inhibitor cocktails to HSC:Lin⁺ niche cell co-culture in autocrine-dominated (3 mg/mL) hydrogel environments showing fold change in cell fractions both without (left column) and with (right column) autocrine inhibitors, with values normalized against 2D co-culture. (C) CFU assay showing effect of paracrine inhibition on progressively mature colonies, GEMM (most primitive) to E (most mature). *: p < 0.05 w.r.t. 2D culture. **: p < 0.01 w.r.t. 2D culture. ***: p < 0.001 w.r.t. 2D culture. NS: Not significant.

uninhibited (*left*) vs. autocrine signal inhibited (*right*) culture (**Fig. 5B**). Interestingly, there was a reduction in the fold change in early progenitor populations, with the greatest reductions seen for the ST-HSC and MPP fractions at low (1:0) niche cell densities as per our hypothesis. Further, the non-significant reduction in the reduced number of LT-HSCs and ST-HSCs in conventional 3mg/ml culture with decreasing niche cell density (1:0 vs. 1:100; Fig. 3A, 3 mg/ml condition) became a significant reduction in the presence of autocrine inhibition ($p < 0.005$) (**Supplementary Fig. 9**). These findings are similar to the significant reduction observed in non-autocrine inhibited high-diffusivity (1 mg/ml) hydrogels (Fig. 3A, 1 mg/ml condition). Despite these changes in early hematopoietic progenitor populations, numbers of late progenitor fractions (e.g., CMP, MEP, GMP; Fig. 5B, Supplementary Fig. 9) as well as colony forming unit assays (GM, G, M, Mk, E; Supplementary Fig. 10) remained relatively unchanged.

4. Discussion

Signaling molecules play a central role in regulating stem cell fate in the *in vivo* HSC niche. Signaling pathways associated with many biomolecules have been evaluated in the context of activated paracrine or autocrine signaling channels. However these interactions have not been translated to 3D culture systems. One main challenge is the interdependence of stiffness, diffusivity, and ligand density. Matrix stiffness is a function of the crosslinking density, which also controls small molecule diffusion through its fiber network with higher stiffness corresponding to lower diffusion [43]. Although decoupling matrix stiffness and diffusion effects is difficult, proper selection of gel properties can help mitigate their biological effects. Discher *et al.* showed CD34⁺ HSCs exhibited different morphologies when cultured on substrates with a 100x difference in stiffness (0.3 kPa vs 34 kPa) [44]. However, Choi *et al.* demonstrated that

1 while HSC bioactivity was impacted by substrate elasticity between 1kPa to 196 kPa, no
2 significant dependence was observed for softer substrates (<100 Pa) [11]. The collagen gel
3 stiffness explored here falls well below these ranges (5.5 Pa, 1 mg/mL; 56 Pa, 3 mg/mL),
4 suggesting a reduced role of matrix stiffness on HSC response in these studies. The influence of
5 ligand density, which increases with collagen density (1 mg/mL vs. 3 mg/mL), on HSC behavior
6 has also been previously documented [11, 45, 46]; while the collagen hydrogel system employed
7 here precludes close control over changes in ligand density, the marked effects of niche cell co-
8 culture as well as paracrine and autocrine inhibition on HSC response observed here suggest an
9 opportunity to design improved biomaterials for selective HSC expansion. Though the
10 importance of niche cell populations on HSC fate decisions *in vitro* is well known, most previous
11 works focus on direct cell-cell contact [47, 48] or the effects of paracrine signals in liquid culture
12 [25]. Through the use of defined 3D hydrogel matrices, we report HSC expansion and myeloid
13 differentiation can be significantly altered by feedback-dependent effects of Lin⁺ niche cells and
14 HSCs themselves.

15
16 Our results reveal the presence of a hydrogel matrix altered the signaling feedback between Lin⁺
17 niche cells (paracrine) and LSK generated (autocrine) feedback. The 3 mg/mL collagen hydrogel
18 environment promoted a higher fraction of the early progenitor fractions at low niche cell
19 densities (**Fig. 3C**). While reducing the number of Lin⁺ niche cells (1:100, 1:10, 1:0) led to a
20 significant reduction in LT-HSC, ST-HSC, and MPP in liquid culture and more diffusive 1mg/ml
21 hydrogels (**Fig. 3A**), the reduction was not significant for the 3mg/ml hydrogel, suggesting the
22 importance of autocrine feedback in dictating primitive HSC fate decisions. Similarly, the

1 increased incidence of late progenitors in the 1 mg/mL gels at high niche cell densities suggest
2 the dominance of paracrine signals.

3
4 We next asked whether the introduction of inhibitor cocktails for known paracrine signaling
5 paths could abrogate effects observed at high niche cell and high matrix diffusivity conditions.
6 Coincident increases in LT-HSC and ST-HSC cell populations, significant reduction in
7 downstream granulocyte-macrophage progenitors (GMPs) (**Fig. 4B**), and reduced numbers of
8 downstream CFU-GM and E colonies is consistent with this hypothesis (**Fig. 4C**). Tellingly, the
9 greatest changes in the late progenitor fractions with paracrine signal inhibition were seen in the
10 highest niche cell density cultures (1:100). This suggests the exciting potential to use expanded
11 time-resolved analyses to examine the impact of paracrine-autocrine signals in future studies.
12 Similarly, inhibition of autocrine signaling pathways, specifically VEGF, PDGF and FGF,
13 altered enhanced early hematopoietic progenitor populations in diffusion-limited or low niche
14 cell density hydrogels (**Fig. 5A**). While late progenitor fractions were not affected by autocrine
15 inhibition, early progenitor fractions were reduced, particularly in the case of HSC only (1:0)
16 cultures (**Fig. 5B**). Hence, disruptions to autocrine feedback via the hydrogel environment may
17 be particularly important for altering expansion of early hematopoietic progenitors. Together,
18 these observations raise a number of exciting questions for future study to investigate dose-
19 dependent effects of singular inhibitors and cocktails, expand the range of hydrogel
20 environments considered, and explore the effects of matrix-remodeling based dynamic changes
21 in biotransport.

1 These observations provide a perspective on the characteristic diffusion time (t_D) of autocrine
2 and paracrine signaling molecules. Given the complex nature of co-culture conditions and the
3 inter-dependent factors influencing cell biology, t_D provides a tunable property capable of
4 decoupling some of these effects. For example, despite only a 1.5-fold difference in matrix
5 diffusivity for the 1 vs. 3 mg/mL collagen hydrogel, vastly different HSC biology was observed
6 even at similar cell concentrations. Characteristic diffusion times on the order of 4400s (3mg/mL
7 hydrogel) were sufficient to promote autocrine signal-mediated expansion of early HSC
8 progenitors. Interestingly, characteristic t_D of order 3000s (1mg/mL hydrogel) did not show the
9 same effect for HSC only cultures, while t_D of order of 540s in the presence of Lin⁺ niche cells
10 was sufficient to promote paracrine signal mediated hematopoietic differentiation. These
11 findings are conceptually consistent with those recently reported by Müller *et al.* for culture of
12 HSPCs in media filled microcavities; autocrine feedback promoted HSC quiescence but could
13 also be abrogated by expanding the size of the microcavity to dilute the effect [26]. In our work,
14 increasing the characteristic t_D of fully three-dimensional hydrogels was able to promote
15 autocrine-mediated HSC expansion, suggesting selective engineering of characteristic diffusion
16 times provides an important avenue for hematopoietic stem cell niche engineering in fully three-
17 dimensional biomaterial environments.

18
19 The ability to concentrate the design of hydrogel networks to alter autocrine/paracrine feedback
20 in heterogeneous niche-stem cell cultures offers an exciting possibility in biomaterial design. The
21 well-defined biological underpinnings of the HSC system [49], along with significant *in vivo*
22 data regarding multiple niche cell populations of potential functional importance motivate this
23 investigation [50]. A broad spectrum of conditions, both in niche cell and matrix concentration,

was chosen to obtain a comprehensive perspective of HSC differentiation trends *in vitro*. Although the variables under study are multi-faceted and co-dependent to draw pointed conclusions, the relative effects and trends observed here suggest engineering a system that can explicitly define these effects will be instrumental for future HSC biomanufacturing applications. It is likely identification of culture conditions to expand early hematopoietic progenitors will require increased resolution of the effect of discrete signaling pathways on HSC fate and whether singular or coordinated inhibition of these pathways can selectively enhance the effect. Further, while we were able to examine a broad spectrum of cell density (1:0 – 1:100), the range of hydrogel environments tested here, while sufficient to resolve important differences in paracrine signals vs. autocrine feedback, represents only a small fraction of available design space. Ongoing efforts are using a methacrylamide-functionalized gelatin hydrogel system that is compatible with analysis of HSC differentiation patterns [10] and provides the capability of generating libraries of hydrogel biomaterials with tunable matrix properties [29, 51]. Further, this study chose to use a Flk-based FACS sorting paradigm as well as CFU assays that allowed us to parse early hematopoietic progenitor populations as LT-HSC vs. ST-HSC vs. MPP. While chosen to allow us to more broadly subtype HSC response to niche cell co-culture, future efforts will focus on more stringent identification of LT-HSCs via surface antigen markers (e.g., Lin⁻ Sca1⁺cKit⁺CD150⁺CD48⁻CD244 [52] and *in vivo* transplantation assays [53]. Finally, the choice of niche cell type provides an opportunity to explore differences in paracrine signals. While Lin⁺ niche cells comprise a broad spectrum of cell populations with a heterogeneous biomolecule profile, incorporation of discrete quiescent versus active niche inspired cell populations such as mesenchymal stem cells or vascular endothelial cells will help accurately classify the role and mechanisms of the biomolecules involved in autocrine and paracrine pathways at different stages

of lineage specification. The overarching objective of this study was to address the critical question of how signaling mechanisms within a complex 3D co-culture environment are influenced by surrounding biophysical and biochemical properties and their resulting effect on HSC biology, and our observations provide a concrete direction for future studies addressing the interplay of HSC – matrix – biomolecule synergy.

5. Conclusions

Interactions between HSCs and niche cells within the bone marrow involve activation of autocrine, paracrine and juxtacrine-contact associated signaling pathways. The nature of these interactions, both complex and dynamic, motivates the design of *ex vivo* culture systems to explore the nature of these interactions. Here we describe an approach co-varying hydrogel diffusivity and niche cell density as a means to alter early HSCs fate decisions. We show that in paracrine-signaling dominated hydrogel regimes, the use of Lin⁺ niche cells can enhance HSC myeloid lineage specification while these effects can be abrogated via inhibitory cocktails of paracrine signaling molecules. Autocrine-dominated regimes, characterized by low diffusivity hydrogel environments, can both insulate HSC response from niche cell co-culture but potentially also enhance the effect of HSC autocrine feedback events. These effects could be reduced by selective inhibition of autocrine signaling molecules. Together, these results suggest the exciting possibility to exploit the balance of paracrine and autocrine signals through selective design of three-dimensional culture tools to gain improved control of HSC fate *in vitro*.

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References

- [1] R. Schofield, The relationship between the spleen colony-forming cell and the haemopoietic stem cell, *Blood cells* 4(1-2) (1978) 7-25.
- [2] D.N. Haylock, S.K. Nilsson, Stem cell regulation by the hematopoietic stem cell niche, *Cell Cycle* 4(10) (2005) 1353-5.
- [3] K. Tokoyoda, T. Egawa, T. Sugiyama, B.I. Choi, T. Nagasawa, Cellular niches controlling B lymphocyte behavior within bone marrow during development, *Immunity* 20(6) (2004) 707-18.
- [4] L.E. Purton, D.T. Scadden, The hematopoietic stem cell niche, *StemBook*, Cambridge (MA), 2008.
- [5] G.B. Adams, D.T. Scadden, The hematopoietic stem cell in its place, *Nat Immunol* 7(4) (2006) 333-7.
- [6] D.T. Scadden, The stem-cell niche as an entity of action, *Nature* 441(7097) (2006) 1075-1079.
- [7] A. Wilson, A. Trumpp, Bone-marrow haematopoietic-stem-cell niches, *Nat Rev Immunol* 6(2) (2006) 93-106.
- [8] J. Isern, S. Mendez-Ferrer, Stem cell interactions in a bone marrow niche, *Current osteoporosis reports* 9(4) (2011) 210-8.
- [9] M.V. Gomez-Gavero, R. Lovell-Badge, F. Fernandez-Aviles, E. Lara-Pezzi, The Vascular Stem Cell Niche, *Journal of cardiovascular translational research* (2012).
- [10] B.P. Mahadik, S. Pedron Haba, L.J. Skertich, B.A. Harley, The use of covalently immobilized stem cell factor to selectively affect hematopoietic stem cell activity within a gelatin hydrogel, *Biomaterials* 67 (2015) 297-307.
- [11] J.S. Choi, B.A. Harley, The combined influence of substrate elasticity and ligand density on the viability and biophysical properties of hematopoietic stem and progenitor cells, *Biomaterials* 33(18) (2012) 4460-8.
- [12] J.E. Nichols, J. Cortiella, J. Lee, J.A. Niles, M. Cuddihy, S. Wang, J. Bielitzki, A. Cantu, R. Mlcak, E. Valdivia, R. Yancy, M.L. McClure, N.A. Kotov, In vitro analog of human bone marrow from 3D scaffolds with biomimetic inverted colloidal crystal geometry, *Biomaterials* 30(6) (2009) 1071-9.
- [13] M.L. Cuchiara, S. Coşkun, O.A. Banda, K.L. Horter, K.K. Hirschi, J.L. West, Bioactive poly(ethylene glycol) hydrogels to recapitulate the HSC niche and facilitate HSC expansion in culture, *Biotechnol Bioeng* 113(4) (2016) 870-881.
- [14] Y. Tajima, E.J. Huang, K. Vosseller, M. Ono, M.A. Moore, P. Besmer, Role of dimerization of the membrane-associated growth factor kit ligand in juxtacrine signaling: the S117H mutation affects dimerization and stability-phenotypes in hematopoiesis, *J Exp Med* 187(9) (1998) 1451-61.
- [15] Y. Jung, J. Wang, A. Havens, Y. Sun, T. Jin, R.S. Taichman, Cell-to-cell contact is critical for the survival of hematopoietic progenitor cells on osteoblasts, *Cytokine* 32(3-4) (2005) 155-62.
- [16] F.W. Ruscetti, S. Akel, S.H. Bartelmez, Autocrine transforming growth factor-beta regulation of hematopoiesis: many outcomes that depend on the context, *Oncogene* 24(37) (2005) 5751-63.
- [17] H.P. Gerber, A.K. Malik, G.P. Solar, D. Sherman, X.H. Liang, G. Meng, K. Hong, J.C. Marsters, N. Ferrara, VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism, *Nature* 417(6892) (2002) 954-8.

- [18] A. Janowska-Wieczorek, M. Majka, J. Ratajczak, M.Z. Ratajczak, Autocrine/paracrine mechanisms in human hematopoiesis, *Stem Cells* 19(2) (2001) 99-107.
- [19] E. Csaszar, W. Wang, T. Usenko, W. Qiao, C. Delaney, I.D. Bernstein, P.W. Zandstra, Blood stem cell fate regulation by Delta-1 mediated rewiring of IL-6 paracrine signaling, *Blood* (2013).
- [20] T. Li, Y. Wu, Paracrine molecules of mesenchymal stem cells for hematopoietic stem cell niche, *Bone marrow research* 2011 (2011) 353878.
- [21] E. Csaszar, D.C. Kirouac, M. Yu, W. Wang, W. Qiao, M.P. Cooke, A.E. Boitano, C. Ito, P.W. Zandstra, Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling, *Cell Stem Cell* 10(2) (2012) 218-29.
- [22] M. Majka, A. Janowska-Wieczorek, J. Ratajczak, K. Ehrenman, Z. Pietrzkowski, M.A. Kowalska, A.M. Gewirtz, S.G. Emerson, M.Z. Ratajczak, Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner, *Blood* 97(10) (2001) 3075-85.
- [23] D.C. Kirouac, C. Ito, E. Csaszar, A. Roch, M. Yu, E.A. Sykes, G.D. Bader, P.W. Zandstra, Dynamic interaction networks in a hierarchically organized tissue, *Mol Syst Biol* 6 (2010) 417.
- [24] R.J. Su, K. Li, X.B. Zhang, P.M. Pan Yuen, C.K. Li, A.E. James, J. Liu, T.F. Fok, Platelet-derived growth factor enhances expansion of umbilical cord blood CD34+ cells in contact with hematopoietic stroma, *Stem cells and development* 14(2) (2005) 223-30.
- [25] D.C. Kirouac, G.J. Madlambayan, M. Yu, E.A. Sykes, C. Ito, P.W. Zandstra, Cell-cell interaction networks regulate blood stem and progenitor cell fate, *Mol Syst Biol* 5 (2009) 293.
- [26] E. Muller, W. Wang, W. Qiao, M. Bornhauser, P.W. Zandstra, C. Werner, T. Pompe, Distinguishing autocrine and paracrine signals in hematopoietic stem cell culture using a biofunctional microcavity platform, *Scientific reports* 6 (2016) 31951.
- [27] B.P. Mahadik, T.D. Wheeler, L.J. Skertich, P.J. Kenis, B.A. Harley, Microfluidic generation of gradient hydrogels to modulate hematopoietic stem cell culture environment, *Advanced healthcare materials* 3(3) (2014) 449-58.
- [28] D.L. Coutu, J. Cuerquis, R. El Ayoubi, K.A. Forner, R. Roy, M. Francois, M. Griffith, D. Lillicrap, A.M. Yousefi, M.D. Blostein, J. Galipeau, Hierarchical scaffold design for mesenchymal stem cell-based gene therapy of hemophilia B, *Biomaterials* 32(1) (2011) 295-305.
- [29] S. Pedron, B.A. Harley, Impact of the biophysical features of a 3D gelatin microenvironment on glioblastoma malignancy, *J Biomed Mater Res A* (2013).
- [30] B.L. Sprague, J.G. McNally, FRAP analysis of binding: proper and fitting, *Trends Cell Biol.* 15(2) (2005) 84-91.
- [31] F.C. Mackintosh, J. Kas, P.A. Janmey, Elasticity of semiflexible biopolymer networks, *Phys Rev Lett* 75(24) (1995) 4425-4428.
- [32] Y.L. Yang, L.M. Leone, L.J. Kaufman, Elastic moduli of collagen gels can be predicted from two-dimensional confocal microscopy, *Biophys J* 97(7) (2009) 2051-2060.
- [33] R.C. Arevalo, J.S. Urbach, D.L. Blair, Size-dependent rheology of type-I collagen networks, *Biophys J* 99(8) (2010) L65-L67.
- [34] J. Brandrup, E.H. Immergut, A. Abe, D.R. Bloch, *Polymer handbook*, Wiley, New York, NY, 1999.
- [35] M. Rubinstein, R.H. Colby, *Polymer physics*, Oxford University, New York, 2003.
- [36] Y. Oono, M. Kohmoto, Renormalization-group theory of transport-properties of polymer-solutions. 1. Dilute-solutions, *J Chem Phys* 78(1) (1983) 520-528.

- [37] J.R. Lawrence, G.M. Wolfaardt, D.R. Korber, Determination of diffusion-coefficients in biofilms by confocal laser microscopy, *Appl Environ Microbiol* 60(4) (1994) 1166-1173.
- [38] I. Leisten, R. Kramann, M.S. Ventura Ferreira, M. Bovi, S. Neuss, P. Ziegler, W. Wagner, R. Knuchel, R.K. Schneider, 3D co-culture of hematopoietic stem and progenitor cells and mesenchymal stem cells in collagen scaffolds as a model of the hematopoietic niche, *Biomaterials* 33(6) 1736-47.
- [39] J.M.T. Ewoldt R.H., Caretta L.M, Experimental challenges of shear rheology with case studies in biological complex fluids, *Complex Fluids in Biological Systems*, Springer Biological Engineering Series (In Press (2014)).
- [40] F. Chambon, H.H. Winter, Linear Viscoelasticity at the Gel Point of a Cross-Linking PdmS with Imbalanced Stoichiometry, *J Rheol* 31(8) (1987) 683-697.
- [41] A. Erikson, H.N. Andersen, S.N. Naess, P. Sikorski, C.D. Davies, Physical and chemical modifications of collagen gels: Impact on diffusion, *Biopolymers* 89(2) (2008) 135-143.
- [42] R. Ishino, K. Minami, S. Tanaka, M. Nagai, K. Matsui, N. Hasegawa, R.G. Roeder, S. Asano, M. Ito, FGF7 supports hematopoietic stem and progenitor cells and niche-dependent myeloblastoma cells via autocrine action on bone marrow stromal cells in vitro, *Biochem Biophys Res Commun* 440(1) (2013) 125-31.
- [43] C.B. Hutson, J.W. Nichol, H. Aubin, H. Bae, S. Yamanlar, S. Al-Haque, S.T. Koshy, A. Khademhosseini, Synthesis and characterization of tunable poly(ethylene glycol): gelatin methacrylate composite hydrogels, *Tissue Eng Part A* 17(13-14) (2011) 1713-23.
- [44] J.W. Shin, A. Buxboim, K.R. Spinler, J. Swift, D.A. Christian, C.A. Hunter, C. Leon, C. Gachet, P.C. Dingal, I.L. Ivanovska, F. Rehfeldt, J.A. Chasis, D.E. Discher, Contractile forces sustain and polarize hematopoiesis from stem and progenitor cells, *Cell Stem Cell* 14(1) (2014) 81-93.
- [45] J.S. Choi, B.A.C. Harley, Marrow-inspired matrix cues rapidly affect early fate decisions of hematopoietic stem and progenitor cells, *Science Advances* 3(1) (2017) e1600455.
- [46] E. Altmann, C.A. Muth, G. Klein, J.P. Spatz, C. Lee-Thedieck, The significance of integrin ligand nanopatterning on lipid raft clustering in hematopoietic stem cells, *Biomaterials* 33(11) (2012) 3107-18.
- [47] S. Mishima, A. Nagai, S. Abdullah, C. Matsuda, T. Taketani, S. Kumakura, H. Shibata, H. Ishikura, S.U. Kim, J. Masuda, Effective ex vivo expansion of hematopoietic stem cells using osteoblast-differentiated mesenchymal stem cells is CXCL12 dependent, *European journal of haematology* 84(6) (2010) 538-46.
- [48] Y. Nakamura, F. Arai, H. Iwasaki, K. Hosokawa, I. Kobayashi, Y. Gomei, Y. Matsumoto, H. Yoshihara, T. Suda, Isolation and characterization of endosteal niche cell populations that regulate hematopoietic stem cells, *Blood* 116(9) (2010) 1422-32.
- [49] S.J. Morrison, D.T. Scadden, The bone marrow niche for haematopoietic stem cells, *Nature* 505(7483) (2014) 327-34.
- [50] P.E. Boulais, P.S. Frenette, Making sense of hematopoietic stem cell niches, *Blood* 125(17) (2015) 2621-9.
- [51] S. Pedron, E. Becka, B.A. Harley, Regulation of glioma cell phenotype in 3D matrices by hyaluronic acid, *Biomaterials* 34(30) (2013) 7408-17.
- [52] I. Kim, S. He, O.H. Yilmaz, M.J. Kiel, S.J. Morrison, Enhanced purification of fetal liver hematopoietic stem cells using SLAM family receptors, *Blood* 108(2) (2006) 737-744.

1 [53] B.M. Sagar, S. Rentala, P.N. Gopal, S. Sharma, A. Mukhopadhyay, Fibronectin and laminin
2 enhance engraftability of cultured hematopoietic stem cells, Biochem Biophys Res Commun
3 350(4) (2006) 1000-5.
4