The use of covalently immobilized stem cell factor to selectively affect hematopoietic stem cell activity within a gelatin hydrogel

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ABSTRACT

Hematopoietic stem cells (HSCs) are a rare stem cell population found primarily in the bone marrow and responsible for the production of the body's full complement of blood and immune cells. Used clinically to treat a range of hematopoietic disorders, there is a significant need to identify approaches to selectively expand their numbers *ex vivo*. Here we describe a methacrylamide-functionalized gelatin (GelMA) hydrogel for *in vitro* culture of primary murine HSCs. Stem cell factor (SCF) is a critical biomolecular component of native HSC niches *in vivo* and is used in large dosages in cell culture media for HSC expansion *in vitro*. We report a photochemistry based approach to covalently immobilize SCF within GelMA hydrogels via acrylate-functionalized polyethylene glycol (PEG) tethers. PEG-functionalized SCF retains the native bioactivity of SCF but can be stably incorporated and retained within the GelMA hydrogel over 7 days. Freshly-isolated murine HSCs cultured in GelMA hydrogels containing covalently-immobilized SCF showed reduced proliferation and improved selectivity for maintaining primitive HSCs. Comparatively, soluble SCF within the GelMA hydrogel network induced increased proliferation of differentiating hematopoietic cells. We used a microfluidic templating approach to create GelMA hydrogels containing gradients of immobilized SCF that locally direct HSC response. Together, we report a biomaterial platform to examine the effect of the local presentation of soluble vs. matrix-immobilized biomolecular signals on HSC expansion and lineage specification. This approach may be a critical component of a biomaterial-based artificial bone marrow to provide the correct sequence of niche signals to grow HSCs in the laboratory.

1. Introduction

Hematopoietic stem cells (HSCs) are of increasing interest in the field of regenerative medicine due to their role in the production and regulation of the body's blood and immune system.

Dysregulations in HSC function can lead to severe blood-related disorders and cancers such as leukemia and lymphoma. These pathologies are typically treated clinically via HSC transplantation where whole donor bone marrow, or in some cases an enriched HSC fraction, is infused into the patient after myeloablative therapy to reconstitute the compromised marrow [1, 2]. Clinical complications associated with both insufficient numbers of isolated donor cells or low homing and engraftment post-transplant require approaches to selectively expand primitive HSC fractions [3, 4]. Bone marrow niches, defined by constellation of neighboring niche cells, the extracellular matrix (ECM), and biomolecules, are believed to significantly affect HSC quiescence, differentiation, migration and hematopoietic lineage specification [5-10]. Mimicking the niche as a coordinated entity of action requires understanding HSC fate decisions in response to multiplexed cell, biophysical, and biomolecular signals. Therefore our mission is to generate an instructive biomaterial able to selectively promote HSC quiescence and self-renewal over differentiation for targeted clinical applications.

While the biomolecular environment within the marrow is complex, a number of factors have been identified as critical regulators of HSC fate. *In vivo* studies that de-functionalize niches by removing cell or matrix constituents have provided insight regarding niche anatomical localization [11-13], putative niche cells [11, 13-17] and niche-regulated signaling pathways (*e.g.*, Jagged-1/Notch, CXCL12/CXCR4) [8, 18-28]. Recently, biophysical signals (matrix mechanical properties, ligand presentation) have been suggested to directly impact HSC lineage

specification and downstream myeloid differentiation [29-31]. The rarity of primitive HSCs within the marrow (<0.005% of marrow) [32] makes it difficult to study HSC-niche interactions *in vivo*. As such, efforts have turned towards developing two and three-dimensional (3D) biomaterials for HSC expansion or directed differentiation [33-36]. Biomaterial-based approaches that functionalize a synthetic niche with defined sequences of cues may provide significant insight regarding the coordinated impact of the niche environment on HSC fate [37]. To meet clinical-scale needs, efforts here concentrate on fully three-dimensional biomaterials.

Biomolecules play a deterministic role in HSC fate decision. Stem cell factor (SCF) is known to be actively involved in HSC survival and maintenance in the niche [38, 39]. While a critical component of HSC culture media, SCF remains active when substrate bound [5, 38-40]. Evidence from *in vivo* studies has implicated niche cells with membrane-bound SCF as being particularly significant in HSC lodging [39]; however it remains unclear whether that effect was due directly due to immobilized SCF or indirectly via other signaling mechanisms. Previous efforts have demonstrated the use of SCF-functionalized 2D substrates to promote expansion of hematopoietic progenitor cell lines [41]. Notably, *Cooper-White et al.*, expanded M-07e human myeloid leukemia cells using physisorbed SCF on tissue culture plastic [38]. Work by *West et al.*, used covalently immobilized SCF on RGD-functionalized PEG surfaces to expand an IL-3-dependent murine myeloid cell line (32D) [42]. While instructive, these efforts were proof-of-concept experiments on 2D substrates using immortalized cell lines, motivating work here that seeks to develop fully 3D biomaterials for selective culture of primary HSCs. Further, light-based immobilization methods have also been recently described for covalently immobilizing biomolecules within 3D biomaterial networks [43-45], suggesting potential to generate patterns

of immobilized biomolecules. However, prior to examining the effect of complex 3D patterns of SCF, here we first explored the effect of bulk immobilization of SCF within a 3D biomaterial on the quiescence vs. expansion of primary murine HSCs. Additionally, the bone marrow is a complex, heterogeneous tissue containing gradients in cellular, biomolecular, extracellular matrix, and mechanical properties [29, 46-48]. This suggests a need to create biomaterials able to mimic multiple distinct niches as well as the gradients linking them. Microfluidic mixing based approaches have previously been shown to generate protein gradients on two-dimensional (2D) substrates [34, 49-51]. Recently, our group described a microfluidic templating tool to create overlapping patterns of HSCs and niche cells across a single 3D hydrogel [34]. Therefore, we explored the use of this microfluidic templating approach to examine the effect of gradients of matrix-immobilized SCF on primary HSCs.

Here we describe an approach to covalently incorporate SCF within a methacrylamide-functionalized gelatin (GelMA) hydrogel for *in vitro* culture of primary murine HSCs. GelMA hydrogel can be UV-immobilized, contains natural ligands (Fn motif RGD), and retains MMP-sensitive degradation sites [52, 53]. We hypothesized that the methacrylamide groups used to crosslink the GelMA hydrogel were also potential sites for covalently incorporating SCF within the matrix. Further, we hypothesized that the mode of SCF presentation (soluble vs. matrix bound) would significantly affect the balance between HSC differentiation and expansion. The primary goal of this project is to demonstrate the selectivity of soluble vs. matrix-immobilized SCF on the proliferation and differentiation of primary murine HSCs. We also describe the use of microfluidic templating to create patterns of immobilized SCF across a single GelMA hydrogel to spatially control HSC response.

2. Materials and Methods

2.1 Synthesis of methacrylamide-functionalized gelatin macromer

Methacrylamide-functionalized gelatin was synthesized as described previously [54] using 10% (w/v) gelatin (Type A, 300 bloom from porcine skin) and 20% (v/v) methacrylic anhydride (MA) (Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS) (Gibco, Grand Island, NY). Following reaction, the GelMA was washed, dialyzed (12,000 – 14,000 M.W, Fisherbrand, Pittsburgh, PA), then lyophilized. The amount of MA added was chosen to create a GelMA macromer with 85% degree of MA functionalization, as previously verified via ^1H NMR [55].

2.2 Synthesis of photoinitiator

The lithium acylphosphinate (LAP) photoinitiator was synthesized as described by *Anseth et al.* [56]. Equimolar 2,4,6-trimethylbenzoyl chloride (Sigma Aldrich) was added to dimethyl phenylphosphonite (Sigma Aldrich) at room temperature and under argon. Fourfold excess lithium bromide in 2-butanone (Sigma–Aldrich) was added then heated to form a solid precipitate. The mixture was cooled to ambient temperature then filtered and washed with 2-butanone to remove unreacted lithium bromide. Excess solvent was removed by vacuum.

2.3 Pegylated stem cell factor (PEG-SCF)

3500 MW Acrylate PEG-NHS ester (JenKem Technology USA, Allen, TX) was reacted at room temperature with recombinant murine SCF (PeproTech, Rocky Hill, NJ) at a molar ratio of 24:1 (PEG-NHS: SCF) in PBS at pH 8.0 [57]. Unreacted PEG-NHS was removed using a 10K MW Pierce Concentrator PES (Thermo Scientific, Waltham, MA). The concentration of PEG-SCF

was determined via ELISA (R&D Systems, Minneapolis, MN). Conjugation was confirmed via Western blot using a 15% Tris-HCl precast polyacrylamide gel (primary: rabbit polyclonal SCF; secondary: goat polyclonal anti-rabbit IgG conjugated to horseradish peroxidase). Detection was via SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Scientific) imaged via ImageQuant LAS 4000 (General Electric). PEG-SCF was suspended in PBS (pH 7.4) with 0.1% BSA, aliquoted and stored at -80 °C for further use.

2.4 GelMA hydrogel synthesis

Hydrogels were generated from a solution of 5% (w/v) GelMA macromer and 0.1% (w/v) LAP photoinitiator in PBS. For PEG-SCF conjugated hydrogels, PEG-SCF was added to the pre-polymer solution at 100 or 400 ng/mL. Hydrogels were formed in an ABS plastic mold (7 mm dia. x 500 μ m thick well) custom printed in our lab (Replicator 2x, Makerbot, Brooklyn, NY). 19 μ L GelMA solution was pipetted into the mold sandwiched between glass slides then exposed to 10 mW/cm² UV light (365 nm) for 20 seconds [54]. Resulting hydrogels were removed from the mold and kept hydrated in PBS (pH 7.4) or StemSpan SFEM media (StemCell technologies, Vancouver, Canada) in a 5% CO₂ incubator at 37 °C for further culture and analysis.

2.5 Fluorescence imaging of PEG-SCF retention within the hydrogel

SCF retention was determined via fluorescent analysis of GelMA hydrogels containing either photopolymerizable PEG-SCF (100 ng/mL), non-functionalized soluble SCF (100 ng/mL) or no SCF (No SCF). The gels were placed in PBS in the incubator (37 °C, 5% CO₂) for 7 days. At days 2, 4 and 7, specimens were embedded in Tissue-Tek O.C.T. Compound (Sakura, Torrance, CA) and frozen (-80 °C). 20 μ m histology slices were cut using a cryostat (Leica CM3050 S) and

deposited onto SuperFrost Excell slides (Thermo Scientific, Waltham, MA). Sections were stained with Biotin-conjugated anti-murine SCF antibody (100 ng/mL; Peprotech, Rocky Hill, NJ) then AlexaFluor 633 conjugated Streptavidin (100 ng/mL; Life Technologies, Grand Island, NY). Sections were then imaged with the Zeiss LSM 710 confocal microscope.

2.6 Time-release profile of SCF from GelMA hydrogels

The elution rates of covalently immobilized (*Covalent*) vs. non-covalently entrapped (*Transient*) SCF from the GelMA hydrogel were determined over 7 days in PBS (37 °C, 5% CO₂ incubator). PBS was extracted after 12h as well as 2, 4, and 7 days, frozen at -80 °C until analysis, then quantified via ELISA (Mouse SCF DuoSet, RnD Systems). SCF retention in the gels was calculated from the initial loading as well as the amount leached into the PBS.

2.7 Primary murine cell isolation

Primary hematopoietic stem cells were isolated from the bone marrow of female C57BL/6 mice (Jackson Labs; Ages 1–3 months) as described previously [34]. All animal experiments were conducted with permission obtained from the University of Illinois Institutional Animal Care and Use Committee (IACUC), Protocols #12-033, 14-1580. Lineage negative marrow was enriched using the EasySep Mouse Hematopoietic Progenitor Cell Enrichment kit (StemCell Technologies, Vancouver, Canada). HSCs were subsequently isolated via FACS as the Lin⁻ Sca-1⁺ c-kit⁺ (LSK) sub-fraction using a cocktail of antibodies (eBioscience, San Diego, CA): PE-conjugated Sca-1 (1:100 dilution), APC-conjugated c-kit (1:100 dilution), and a 1:100 dilution of an FITC-conjugated Lineage (Lin) cocktail (CD5, B220, Mac-1, CD8a, Gr-1, Ter-119). LSK cells were sorted using a BD FACS Aria II flow cytometer (BD FACS Diva software).

2.8 SCF supplementation of LSK-laden hydrogels

Freshly isolated LSK cells were mixed with the GelMA pre-polymer solution at a density of 5×10^5 cells/mL, then photopolymerized as described previously. LSKs were encapsulated in one of four GelMA hydrogel variants (**Fig. 1**) then maintained for up to 1 week in culture in StemSpan SFEM media (StemCell Technologies) replaced every 2 days. **No SCF**: GelMA hydrogel alone; cultured in SCF-free media. **Transient SCF**: GelMA hydrogel fabricated with 100 ng/mL soluble (freely-diffusible) SCF; cultured in SCF-free media. **Continuous SCF**: GelMA hydrogel fabricated with 100 ng/mL soluble SCF; cultured in media containing 100 ng/mL SCF. **Covalent SCF**: GelMA hydrogel fabricated with photoimmobilized SCF (Covalent Lo: 100ng/mL; Covalent Hi: 400 ng/mL); cultured in SCF-free media.

2.9 Analysis of HSC bioactivity

Cell viability was tracked via a Live/Dead Cytotoxicity kit (Invitrogen Carlsbad, CA) [30]. HSC-seeded hydrogels were incubated in a 2 μ M Calcein AM and Ethidium homodimer-1 solution. Viability was determined at 12h post-fabrication as well as after 2, 4 and 7 days of culture using a Zeiss LSM 710 Multiphoton Confocal Microscope, with post-imaging analysis via ImageJ.

HSC phenotype was determined via surface antigen expression using a LSRII flow cytometer (BD Bioscience). Prior to analysis, gels were digested with 50 U/mL of Collagenase type 2 (Worthington Biochemicals, Lakewood, NJ). The cell solution was filtered (40 μ m nylon mesh; Fisher Scientific), spun down, then resuspended in PBS plus 5% fetal bovine serum (PBS/FBS) with 0.1% sodium azide, then stained for the following antibodies: APC-conjugated CD34 (1:20), PE-conjugated Sca-1 (1:100), APC-conjugated c-kit (1:100), FITC-conjugated lineage

cocktail (1:100). All antibodies were purchased from eBioscience (San Diego, CA). Microbeads (5 μ m, Spherotech, Lake Forest, IL) were added to each sample as a concentration standard. Data was analyzed using FCS Express 4.0 software [58]. The number of cells in each sample was calculated based on the microbead concentration using the “Concentration Calculator” function.

The functional capacity of cultured HSCs was assessed via the Colony-forming unit (CFU) assay using Methocult GF M3434 methylcellulose-based medium (StemCell Technologies). Cell-seeded hydrogels were digested as previously described. Recovered cells were resuspended in StemSpan SFEM media, aliquoted into Methocult media (total volume, 1.1 mL per sample), then cultured on gridded 35 mm culture dishes (StemCell Technologies) for 12 days (5% CO₂, 37°C). The number of resultant GEMM (Granulocyte-Erythrocyte-Megakaryocyte-Macrophage), GM (Granulocyte-Macrophage), G (Granulocyte), M (Macrophage), Mk (Megakaryocyte), and E (Erythrocyte) colonies were counted using an inverted microscope [59]. Results are reported as percentage of the total number of colonies per gel.

2.10 Design of a silicon (Si) master for integrated microfluidic mixer

We have previously described a 2-part microfluidic mixer and culture chamber system for creating spatially-graded hydrogels [34, 60, 61]. Here, the identical mixer geometry was used, but the mixer outlet was modified to both reduce the size of the final hydrogel construct (72 μ L from 180 μ L) and to integrate the mold for hydrogel gelation and microfluidic mixer into a single construct (**Supplemental Fig. 1A**). Fabrication of the silicon master based on this design followed previously described methods [34, 60, 61].

2.11 Fabrication of the integrated microfluidic mixer and cell culture device

Microfluidic devices were created from RTV (RTV 615 Part A/B, General Electric, Waterford, NY) from the Si master as described previously [60]. Access ports were punched into the device inlets using a 20-gauge blunt syringe needle. A blank Si wafer was used to create a cell culture media reservoir PDMS layer. The PDMS pieces were cleaned with tape (Scotch™ 3M), treated with oxygen plasma (Harrick Plasma, Ithaca, NY), then immersed in a 1% solution of (3-aminopropyl)-trimethoxysilane (APTMS; Sigma-Aldrich). A transparent polycarbonate membrane (5 µm pores; EMD Millipore, Billerica, MA) that acts as a porous barrier between the hydrogel and cell culture media PDMS layers was treated with oxygen plasma then immersed in a 1% solution of (3-Glycidoxypyl)-methyldiethoxysilane (GPTMS; Sigma-Aldrich). All components were washed with DI water and dried on a lint-free cloth. A 3-layer sandwich was created from the PDMS mixer layer, the polycarbonate membrane and the PDMS media well linked by spontaneous APTMS-GPTMS bonds (**Supplementary Fig. 1B**) [60].

2.12 Hydrogels containing gradients in SCF content

GelMA hydrogels containing a gradient in SCF were created by mixing two pre-polymer solutions through the microfluidic device (5% w/v GelMA; 5% w/v GelMA + 400 ng/mL PEG-SCF) using a computer-controlled syringe pump (Harvard Apparatus, Micro-Liter OEM syringe pump, Holliston, MA) [34], followed by UV exposure (365 nm UV light; 10 mW/cm²; 30 seconds) (3). Gradients of SCF within the matrix were quantified via fluorescence. Gels were stained with Biotin conjugated anti-murine SCF antibody (100 ng/mL, Peprotech, Rocky Hill, NJ) then Streptavidin-conjugated Fluorescein isothiocyanate (FITC; 100 ng/mL; Life Technologies, Grand Island, NY) at room temperature. Stained hydrogels were scanned using a Typhoon 9400 Image Scanner and quantified as mentioned previously [34].

HSC seeded GelMA hydrogels containing graded-SCF were generated by adding LSK cells (2.5×10^5 cells/mL) to the pre-polymer solution in both mixer inlets. Excess media was added into the media reservoir layer and the entire assembly maintained in a 5% CO₂ incubator at 37 °C. Cell bioactivity was assessed after 1 and 7 days in culture. The media reservoir and polycarbonate membrane were gently cut away. The hydrogel was removed from the PDMS device and subdivided into five equal regions across the gradient. Cell viability within each sub-region was measured using the Live/Dead Cytotoxicity kit, with total number of live vs. dead cells in each region evaluated via the Zeiss LSM 710 microscope.

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 time, SCF type (PEG-SCF, SCF) and concentration. SCF immobilization and release experiments used at least $n = 5$ constructs per group. Analysis of LSK cell bioactivity (viability, proliferation, surface antigen expression, CFU assay) used at least $n = 3$ 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. SCF is robustly tagged with PEG and retains its bioactivity

Western Blot analysis of unmodified SCF showed a discrete band at 18.3 kDa (the known size of SCF). Alternatively, PEG-modified SCF (PEG-SCF) showed no appreciable band at 18.3 kDa, but rather a broad smear at 40 – 60 kDa (**Fig. 2A**). This suggests both high efficiency of PEG

functionalization and the presence of multiple acrylated PEG linkers per SCF molecule, likely owing to the polydispersity of the PEG chains [42]. To demonstrate that PEG-functionalized SCF was still bioactive, HSC proliferation in liquid media culture was compared for media supplemented with unmodified SCF (*Soluble SCF*) alone vs. PEG-functionalized SCF (*PEG-SCF*) (**Fig. 2B**). HSCs cultured with *Soluble SCF* or *PEG-SCF* showed equivalent increases in proliferation after 48 hours. HSCs in media lacking SCF (*No SCF*) showed significantly reduced cell numbers, confirming the PEG-SCF molecule retained bioactivity.

3.2. PEG-modified SCF is retained in the GelMA hydrogel

Retention of SCF within the GelMA hydrogel was monitored via ELISA analysis of SCF eluted into the media (**Fig. 2C**) and fluorescence imaging of SCF retained within the matrix (**Fig. 2D**, **Supplementary Fig. 2**) for *Transient SCF* vs. *Covalent Lo* hydrogels. Rapid (<12 hours), significant elution of SCF into the media (~60% of initially incorporated SCF) was observed for Transient encapsulated SCF. Comparatively, greater than 80% of the PEG-SCF covalently incorporated within the matrix was retained for 7 days in culture. Immunofluorescence analysis of GelMA hydrogels at day 7 also demonstrate significant retention of SCF with Covalent incorporation compared to Transient encapsulation. Together, these demonstrate PEG-SCF remains bioactive and can be covalently incorporated and retained within the GelMA hydrogel for up to 7 days.

3.3. Mode of SCF presentation in the GelMA hydrogel significantly affects HSC response

HSC viability was significantly reduced as early as the second day in culture in GelMA hydrogels with either *No SCF* or *Transient SCF* (**Figs. 3, 4A**). Comparatively, HSCs maintained

in the *Continuous SCF* or *Covalent SCF* GelMA hydrogels demonstrated enhanced cell viability (**Figs. 3, 4B**). By day 7, *Continuous SCF* GelMA hydrogels showed significantly increased viability (**Fig. 4B**; $p < 0.001$) and cell numbers (calculated relative to the number of initially seeded HSCs; **Fig. 4C**; $p < 0.001$). While HSC viability and cell number increase with covalently immobilized SCF dose, the effects were not significant. We subsequently examined whether the mode of SCF presentation affected functional HSC phenotype via surface antigen expression and colony forming unit (CFU) assays. Given the poor viability of *No SCF* and *Transient SCF* conditions, functional phenotype were only determined for *Continuous SCF* and *Covalent SCF* conditions. Whereas *Continuous SCF* led to the greatest increase in overall number of cells after 7 days, this expansion came at a cost, with the resultant cell population showing significantly ($p < 0.001$) reduced maintenance of both the initial LSK phenotype and the more primitive CD34⁺LSK cell fraction (**Fig. 5**). In contrast, GelMA hydrogels containing *Covalent SCF* show significantly higher selectivity for maintaining both LSK and CD34⁺LSK populations. Additionally, while the fraction of LSK cells retained in GelMA hydrogels with *Continuous SCF* reduced significantly with time, it was maintained throughout the 7 day experiment in hydrogels containing *Covalent SCF* (**Fig. 5C**).

We subsequently determined the functional capacity of cells remaining in the GelMA hydrogels via CFU assay (**Fig. 6**). Results were consistent with findings from fold-expansion and surface antigen expression. Total number of CFU colonies, indicative of the proliferation capacity of the cells in culture, were significantly higher, and increased significantly with time ($p < 0.001$ at day 7), for *Continuous SCF* hydrogels relative to *Covalent SCF* conditions (**Fig. 6B**). GEMM colonies, indicative of selective maintenance of a more primitive HSC population, decreased

significantly with time for HSCs maintained in *Continuous (soluble) SCF* conditions. However, the fraction of GEMM colonies increased significantly for covalently-immobilized SCF (**Fig. 6C**). Further, GM, G and M, as well as MK and E colonies, all indicative of the presence of increasingly differentiated HSCs within the GelMA hydrogels showed less defined trends as a function of mode of SCF presentation. However, in many cases (*e.g.*, CFU-G, E), the number of differentiated colonies was increased with exposure to soluble SCF supplemented hydrogels (**Supplementary Fig. 3**).

3.4. Spatial-gradients in immobilized SCF induce gradated HSC response

Given the selective effect of immobilized SCF on HSC bioactivity, we used a previously described [34, 61] microfluidic platform to create HSC-seeded GelMA hydrogels containing a linear gradient of immobilized SCF (mixer inlets: 0, 400 ng/mL SCF; **Fig. 7A**). The mean fluorescence intensity of hydrogels immunofluorescently (FITC) labeled against SCF showed a linear gradient in local SCF content across the construct (**Fig. 7B**). HSC viability was found to be strongly linked to regional SCF content. While HSC viability in Region 1 (lowest SCF content) was non-significantly reduced after 1 day in culture, HSCs in all other regions showed overall high (50 – 60%) viability. After 7 days in culture a significant relationship was observed between local SCF content and maintenance of HSC viability (**Fig. 7C**). Linear increases in cell number with increasing local SCF content were also observed via local confocal analysis (**Supplementary Fig. 4**) as well as when quantifying overall number of viable cells per sub-region (**Fig. 7D**).

4. Discussion

The structural and biomolecular environment of the niches supporting HSCs within the bone marrow is complex [62-64]. The rarity of HSCs in the marrow as well as the dynamics associated with their behavior make studying HSC-niche interactions *in vivo* difficult. An artificial bone marrow would have significant clinical value for therapeutic expansion of HSCs as well as to facilitate the study of the etiology and treatment of hematologic diseases. Here a critical bottleneck remains identifying material systems able to selectively promote expansion of early HSC progenitor fractions while preventing subsequent lineage specification. Previous work in our lab has shown HSCs can be cultured in 3D hydrogel constructs [34] and that the dimensionality, stiffness, and ligand content of the biomaterial microenvironment significantly affect HSC morphology [30]. While stem cell factor (SCF) is a common media additive for HSC culture, this study examined whether the mode of SCF incorporation (traditional soluble vs. matrix-immobilized) within a model GelMA hydrogel selectively impacted HSC fate. We hypothesized covalent incorporation within the GelMA hydrogel would reduce diffusive loss during long-term culture as well as affect the balance between HSC differentiation and expansion.

SCF is a critical niche component responsible for maintaining a population of quiescent and activated HSCs [5, 15, 17]. *In vivo* SCF is presented to HSCs in both soluble and insoluble forms [38]. Multiple efforts aimed at expanding HSCs *ex vivo* have identified the potential for incorporating low doses of soluble SCF into cell culture media [65, 66]. However, the membrane-bound version of SCF is known to play a critical role in HSC mobilization, homing, and lodgment [39]. As such, recent efforts have begun to explore immobilized forms of SCF *in vitro*. *Cooper-White et al.* demonstrated SCF physisorbed onto 2D substrates showed

significantly enhanced bioactivity versus soluble SCF [38]. These observations have been extended to demonstrate improved expansion of embryonic stem cells and 32D hematopoietic cell lines on poly ethylene glycol (PEG) surfaces [42, 67], as well as promote differentiation of megakaryocytic lineages from CD34⁺ human hematopoietic stem and progenitor cells [68]. However, these efforts used 2D substrates and have not examined the effect of matrix-immobilized SCF on primary HSCs in fully 3D biomaterial microenvironments, critical steps needed for the development of HSC biomanufacturing applications.

Efforts within our group by *Pedron et al.* have previously demonstrated the use of the methacrylate-based photoimmobilization to selectively incorporate brain-mimetic hyaluronic acid within a GelMA hydrogel, but had not yet demonstrated the incorporation of a growth factor [61, 69]. Inspired by work by *West et al.* [57] using acrylate PEG-NHS esters, we generated an acrylated-SCF molecule which was able to retain its overall bioactivity and promoted equivalent expansion of HSCs compared with unmodified SCF (**Fig. 2**). The acrylated-SCF could be incorporated and retained in the GelMA hydrogel at significantly higher levels than SCF encapsulated during hydrogel gelation (*Transient SCF*), consistent with observations by *Zandstra et al.* [67]. The initial release (< 2 days) of SCF from the *Covalent SCF* hydrogel constructs (**Fig. 2C**) is likely due to acrylated-SCF not incorporated within the GelMA network during polymerization. While increasingly harsh photoimmobilization might yield better incorporation, the cytotoxicity of such protocols towards primary HSCs, already demonstrated for a range of stem cell populations [70], precludes such a modification. This finding highlights the significant difference between hematopoietic cell lines which are relatively insensitive to UV intensity, and primary HSCs which are extraordinarily sensitive. While acrylated-SCF retained

its bioactivity and could be covalently incorporated into the GelMA network, the bulky multi-PEG modifications to the SCF molecule likely affect the overall activity of the immobilized SCF [42]. Therefore, ongoing efforts are exploring the use of more selective and potentially orthogonal chemistries (*e.g.*, thiol-based) [71, 72] to increase the efficiency of biomolecule incorporation.

Nonetheless, we found SCF is a critical factor for *in vitro* culture of primary HSCs, consistent with previous findings regarding the design of soluble media formulations for hematopoietic cell expansion [65, 66, 68]. HSCs cultured in *No SCF* or *Transient SCF* showed rapid cell death (**Figs. 3, 4A**). Not surprisingly, for the *Covalent SCF* conditions, the higher dose of covalently incorporated SCF (400 ng/ml) led to increased viability and cell numbers, compared to the lower (100 ng/mL) dose, possibly due to increased availability of SCF in the matrix. The *Continuous SCF* showed significantly enhanced viability (**Figs. 3, 4B**) as well as expansion of the initial HSC population (**Fig. 4C**) compared to the *Covalent (Lo/Hi) SCF* conditions. However, it is important to note that this condition also required significantly greater total SCF (SCF added to the media bath for repeated media changes). On a per construct basis, *Continuous SCF* required 92 ng SCF over the full week of culture while *Covalent Lo* and *Hi* required 2 ng and 8 ng total SCF respectively. SCF is an expensive cytokine and the high cost associated with its continuous usage is detrimental from the perspective of a biomaterial platform for stem cell engineering. Further, observed differences between *Continuous SCF* and *Covalent SCF* may be influenced by differential proliferation rates; discerning greater mechanisms by which these different forms of SCF acts is the subject of ongoing efforts.

Inspired by previous efforts in the literature regarding the effect of the mode of SCF presentation (soluble, matrix/cell immobilized) on HSCs (*in vivo*) and hematopoietic cell lines (2D substrates, *in vitro*) [38, 39, 42, 65], we explored whether matrix-immobilized SCF was able to selectively promote better maintenance of hematopoietic progenitor cells in a fully-3D GelMA hydrogel. This effort was also inspired by a larger body of literature suggesting that biomolecular supplementation strategies that enhance (stem) cell expansion may do so at the expense of loss of desired phenotype [73-75]. A critical finding of this work is that while *Covalent SCF* immobilized within the GelMA hydrogel does not promote overall expansion of the total number of cells in the biomaterial, it is able to selectively maintain a more primitive fraction (CD34⁺ LSK; improved CFU-GEMM colonies) of hematopoietic stem and progenitor cells. (**Figs. 5, 6**) compared to the *Continuous SCF* condition. At first glance, it may likely be possible to elicit similar response using lower dosages of soluble SCF to promote reduced proliferation and increased maintenance; however, such a finding will not overcome the need for continuous media supplementation and concerns of insufficient biotransport through fully-3D culture environments. Further, it is likely that the soluble and matrix bound forms of SCF have distinct optimal concentrations to promote a desired HSC phenotype. Indeed, mechanisms via which the soluble and bound forms of cytokines interact with cells need not be similar. It is well-established that the c-kit receptor is able to internalize when conjugated with soluble SCF [76, 77]; however, *in vivo* studies suggest that membrane-bound SCF may be particularly important in niches that promote HSC maintenance and expansion [39]. While not clear whether the effect was due to the presence of membrane-bound SCF or due to other paracrine factors associated with HSC-niche cell interactions, results here suggest matrix-bound SCF may play a critical role in engineered bone marrow biomaterials. Lack of internalization of the c-kit/SCF complex in

case of the immobilized SCF may promote HSC fate specification via distinct signaling mechanisms. Similar results were observed by *Anderson et al.* [78] where internalization of VEGF was reduced in case of covalently bound VEGF and altered the downstream signaling profiles of the human umbilical vascular endothelial cells. Together, our results suggest it may likely be possible to co-optimize soluble and matrix-bound dosages of SCF to promote the expansion of the desired primitive HSC phenotype. While beyond the scope of this effort, ongoing work is examining relative activation of signaling pathways so as to identify optimal combinations of immobilized and soluble SCF. However, this future work does not take away from the critical finding here that matrix-immobilized SCF can be used within a fully-3D hydrogel to selectively promote maintenance of primary, marrow-isolated HSCs in the absence of other cytokines.

Anatomical gradients exist across the marrow, suggesting a need for biomaterials that replicate distinct both discrete niches and the gradations linking them. A variety of techniques have been described in the literature to create spatially-heterogeneous environments for HSC culture such as photolithography or microprinting [36, 79] as well as microfluidic patterning [49, 67] to create pattern or gradients of biomolecules on substrates. However, the majority of these techniques have limited capacity to generate biophysical and biochemical properties of fully 3D biomaterials. We previously described microfluidic templating tools to create 3D collagen and GelMA hydrogels containing overlapping patterns of cells and matrix proteins inspired by both the marrow and other spatially-graded tissues such as the tumor microenvironment [34, 61]. Using this approach we created stable gradients of immobilized SCF across GelMA hydrogels (**Fig. 7B**). A gradient in covalent SCF induced dose-dependent changes in HSC viability and cell

numbers over a period of 7 days (**Fig. 7C, D**). While the initial cell viability within the gradient construct is low (~50%), this effect is likely due to flow-induced shear stresses [34, 80], and future efforts are working to optimize flow conditions to improve initial cell viability. Given the wealth of literature associated with biomolecules to promote HSC lineage specification [38, 81, 82], the immobilization and patterning strategies described here therefore have the potential to be used in conjunction with soluble supplementation to promote both expansion and maintenance of primitive HSCs.

5. Conclusions

SCF is a critical biomolecular component of the HSC niche *in vivo* and culture media for *ex vivo* HSC expansion. Here, we demonstrated a methacrylate photochemistry based method to covalently immobilize SCF within a fully-3D GelMA hydrogel. We subsequently adapted a microfluidic approach to create patterns of immobilized SCF within these hydrogels. The mode of SCF incorporation (Soluble: transient, continuous; Covalent) within the hydrogel significantly affected HSC viability, expansion, and maintenance of primitive phenotype. Taken together, our results underlie two critical findings. Matrix immobilized SCF is more selective in its ability to maintain primitive HSC fractions within a fully-3D hydrogel biomaterial as compared to soluble SCF in the media, likely due to exclusion of soluble SCF-induced differentiation. Further, methods able to locally control the density of immobilized SCF may have particularly significant potential in biomaterial platforms for *ex vivo* expansion and maintenance of HSCs for a range of research and clinical applications.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure Captions

Figure 1. Schematic of GelMA hydrogel environment. HSCs were encapsulated within a GelMA hydrogel network (gray) and maintained for up to 7 days in culture in one of 4 biomolecular environments. *No SCF*: HSCs were cultured in GelMA hydrogels in SCF-free media. *Transient SCF*: HSCs were encapsulated in GelMA hydrogels that were fabricated with 100 ng/mL (soluble; freely-diffusible) SCF; constructs were subsequently cultured in SCF-free media resulting in diffusive loss of SCF over time. *Continuous SCF*: HSCs were encapsulated in GelMA hydrogels that were fabricated with 100 ng/mL (soluble; freely-diffusible) SCF; constructs were subsequently cultured in media also containing 100 ng/mL SCF. *Covalent SCF*: HSCs were encapsulated in GelMA hydrogels that had 100 (Lo) or 400 (Hi) ng/mL PEG-SCF added to the hydrogel precursor solution prior to photopolymerization leading to covalent immobilization; constructs were subsequently cultured in SCF-free media.

Figure 2. (A) Reaction schematic combining SCF with PEG-NHS ester to create a PEG-SCF complex. Western blot showing unmodified SCF and PEG-SCF complexes. (B) Fold-expansion of HSCs after 48 hours in liquid culture in either the absence of SCF (No SCF) or in the presence of unmodified (Soluble SCF) or PEG-functionalized (PEG-SCF) SCF at 100ng/mL. Results are normalized by the initial number of HSCs. (C) Retention of SCF within the GelMA hydrogel up to 7 days in culture. 100 ng/mL SCF (*Transient*) or SCF-PEG (*Covalent Lo*) was added to the GelMA suspension prior to photopolymerization. While matrix-immobilized SCF was retained within the matrix, rapid loss of SCF was observed for *Transient SCF* hydrogels. (D) Characteristic fluorescence image of histology sections taken of the GelMA hydrogel network after 7 days, showing SCF (AlexaFluor 633) retained within the GelMA network. *: $p < 0.05$.

Figure 3. Representative live/dead (green/red) confocal images of HSCs encapsulated in GelMA hydrogels as a function of time in culture (12 hours, 2 days, 4 days, 7 days) and mode of SCF presentation (No SCF; Transient SCF; Continuous SCF; Covalent Lo SCF; Covalent Hi SCF). Scale bar: 400 μ m.

Figure 4. Viability of HSCs encapsulated in GelMA hydrogels with (A) no (*No SCF*) or only initially available soluble SCF (*Transient SCF*) versus (B) continuously available soluble (*Continuous SCF*) or matrix immobilized (*Covalent*) SCF. Lack of sustained SCF exposure leads to a rapid decrease in cell viability within 2 days of culture, while constant availability of SCF induces significantly enhanced HSC viability through the 7 day culture period. (C) Number of HSCs normalized by the initial number of encapsulated HSCs. Increased proliferation is seen in the presence of continuous soluble doses of SCF (*Continuous SCF*) while matrix-immobilized SCF (*Covalent Lo/Hi*) induced stable maintenance of a population of cells. The greatest reduction of cell number was seen for *Transient SCF*. Viability and cell number increase with the total overall dose of SCF used per construct for the entire 7 day culture period (*Continuous SCF*: 92 ng/construct; *Covalent Hi*: 8 ng/construct; *Covalent Lo*: 2 ng/construct). ^: significant differences between days for the same group ($p < 0.05$ unless noted in text); *: significant differences between groups for the same day ($p < 0.05$ unless noted in text)

Figure 5. (A) Schematic of HSC differentiation hierarchy indicating Lin⁻Sca1⁺cKit⁺ (LSK) as well as more primitive CD34⁺LSK hematopoietic stem cell populations. (B) Fraction of primitive

CD34⁺LSK cells retained in GelMA constructs as a function culture time and mode of SCF presentation (*Continuous SCF* vs. *Covalent Lo/Hi SCF*). (C) Fraction of LSK cells retained in GelMA constructs as a function culture time and mode of SCF presentation (*Continuous SCF* vs. *Covalent Lo/Hi SCF*). Increased maintenance of both the LSK and CD34⁺LSK fractions is seen for GelMA constructs containing covalently-immobilized SCF. ^: significant differences between days for the same group ($p < 0.05$ unless noted in text); *: significant differences between groups for the same day ($p < 0.05$ unless noted in text).

Figure 6. (A) Schematic of HSC differentiation hierarchy depicting the cells responsible for the formation of hematopoietic cell colony forming units (CFU-GEMM, CFU-GM, CFU-G, CFU-M, CFU-Mk, CFU-E). (B) The total number of colonies formed by cells recovered after culture for 2, 4, or 7 days in GelMA hydrogels, indicative of overall expansion of the hematopoietic progenitor cell population. CFU-Total increases with the total overall dose of SCF used per construct over the entire 7 day culture period. (C) The total fraction of GEMM colonies formed by cells recovered after culture for 2, 4, or 7 days in GelMA hydrogels, indicative of improved maintenance of a more primitive hematopoietic progenitor population. CFU-GEMM fraction is significantly higher in GelMA hydrogels containing matrix-immobilized rather than soluble SCF. ^: significant differences between days for the same group ($p < 0.05$ unless noted in text); *: significant differences between groups for the same day ($p < 0.05$ unless noted in text)

Figure 7. (A) Schematic of integrated microfluidic device containing 2 inlets (left), a microfluidic mixing zone (middle), and the integrated chamber for hydrogel formation and subsequent culture (right). (B) Linear opposing gradient of fluorescently labeled SCF across a GelMA hydrogel (Inlet 1: GelMA precursor suspension + 0 SCF; Inlet 2: GelMA precursor suspension + 400 ng/mL SCF). Results are reported as mean fluorescent intensity of SCF signal for five equally-sized regions across the hydrogel (inset: representative image of whole GelMA construct). (C) Viability of HSCs encapsulated in GelMA hydrogels containing gradient of SCF after 1 and 7 days in culture. HSC viability is reduced as early as 1 day for regions of the hydrogel containing the least matrix-immobilized SCF. A gradient in HSC viability is observed after 7 days across the GelMA hydrogels, with HSC viability remaining high in regions of the constructs containing the greatest matrix-immobilized SCF. (D) Fold-expansion of HSCs encapsulated in GelMA hydrogels containing gradients of SCF after 1 and 7 days in culture (results normalized to region 1). A gradient in HSC expansion is observed across the GelMA hydrogels, with highest expansion observed for regions containing the greatest matrix-immobilized SCF. #: significant differences between days for the same group (#: $p < 0.05$; ##: $p < 0.01$). *: significant differences between groups on the same day (*: $p < 0.05$; **: $p < 0.01$).