

Developing an Optimal Biofunctional Scaffold for Hematopoietic Stem Cell Quiescent Maintenance and Expansion

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Hematopoietic stem cells (HSCs), characterized by their CD34 glycoprotein expression, can be extensively exploited in a variety of clinical applications to treat bone-marrow related disorders and cancers, which affect hundreds of thousands worldwide. HSCs are known to efficiently self-renew and maintain their quiescent state in their *in vivo* microenvironment, but rapidly lose their multipotency *in vitro* due to quick onsets of differentiation. Shortages of available donor cells have led to scientific interest in developing biofunctional scaffolds – such as cross-linked polymer hydrogels – that mimic the natural stem cell niche. Here we show that a firm, gelatin-based hydrogel cross-linked by microbial transglutaminase (mTG) (in a ratio of 1:25 mTG to gelatin) is ideal for quiescent self-renewal, and that the purine derivative, Stemregenin 1 (SR1), aids in directing cell migration, proliferation, and stemness. The 1:25 ratio with exposure to SR1 yielded a promisingly high stemness level of 94.53%. Our results demonstrate the previously undocumented effectiveness of gelatin hydrogels as biomimetic scaffolds suitable for HSC expansion. Furthermore, our findings and the culture system we have developed are expected to facilitate bone marrow disease treatment by providing large quantities of quiescent HSCs for medical applications and potentially diminishing the high demand for marrow donors.

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INTRODUCTION

Serious bone marrow disorders such as leukemia, lymphoma, aplastic anemia, and multiple myeloma affect over 100,000 Americans each year, and leukemia alone kills half of all adults and one-fifth of all children diagnosed.¹ Conventional treatments primarily involve clinical transplantation of hematopoietic stem cells (HSCs). These stem cells, which are mobilized from donor marrow into the peripheral blood with hematopoietic cytokines,² may be extracted and are able to reconstitute a patient's entire hematopoietic system.³ However, a shortage of donor cells limits therapeutic applications.

Like all multipotent stem cells, HSCs – characterized by their CD34 surface glycoprotein expression – can both proliferate to increase stem cell population and differentiate to replenish all blood cell types.³ This is accomplished through the maintenance of long term quiescent true stem cells (capable of extensive self-renewal) and the expansion of lineage specific hematopoietic progenitor cells (capable of proliferating rapidly but limited in their capacity to

differentiate into more than one mature blood cell type).⁴ As of now, it is known that HSCs can generate a large number of daughter cells *in vivo* and efficiently self-renew in their natural microenvironment, but this phenomenon is significantly diminished when proliferation is attempted *in vitro* due to a rapid loss of multipotency and quick onset of differentiation.⁵ The inability to regulate differentiation and the current lack of donor cells have led to scientific interest in maintaining HSC properties of self-renewal in culture for medical applications.

Previous studies have shown that the specific stem cell microenvironment itself, or niche, plays a critical role in the maintenance of stem cell function.^{6,7,8} The HSCs reside in specialized bone marrow (BM) niches,^{4,9} where they are maintained in a quiescent state by interaction with the extracellular matrix, as well as by various cytokines, growth factors, and other mediators comprising the BM niche.^{10,11} In addition, a recent study has shown that the exogenous purine derivative, Stemregenin 1 (SR1), antagonizes the aryl hydrocarbon receptor and can result in a significant increase in cells expressing CD34.¹² However, the understanding of the niche components and their roles in regulating the balance between proliferation and differentiation still remain

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generally uncertain and debatable.⁸ Thus, it becomes necessary to develop a culture system that closely parallels marrow physiology in order to both study the niche-mediated orchestration of HSC fate and develop a model to maximize stemness, i.e. maintaining the stem cell characteristics present before differentiation occurs.

Hydrogels (as artificial scaffolds for cell culture) are highly water-swollen networks consisting of cross-linked hydrophilic polymers; their 3D insoluble structure, ability to swell, and high water content make them ideal for biomedical applications.¹³ Hydrogels closely resemble soft tissue and generally exhibit good biocompatibility and permeability for oxygen and nutrients. These biofunctionalized surrogate niches have been used to assess the effects characteristic of the *in vivo* microenvironment and mimic the spongy architecture of the marrow from which HSCs are derived.¹⁴ In this study, we used gelatin hydrogels cross-linked by the enzyme microbial transglutaminase (mTG), with a plastic petri dish and polymer thin film as controls. We hypothesized that the previously undocumented biomimetic gelatin hydrogels would be optimal for culture conditions; our results supported our hypothesis and additionally indicated that SR1 not only induced proliferation,¹² but also promoted high stemness and quiescence levels, which had not been demonstrated thus far.

METHODS

Hydrogels and Viscoelastic Characterization

We first tested the properties of various gelatin based hydrogels cross-linked by microbial transglutaminase (mTG) – an enzyme which catalyzes the formation of polymer bonds – to examine optimal surfaces. Gelatin from porcine skin (type A) was acquired from Sigma-Aldrich (St. Louis, MO, USA), and ACTIVA mTG was acquired from Ajinomoto (Fort Lee, NJ, USA). Gelatin and mTG were both prepared in 10% solutions with Gibco Dulbecco's Phosphate Buffered Saline (DPBS), obtained from Life Technologies (Carlsbad, CA, USA).

Three milliliter hydrogels were prepared in ratios of 1:3, 1:5, 1:25, 1:125, 1:150, 1:250, and 1:300 of mTG solution volume to gelatin solution volume (six samples were prepared for each individual ratio). Gelatin and mTG solutions were mixed via pipetting in a Falcon 15 mL Conical Centrifuge Tube before being transferred to petri dishes and allowed to incubate for 24 hours in a Napco CO₂ 6000 incubator at 37 °C and 5% CO₂.

Frequency and amplitude sweeps through rheology analysis were performed with a Bohlin Gemini HRnano Rotational Drive 2 by Malvern Instruments. Oscillation test parameters were set with ETO temperature control of 37 °C, Amplitude Sweep and Stress Control, a frequency of 1 Hertz, a minimum stress of 1 Pa and maximum stress of 5000 Pa, and 25 sample points, each 6 seconds (4 second integration time, 2 second delay time), of increasing stress. We adjusted the gap size of each sample to ensure three bars of pressure before running the oscillation tests. This was done to

guarantee consistency between different samples during the frequency and amplitude sweeps. The sterile 1.5 mL hydrogels for cell culture, using a sterilized Purifier Class II Biosafety Cabinet from Labconco. Gelatin and mTG solutions were pressed through a BD non-pyrogenic Sterile-R filter with a BD 5mL syringe with a Luer-Lok tip. Gelatin and mTG were mixed, transferred to cell culture wells, and incubated for 24 hours in the same conditions mentioned above. Following incubation, hydrogels were heated to 65 °C for 7.5 minutes in a VWR Incubating Waver. This process does not affect mTG which has already been cross-linked and only anneals the proteins of excess mTG so that it does not cross-link the HSCs.

A spincoated poly(methyl methacrylate) (PMMA) thin film was also created as a negative control; we wanted to verify that biomimetic hydrogel surfaces would prove more compatible to cell growth and stemness than a hard, thin film surface. A 20 mL vial was prepared with 150 mg of PMMA (Sigma-Aldrich) and 5 mL of toluene solvent (Sigma-Aldrich) for a concentration of 30 mg/mL. Vials were sealed with parafilm and left overnight for the PMMA to effectively dissolve. Glass cover slips were covered with a layer of solution (approximately 4 drops from a disposable glass pipette) and spincoated at a rate of 2500 rpm for thirty seconds, using a PWM32 spincoater from Headway Research. As the cover slips are spun, the toluene evaporates and leaves only a PMMA thin film on the surface. The glass cover slips were then placed in the culture wells.

Two samples for each various substrate used – the 1:25 mTG to gelatin ratio, 1:300 mTG to gelatin ratio, PMMA thin film, and plastic culture well – were prepared, for a total of eight test scaffolds. Substrates were prepared for cell incubation through the addition of 2 mL of a mixture of cell media from StemSpan nourished by fetal bovine serum (FBS) in a ratio of 9:1 cell media to FBS. The cytokines thrombopoietin, stem cell factor, and fms-related tyrosine kinase 3 ligand – all three of which known for their ability to maintain effect quiescent HSC proliferation^{10,11,12,15} – were added in 1:1000 ratios each (2 µL each). (+) 10,000 units/mL Penicillin and (+)10,000 µg/mL Streptomycin were added through gibco Pen Strep by Life Technologies. All substrates were incubated overnight in a VWR Symphony Incubator at 37 °C and 5% CO₂.

CD34+ hematopoietic stem cells obtained from AllCell LLC (Alameda, CA, USA) were washed with media and centrifuged for 6 minutes at 300xg speed in a Thermoscientific Sorvall ST16R centrifuge. 500 µL of cells, additional medium, and additional cytokines (still each in a 1:1000 ratio) were plated onto every sample. SR1 was diluted in a 1:10 ratio of SR1 to DPBS, and 5 µL of the diluted solution was added to one sample of each substrate. HSCs were incubated for five days in a VWR Symphony Incubator with the same conditions above. Cells were briefly removed from incubation to monitor cell velocity; pictures were taken with a Nikon camera connected to an Olympus CKX48 inverted microscope.

Examination of HSCs and Flow Cytometry Labeling

Following the 5 day incubation, cells were mixed in the culture wells via pipetting and counted with a hemocytometer and Olympus CKX48 inverted microscope using standard procedure.¹⁶ Two counts were performed for each test sample. A Nikon camera connected to the microscope was used to take pictures of the cell culture wells to examine the cell migration.

HSCs that were not cultured for the incubation were labeled as input for the flow cytometry. 10 μ L of BD Pharmingen PE Mouse Anti-Human CD38 antibodies, 10 μ L of BD Pharmingen APC Mouse Anti-Human CD34 antibodies, and 100 μ L of FACs buffer (used to prevent non-specific binding) were added to the cells, mixed, stored in the dark at 4 °C (to incubate and prevent death of the antibodies), centrifuged, and fixed with formaldehyde. The protocol was repeated for the cells on each sample as well as in creating an unlabeled sample.

During the flow cytometry, the unlabeled sample was run first to effectively gate the cells by readjusting the axes. The input was run next to determine the original stemness of the HSCs, and this was followed by the remaining samples.

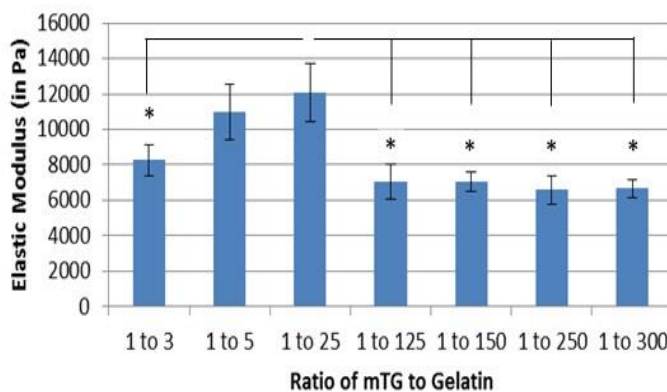


Figure 1. Elastic moduli of various hydrogel ratios. Bars in the graph represent the average elastic moduli of each ratio run at 37 °C. Error bars represent the standard deviation resulting from the multiple trials (n=6). The elastic modulus in the 1:25 ratio was statistically significant ($p < 0.05$) to those in all other ratios except for the 1:5, which was significant to none. The 1:3, 1:125, 1:150, 1:250, and 1:300 were all only significant to the 1:25.

RESULTS

Since the variation of substrates was thought to affect HSC behavior and growth, scaffolds were examined for their individual properties (through rheology analysis with a Bohlin rheometer) before tested in cell culture. Scaffolds were examined again after HSCs were cultured to monitor the cell migration patterns, the cell count and proliferation, and the level of stemness maintained.

Properties of Various hydrogels were prepared in the ratios of 1:3, 1:5, 1:25, 1:125, 1:150, 1:250, and 1:300 of microbial transglutaminase (mTG) to gelatin (see Methods and Materials). Inspection of hydrogels with amplitude and frequency sweeps through rheology analysis indicated a

correlation between the presence of mTG and the corresponding stiffness (**Figure 1**). As expected, the elastic moduli (the resistance to being deformed elastically) of ratios with greater amounts of mTG - i.e. 1:3, 1:5, and 1:25 - remained generally high (stiffer), while the elastic moduli of ratios with lower amounts of mTG - i.e. 1:125, 1:150, 1:250, and 1:300 - remained noticeably lower (softer). The greater presence of the mTG enzyme led to higher amounts of cross-linking, tightening the bonds within the gelatin and causing the formation of a much firmer substance. However, the data were not linked exactly to only the concentration of mTG to gelatin; the 1:3 yielded a lower modulus (an average of 8250 Pa) than both the 1:5 and the 1:25 (with averages of 11000 and 12100, respectively), and we attributed this deviation from our expectations to the excessive presence of mTG. After all bonds had been cross-linked, the presence of mTG had a counterproductive effect, weakening the hydrogels by lending them instability.

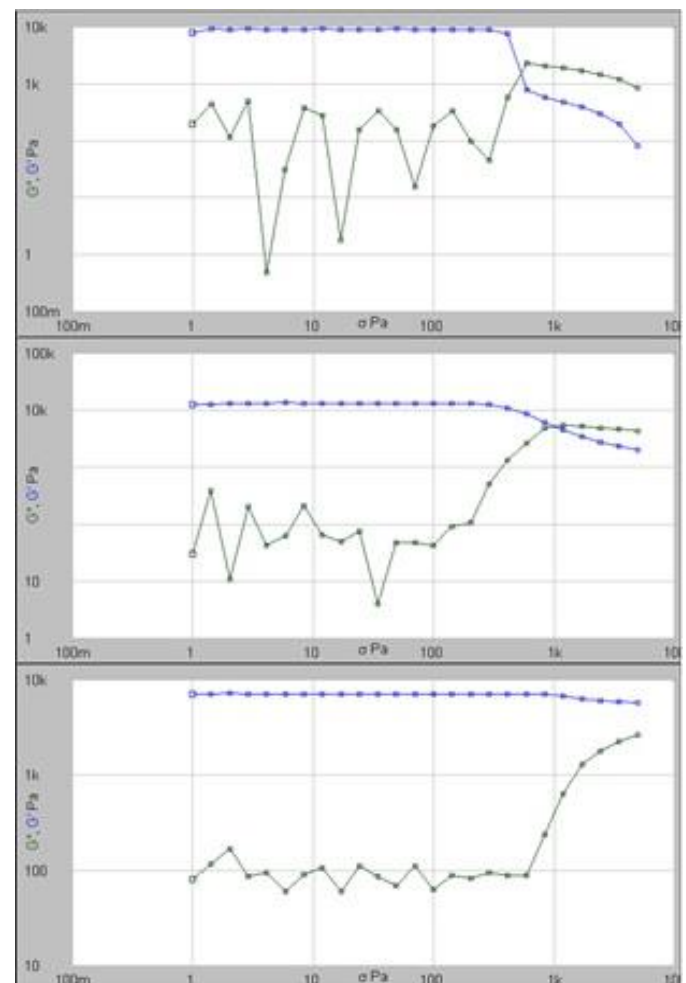


Figure 2. Sample rheology data from 1:3 (top), 1:25 (middle), and 1:300 (bottom). Greater concentrations of mTG led to greater fluctuation between points (in both the elastic and viscous moduli) and sharp declines of the elastic moduli. Fewer concentrations of mTG led to a smoother, linear plateau and very gradual decline of the elastic modulus. The blue line is the elastic modulus while the green is the viscous. The y-axis is the moduli in Pascals; the x-axis is the shear stress in Pascals.

In addition to the elastic modulus and the corresponding stiffness of the gels, it is also critical to determine other properties derived from the varying ratios of mTG to gelatin. In the rheology data and graphs for each individual sample (which characterized viscoelasticity, i.e. the elastic and viscous moduli), there were different patterns according to the ratio of mTG to gelatin as to how quickly the hydrogel would collapse from shear stress and how sharp the decline would be. **Figure 2** shows three sample graphs - 1:3, 1:25, and 1:300 from the rheology data. The shear stress at which the elastic and viscous moduli cross typically reflects the point at which the gel loses its properties and becomes more liquid than solid, although the point at which the elastic modulus declines is when the gel begins breaking down. The 1:3 ratio (top panel) revealed much more fluctuation between sample points and a sharp decline of the elastic modulus (blue), which quickly crossed the viscous modulus (green) at a shear stress generally between 417.0 and 594.6 Pascals. The 1:25 ratio (middle panel) with a higher elastic modulus, was more stable with a smoother curve and a much more gradual decline in the elastic modulus, crossing the viscous modulus at a shear stress generally between 847.9 and 1209.0 Pascals. The 1:300 ratio (bottom panel) showed the flattest graph with the slowest and least noticeable decline, with the elastic and viscous moduli not crossing in the shear stress range tested.

Once the HSCs are plated, it becomes necessary to find proper hard and soft gels with the greatest consistency for optimal cell behavior. The elastic modulus in the 1:25 ratio was statistically significant ($p < 0.05$) compared to the 1:125, 1:150, 1:250, and 1:300 ratios, which were all statistically insignificant compared to each other ($p > 0.05$). For the hard hydrogel, we chose the 1:25 ratio due to its significantly higher elastic modulus and its stability in response to rheometer shear stress (providing a more uniform surface and a smoother decline of its elastic modulus at a high shear stress than its over-crosslinked counterparts, the 1:3 and 1:5 ratios). For the soft hydrogel, we chose the 1:300 due to its lowest elastic modulus (thus its softest, rubbery properties) and its flattest graph curve (lending it more consistency). Because the 1:300 ratio was not statistically significant when compared to ratios other than the 1:25, it was chosen as representative of soft hydrogels in general; the cell data for the 1:300 will likely be similar to those in the 1:125, 1:150, and 1:250 ratios, as well as in other similarly soft hydrogel substrates.

Cell Velocity and Migration

After rheology inspection was complete, CD34+ HSCs were plated on four different test substrates (a 1:25 mTG to gelatin hydrogel, a 1:300 mTG to gelatin hydrogel, a poly(methyl methacrylate) (PMMA) thin film, and a plastic petri dish) for a 5-day incubation experiment. For each test substrate condition, two physical scaffolds were created – one with the presence of SR1, and one without – for a total of 8 conditions.

Regarding cell velocity, **Figure 3** shows the resulting speeds, in $\mu\text{m/hr}$, of the HSCs cultured on the two hydrogels and the

petri dish. The quickest velocities were observed on the 1:25 hard hydrogel at $287.17 \mu\text{m/hr}$ and the slowest on the control petri dish at $140.57 \mu\text{m/hr}$. Both the velocities observed on the 1:25 and 1:300 were statistically significant compared to the velocity on the control ($p < 0.01$ and $p < 0.05$, respectively), as well as statistically significant compared to each other ($p < 0.01$). Cell velocity may be an indicator of cell adhesion, which is important for biochemical and mechanical cues in regulating cell behavior (see Discussion).

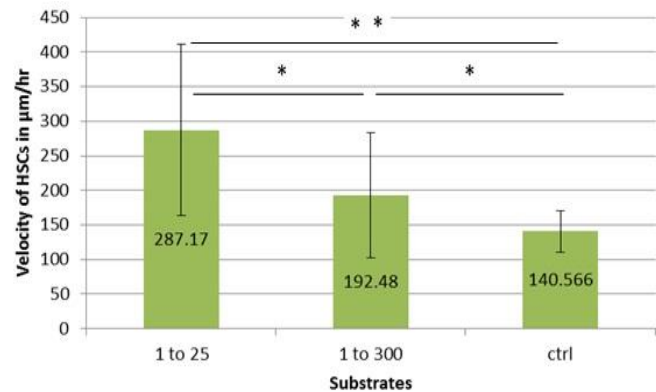


Figure 3. Effect of various substrates on cell migration velocities. Stiff hydrogels appeared to have the greatest migration speeds, followed by soft hydrogels. The slowest were observed on the control plastic petri dish. Error bars represent the standard deviation ($n=20$). The velocity of migration on the 1:25 ratio was statistically significant compared to the velocity on both the 1:300 ratio and control ($p < 0.01$); the velocities on the 1:300 and controls were also statistically significant ($p < 0.05$) compared to each other.

Regarding cell migration, the microscopy images of the plastic petri dish and PMMA samples, both with and without SR1, display little to no particular cell migration patterns. In contrast, HSCs seeded on the hydrogels moved and clustered according to the particular ratio, and these patterns were both amplified upon exposure to SR1 (**Figure 4**).

More specifically, the HSCs in the 1:25 (-) (i.e. the 1:25 ratio without SR1) generally migrated to the center in an attempt to form one primary cluster, although not well defined. In the 1:300 (-), the general migration pattern was distinguished by a large block of cells surrounded by individual, smaller clusters. Interestingly, with the presence of SR1, the 1:25 (+) formed two distinct clusters in the center and upper left corner, both of which were better defined than the 1:25 (-). The 1:300 (+) also seemed to have better results as the cells had formed only one large aggregation, with a very smooth edge and very few cells lying outside of the cluster. The degree of order was higher on soft matrices than on hard ones, and higher on substrates with SR1 than on substrate without.

Based on this data, we can draw three tentative conclusions. 1) Maximal HSC migration speeds occur on intermediately firm surfaces; 2) HSCs have a natural tendency to migrate towards the center of hydrogels and form larger, denser, and better defined clusters on softer surfaces; 3) SR1 appears to direct cell migration and result in closer cell to cell interactions, evidenced by clearer clusters on hydrogel (+) samples.

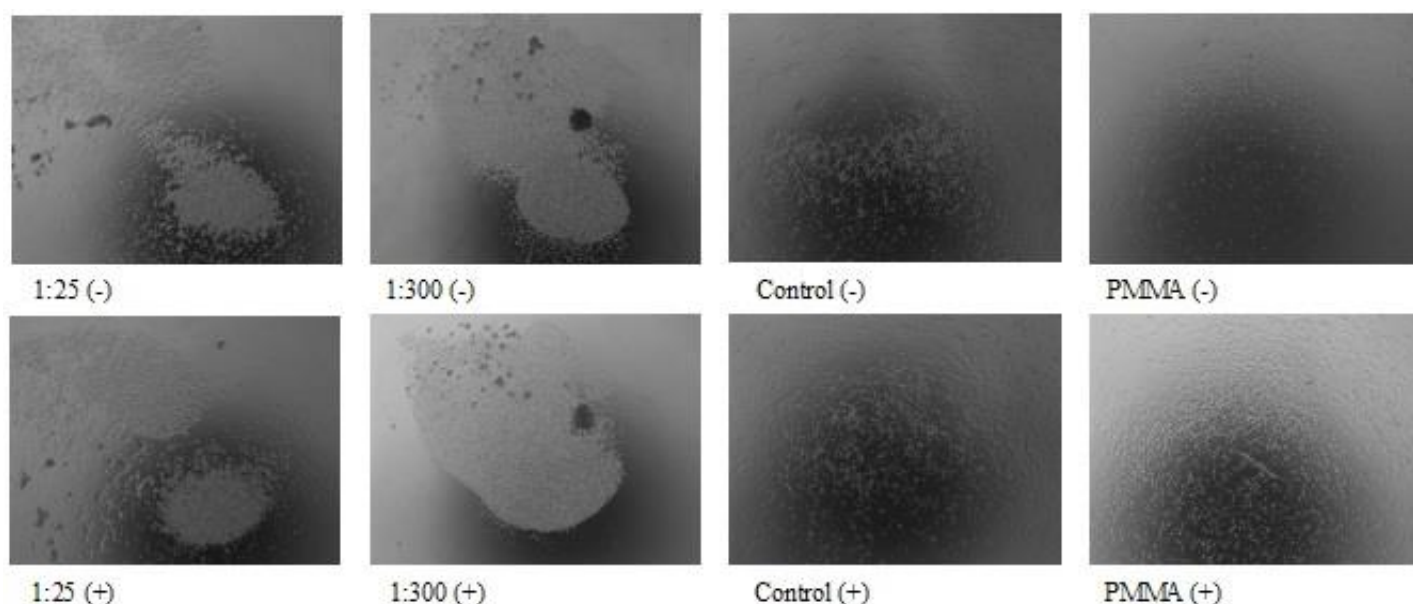


Figure 4. Microscope pictures of Day 5 HSCs cultured on test substrates with and without SR1. The scaffolds are as follows: from left to right, 1:25 mTG to gelatin, 1:300 mTG to gelatin, the plastic petri dish, and the PMMA thin film. The top row are all samples without SR1; the bottom row are all samples with SR1. We observed that the degree of order and clustering was higher on soft matrices than on hard ones, and higher on substrates with SR1 than on substrates without.

Cell Count and Proliferation

Following the incubation period and observation of cell migration, cells were lifted and two 10 μ L samples of cells and cell medium were transferred from each individual culture well to a hemocytometer via a micropipette (providing two readings of cell count for each test scaffold). The data are displayed in **Figure 5**. Our data indicate that SR1 has a generally positive effect on proliferation - as reported by an earlier study¹² - in all substrates except the 1:300 ratio. However, the results also show that there was no statistical significant difference ($p > 0.05$) between the PMMA (+) and the PMMA (-), or between the 1:300 (+) and the 1:300 (-). Thus, the only notable proliferation increase in CD34 expressing HSCs occurred in the 1:25 ratio and the control plastic petri dish, which is encouraging because these two substrates also yielded nearly 100% stemness with exposure to SR1 (see data in next section).

Flow Cytometry and Stemness

Flow cytometry was used to determine the percentage of stemness maintained by tracking CD34 expressing and CD34/CD38 double expressing HSCs tagged with fluorescent antibodies, and then comparing the numbers to the overall population of cells, which included cells without the CD34 glycoprotein (either differentiated or dead). We first ran an unlabeled sample to gate the HSCs before proceeding to run the labeled input and then all test scaffolds.

We examined the data in the UR (upper right - where the CD34+/CD38+ cells are pictured) and the LR (lower right - where the CD34+ cells are pictured) corners under the “% Gated” column. To obtain total percentage of stemness, we added together the values from the UR and LR. **Figure 6** shows the results for each individual test sample.

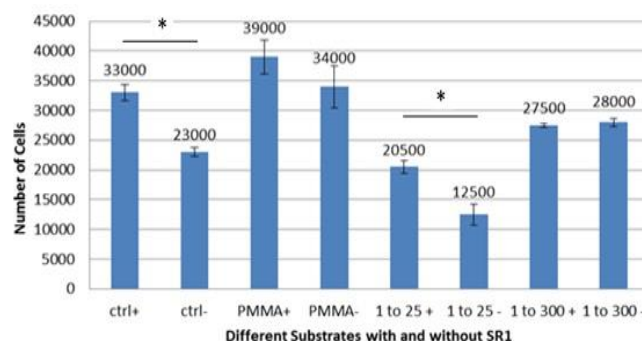


Figure 5. Cell count on various substrates. Bars represent the counts of HSCs seeded on different test scaffolds conditions after a 5 day incubation. Error bars represent the standard deviation obtained ($n=2$). The presence of SR1 induced greater proliferation in all substrates except for the 1:300. After a t-test was performed, the only significant difference in cell counts between (+) substrates and (-) substrates was observed in the 1:25 ratio and the control plastic surface ($p < 0.05$).

Scaffolds with SR1 showed a notably higher level of stemness than their no-SR1 counterparts in all substrates. Among samples without SR1, the 1:25(-) ratio yielded the peak percentage (45.22%), while the 1:300 (-) ratio yielded the lowest percentage (32.51%). Meanwhile, among samples with SR1, the 1:25 (+) ratio yielded the peak percentage (60.98%), while the PMMA thin film yielded the lowest percentage (41.62%). Surprisingly, we observed that the control plastic petri dish produced a high stemness in the presence of SR1, with a total percentage of 60.42%. The 1:25 ratio ended up as the best substrate in terms of overall stemness maintained, both with and without SR1. These data reflect well on both the overall effectiveness of SR1 in promoting stemness and the usefulness of firm hydrogel scaffolds to induce high stemness and quiescence levels.

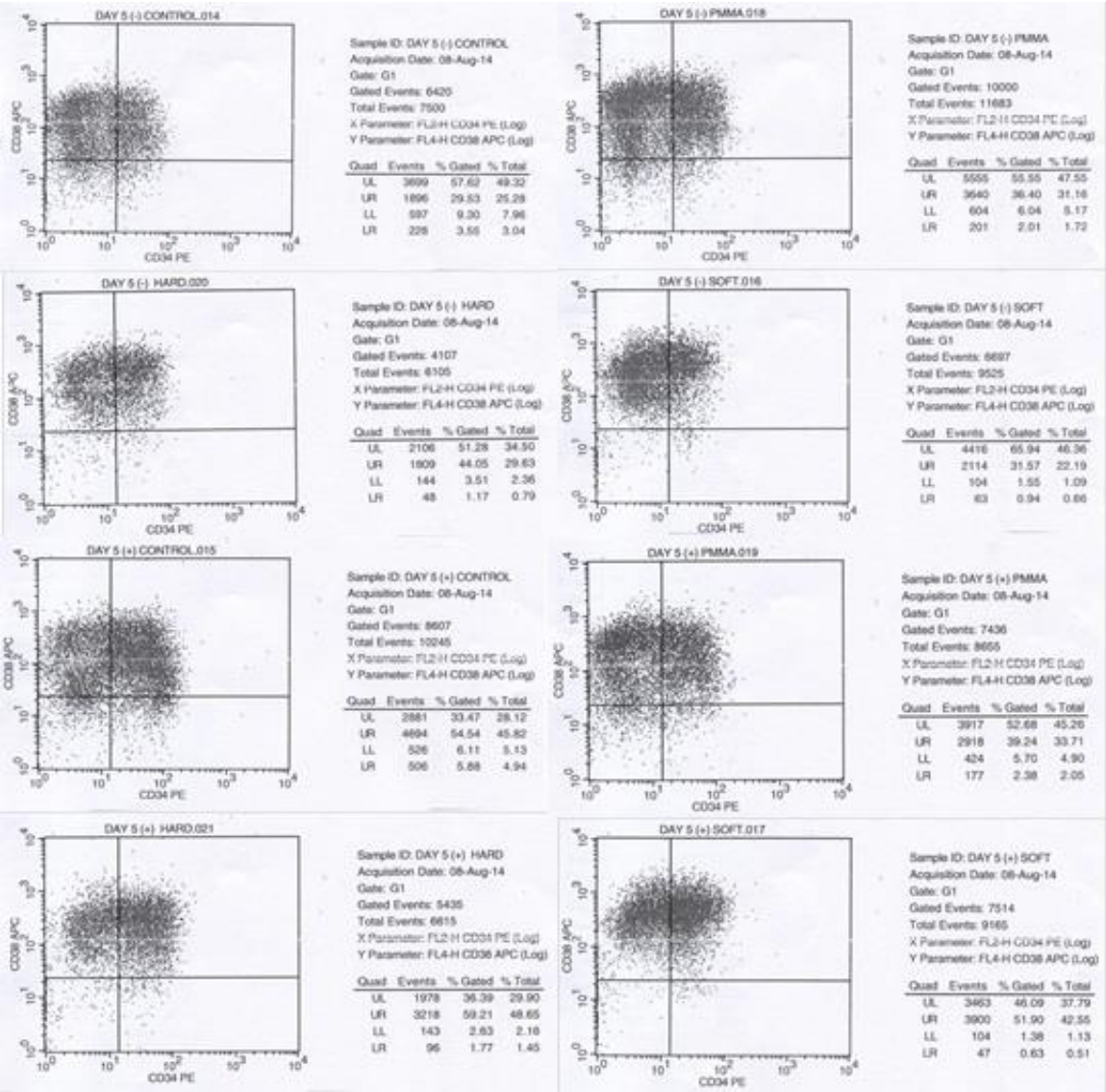


Figure 6. Flow cytometry of all eight test scaffolds. The data presented reveal substrate and SR1 effects on stemness. The lower right (LR) indicates the relative percent of CD34+ HSCs, and the upper right (UR) indicates the relative percent of CD34+/CD38+ HSCs. We used the percentages under the column “% Gated.” Scaffolds with SR1 resulted in higher levels of stemness throughout all substrates, most notably in the control plastic petri dish and the 1:300 ratio. The 1:25(+) and control (+) yielded the best results; the 1:300 (-) and control (-) yielded the worst results.

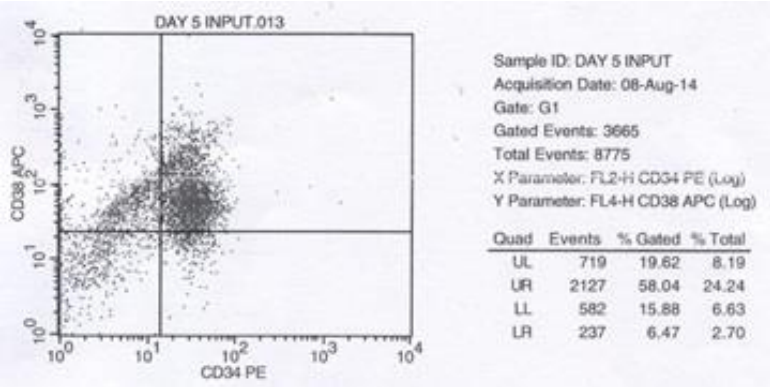


Figure 7. Flow cytometry of the input. Through examination of the upper right (UR) and lower right (LR) corners under the “% Gated” column, we observed that only 64.51% of the original HSCs were true stem cells.

It is also necessary, however, to set the percentage of stemness maintained relative to the input, which shows that only 64.51% of the original cells were true stem cells (**Figure 7**). Thus, we adjust the percentages of each test scaffold relative to the input and find that the 1:25 (+) and control (+) resulted in 94.53% and 93.66% stemness, respectively (**Figure 8**). However, the high proliferation rates observed in the control (+) are not indicative of quiescent self-renewal, the most desirable feature in our study. Therefore, although appearing to yield good results, the control petri dish may not be an ideal substrate (see Discussion). Meanwhile, the consistency of the 1:25 ratios as the best scaffolds supports our hypothesis of gelatin-hydrogels as good artificial niches.

DISCUSSION AND CONCLUSION

This study sought to develop a biocompatible scaffold that could closely imitate the bone marrow niche and extracellular matrix (ECM) to increase the population of quiescent HSCs and thereby increase their potency and application possibilities. We found that: 1) the degree of cell clustering is proportional to the softness of the scaffold; 2) SR1 directs cell migration and enhances HSC proliferation in all scaffolds; and 3) SR1 promotes stemness with the best results found in the 1:25(+).

Hydrogels: Cell Migration and Adhesion

We observed in our cell velocity data that HSCs moved at varying velocities according to the scaffold upon which they were plated (see **Figure 3**), with the hard hydrogel witnessing the fastest speeds, followed by the soft and then by the plastic control. We attribute these data to the dissimilar properties and varying elastic moduli of each scaffold, resulting in different cell-substrate adhesivity that affects cell migration. The differences in velocity are likely caused by the attachment strength of cells in a biphasic manner, with maximal migration occurring at an intermediate level of adhesiveness¹⁷ - i.e. the 1:25 hydrogel. HSCs seeded on strongly adhesive surfaces (control plastic) attach and remain immobilized, making cell substratum detachment difficult. HSCs seeded on weakly adhesive surfaces (soft hydrogel), witness low motility due to cell inability to form sufficient stable adhesions to support contractile forces generated during migration.¹⁷ Cell velocity was used to gauge cell adhesion on different substrates. Because cell adhesion directs signal transduction for cell behavior via an activation of tyrosine kinases or mitogen activated protein kinases,¹⁸ we can conclude that intermediate adhesion on firm hydrogel surfaces (providing both the mechanical support and the control of biological signaling) is ideal for regulation of stemness.

Hematopoietic Stem Cells: Migration, Proliferation, Stemness

Stem cell fate is currently attributed to a plethora of factors, the most identified of which is the ECM and microenvironment – more specifically, substrate stiffnesses, ECM ligands, and genetic and molecular mediators such as growth and transcription factors.¹⁹ We observed that our HSCs distributed randomly on the plastic petri dish and

clustered together best on the 1:300 hydrogel. This is similar to other cell type behavior; Guo et al²⁰ show the fundamental relationship between fibroblastic and epithelial cell migration and surface stiffness by using substrates of identical chemical composition but different flexibilities. It was observed that these cells migrate away from each other in hard surfaces, but merge to form tissue like structures in soft surfaces. The compaction of HSCs on soft surfaces are reminiscent of the formation of tissues by fibroblastic cells²⁰ and mesenchymal condensation by mesenchymal stem cells (MSCs), which is the process by which MSCs group together to differentiate into a single tissue type.²¹ This may be a reason as to the higher levels of differentiation observed on the 1:300 (see **Figure 8**). It is possible that like these other types of cells, biochemical signaling causes HSCs to migrate and group together to form a specialized tissue - thereby promoting differentiation into a particular lineage of blood cells.

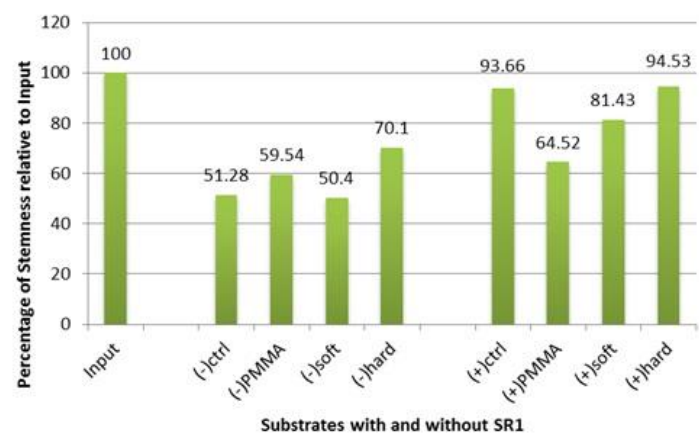


Figure 8. Substrate and SR1 effects on stemness relative to the input. Each percentage taken from the flow cytometry was divided by the stemness percentage of the input (64.51%) to find to actual level of stemness maintained, as shown by the bars in this graph.

The ECM stiffness itself has also been shown in previous studies to have significant effects on general cell proliferation and differentiation.^{22,23} It has been demonstrated that lower stiffnesses result in generally higher cell differentiation. For example, mouse mammary epithelial cells cultured on soft collagen gel substrates resulted in increased differentiation in comparison to plastic substrates.²² A study conducted by Hadijpanayi et al²³ also revealed that human dermal fibroblasts proliferated rapidly on the stiffest matrices and resulted in a population doubling time of only 2 days, while the fibroblasts experienced a four day lag period on comparatively softer surfaces. However, research regarding these occurrences for hematopoietic stem cells is currently few or non-existing. Our migration and stemness results suggest that HSCs seem to act in accordance with other types of cells and are likely affected by similar biochemical and mechanical cues. This becomes significant as we can now culture HSCs as fundamentally comparable to epithelial cells or MSCs, both of which are better studied and documented. Our research is also notable for the discovery of the formation of two clear but undefined clusters on the 1:25 (+) and the correlated high stemness. The reasons behind this

unreported, unusual cell patterning and the interaction between HSCs and hydrogels that facilitate good stemness levels are unclear and remain promising areas of future research.

It is important to note the cell count data in comparison to the flow cytometry data. The 1:25 (+) resulted in an overall 94.53% stemness maintained (the highest value among all substrates), but we counted only 20,500 cells (the lowest value among all substrates, not including the 1:25(-)). This data, however, is still encouraging due to the fact that true, quiescent stem cells divide and replace themselves slowly in bone marrow, and large increases in HSC population in culture are due to differentiation of cells into hematopoietic progenitor cells (HPCs), which also express CD34 but proliferate rapidly and are undesirable as they are already lineage restricted.³ This means that cells grown under the 1:25 conditions, both with and without SR1, are likely dominantly true HSCs. Although the control (+) also resulted in high stemness at 93.66%, its high cell count makes it difficult to ascertain whether the observed cells were true stem cells. Likewise, the anomaly of the 1:300 (-) cell count being higher than the 1:300 (+) cell count may be attributed to the very low stemness of the 1:300 (-) and the significantly higher stemness of the 1:300 (+). Our data also reveals that PMMA is not an effective scaffold due to its highest cell count and generally low stemness. These phenomena provide strong evidence to the potential and effectiveness of firm, biomimetic gelatin-based hydrogel cultures.

Previous research has demonstrated that the development of hydrogels as stem cell scaffolds is currently a popular field of tissue engineering. Thus far, studies have characterized the properties of hydrogel scaffolds;¹³ attempted to mimic the functional hematopoietic niche and imitate marrow physiology with various support cells;¹⁴ and created macroporous poly(ethylene glycol) (PEG) and collagen based hydrogels for the multiplication of hematopoietic stem and progenitor cells.^{5,6} However, the effective exploitation of simple, cheap gelatin based hydrogels with mTG as a crosslinking agent remains largely overlooked, and studies which do examine these hydrogels focus primarily on mechanical properties²⁴ or cell compatibility.^{25,26} Our research serves as a translational bridge between these two previously unconnected ideas. We also extend the usefulness of the exogenous signaling molecule SR1, an antagonist of the aryl hydrocarbon receptor (AhR), which has only been recently discovered and whose characteristics have only been previously related to inducing proliferation and have not been yet linked to promoting stemness or quiescence.¹² Through the pronounced, positive effects of SR1 on stemness, we suggest that inhibition of the AhR is not only important in inducing proliferation of HSCs,¹² but also in orchestrating quiescence of HSCs and minimizing active differentiation of HPCs.

Our research has potential far reaching implications. Expansion of HSCs *in vitro* is currently important in the treatment of bone-marrow related disorders and cancers, including leukemia, lymphoma, aplastic anemia, and multiple

myeloma, among others. However, treatment options - which primarily involve clinical transplantation of HSCs - are limited by an inadequate supply of HSCs due to a shortage of appropriate donors and a need for an effective culture method, which is what we are aiming to resolve. Quiescent HSCs are especially fundamental in the reconstruction of marrow-recipient hematopoietic systems. Differentiation of HPCs effectively renders the cells useless as they are too lineage specific to be transplanted and cannot adequately replenish certain types of blood cells. HSCs are usually maintained in their quiescent state through interactions with the natural bone marrow niche, but may be activated by growth factors and cytokines (such as granulocyte-colony stimulating factor) during the cell cycle to extensively repair target sites.^{3,4} In the case of a marrow recipient, the quiescent HSCs are mobilized and begin to rebuild the hematopoietic system upon transplantation.⁴ Hematopoietic stem cells may also be experimentally utilized in treatment for diabetes, rheumatoid arthritis, and system lupus erythematosus, as well as in gene therapies and scientific research.³ Our results indicate that we have successfully delayed the onset of differentiation on the 1:25 (+) scaffold; this can be utilized to amass large quantities of quiescent HSCs for long term storage prior to transplantation, diminishing the current high demand for donor cells.

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CONFLICT OF INTEREST

None.

ETHICAL APPROVAL

This work meets all the ethical guidelines.

REFERENCES

1. Bone Marrow Statistics. Institute for Justice. <http://www.ij.org/bone-marrow-statistics>. Accessed September 17, 2014.
2. Gunsilius E, Gastl G, Petzer AL. Hematopoietic stem cells. *Biomed Pharmacother*. 2001;55:186-194.
3. Hematopoietic Stem Cells [Stem Cell Information]. National Institutes of Health. <http://stemcells.nih.gov/info/scireport/pages/chapter5.aspx>. Accessed September 17, 2014.
4. Pietras EM, Warr MR, Passegue E. Cell cycle regulation in hematopoietic stem cells. *J Cell Biol*. 2011;195:709-720.
5. Raic A, Rödling L, Kalbacher H, Lee-Thedieck C. Biomimetic macroporous PEG hydrogels as 3D scaffolds for the multiplication of human hematopoietic stem and progenitor cells. *Biomaterials*. 2014;35:929-940.
6. Leisten I, Kramann R, Ventura Ferreira MS, et al. 3D co-culture of hematopoietic stem and progenitor cells and mesenchymal stem cells in collagen scaffolds as a model of the hematopoietic niche. *Biomaterials*. 2011;33:1736-1747.
7. Muth CA, Steinl C, Klein G, Lee-Thedieck C, Tjwa M. Regulation of Hematopoietic Stem Cell Behavior by the Nanostructured Presentation of Extracellular Matrix Components. *PLoS One*. 2013;8:e54778.
8. Lutolf MP, Doyonnas R, Havenstrite K, Koeckar K, Blau HM. Perturbation of single hematopoietic stem cell fates in artificial niches. *Integr Biol (Camb)*. 2008;1:59-69.
9. Wilson A, Trumpp A. Bone-marrow Haematopoietic-stem-cell Niches. *Nat Rev Immunol*. 2006;6:93-106.
10. Krause DS. Regulation of hematopoietic stem cell fate. *Oncogene*. 2002;21:3262-3269.
11. Zandstra PW. Cytokine manipulation of primitive human hematopoietic cell self-renewal. *Proc Natl Acad Sci USA*. 1997;94:4698-4703.

12. Boitano AE, Wang J, Romeo R, et al. Aryl Hydrocarbon Receptor Antagonists Promote the Expansion of Human Hematopoietic Stem Cells. *Science*. 2010;329:1345-1348.
13. Zhu J, Marchant RE. Design Properties Of Hydrogel Tissue-engineering Scaffolds. *Expert Rev Med Devices*. 2011;8:607-626.
14. Sharma MB, Limaye LS, Kale VP. Mimicking the functional hematopoietic stem cell niche in vitro: Recapitulation of marrow physiology by hydrogel-based three-dimensional cultures of mesenchymal stromal cells. *Haematologica*. 2012;97:651-660.
15. Henschler R, Brugger W, Luft T, Frey T, Kanz L. Maintenance of transplantation potential in ex vivo expanded CD34(+)-selected human peripheral blood progenitor cells. *Blood*. 1994;84:2898-2903.
16. Grigoryev Y. Cell Counting with a Hemocytometer: Easy as 1, 2, 3. <http://bitesizebio.com/13687/cell-counting-with-a-hemocytometer-easy-as-1-2-3/>. Accessed September 17, 2014.
17. Gobin A. Cell Migration Through Biomimetic Hydrogel Scaffolds [master's thesis]. Houston, TX: Rice University; 2003. Miyajima A, Ito Y, Kinoshita T. Cytokine signaling for proliferation, survival, and death in hematopoietic cells. *Int J Hematol*. 199;69:137-146.
19. Guilak F, Cohen DM, Estes BT, Gimble JM, Liedtke W, Chen CS. Control of Stem Cell Fate by Physical Interactions with the Extracellular Matrix. *Cell Stem Cell*. 2009;5:17-26.
20. Guo W, Frey MT, Burnham NA, Wang Y. Substrate Rigidity Regulates the Formation and Maintenance of Tissues. *Biophys J*. 2006;90:2213-2220.
21. Mammoto T, Mammoto A, Torisawa YS, et al. Mechanochemical Control of Mesenchymal Condensation and Embryonic Tooth Organ Formation. *Dev Cell*. 2011;21:758-769.
22. Emerman JT, Burwen SJ, Pitelka DR. Substrate properties influencing ultrastructural differentiation of mammary epithelial cells in culture. *Tissue Cell*. 1979;11:109-119.
23. Hadjipanayi E, Mudera V, Brown RA. Close dependence of fibroblast proliferation on collagen scaffold matrix stiffness. *J Tissue Eng Regen Med*. 2009;3:77-84.
24. McDermott MK, Chen T, Williams CM, Markley KM, Payne GF. Mechanical Properties of Biomimetic Tissue Adhesive Based on the Microbial Transglutaminase-Catalyzed Crosslinking of Gelatin. *Biomacromolecules*. 2004;5:1270-1279.
25. Chen PY, Yang KC, Wu CC, Yu JH, Lin FH, Sun JS. Fabrication of large perfusable macroporous cell-laden hydrogel scaffolds using microbial transglutaminase. *Acta Biomater*. 2014;10:912-920.
26. Yung CW, Wu LQ, Tullman JA, Payne GF, Bentley WE, Barbari TA. Transglutaminase crosslinked gelatin as a tissue engineering scaffold. *J Biomed Mater Res A*. 2008;83:1039-1046.