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Extracellular Matrix Composition and Stiffness Differentially Regulate Hematopoietic Stem Cell and Myeloid Progenitor Fate



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# Extracellular Matrix Composition and Stiffness Differentially Regulate Hematopoietic Stem Cell and Myeloid Progenitor Fate

by

Nathan Edwards

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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of the

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[redacted]

This thesis is dedicated to the memory of Gordon Edwards

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# ABSTRACT

## Extracellular Matrix Composition and Stiffness Differentially Regulate Hematopoietic Stem Cell and Myeloid Progenitor Fate

**Nathan Edwards**

**Supervisor: Dr. Emmanuelle Passegué**

The hematopoietic stem cell (HSC) and the granulocyte/macrophage progenitor (GMP), a downstream myeloid progenitor, reside within distinct bone marrow (BM) niches. The biophysical properties of these BM niches and the effect of these biophysical properties on the regulation of HSCs and GMP cell function remain unknown. We determined the mechanical properties of the endosteum, the perivasculature and non-vascularized central marrow, three specific BM niches that HSCs or GMPs have been shown to reside. We then used *ex vivo* adhesion assays to determine the extracellular matrix (ECM) ligands to which HSCs and GMPs preferentially adhere. *Ex vivo* culture on polyacrylamide (PA) hydrogels mimicking distinct BM niche microenvironments were then used to assay the effect of ECM elasticity on cell expansion and lineage differentiation capacity. We also performed transplantation assays after HSC expansion on PA gels to determine the role of ECM

elasticity on engraftment potential. We found that GMPs preferentially bound to fibrillar collagen, but also adhered to fibronectin, and showed greater expansion on soft, compliant environments. Conversely, HSCs adhered only to fibronectin, and after growth on stiff environments, had greater cell expansion and higher engraftment potential due to increased expression of homing receptors. Myeloid differentiation was found to be affected by ECM elasticity such that compliant ECM niches enhanced granulocyte production. Conversely, stiff environments enhanced macrophage lineage commitment. Mechanotransduction of ECM elasticity was determined to be dependent on actomyosin contraction and activation of focal adhesion kinase (FAK) likely through integrin engagement. Together, our data show that the biophysical properties of specific BM niches function in controlling the differential growth and differentiation of HSCs and progenitors during homeostasis, and may even have the potential to regulate hematopoietic regeneration following insult, injury or transplantation.

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# CHAPTER 1

## Introduction to biomechanics and hematopoiesis

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The hematopoietic system is a complex hierarchy of stem and progenitor compartments of increasingly restricted differentiation capacity. At the top of this hierarchy is a small pool of hematopoietic stem cells (HSC) capable of replenishing the entire blood system.[1] At steady state, HSCs are mostly quiescent and infrequently proliferate, either self-renewing or giving rise to non-self-renewing early multipotent progenitors (MPPs). During homeostasis, MPPs largely sustain adult hematopoiesis giving rise to more abundant and increasingly lineage-restricted lymphoid or myeloid progenitors.[2] One such myeloid-committed progenitor is the short-lived, highly proliferative granulocyte/macrophage progenitor (GMP), which is limited to the production of granulocytes and macrophages. Controlling the fate of hematopoietic stem and progenitor cells (HSPCs) is essential to adapt blood production to the needs of an organism both during homeostasis, and during emergency hematopoiesis following physiological and/or pathological perturbation.[3] While the exact mechanisms controlling HSPCs remain to be fully elucidated, it is known that bone marrow (BM) niche microenvironments, where HSPCs reside, provide specific instructive cues to control cell fate.

The BM can be divided into three structurally distinct niches in which HSCs, as well as more differentiated progenitors, like the GMP, reside.[4] The endosteum is structurally defined as being within 15 μm from the inner bone surface. The perivasculature is defined here as being within 5 μm of blood vessels, and the central marrow is defined here as everything non-endosteal or non-perivascular. Recent advances in deep immunofluorescent (IF) imaging of the BM cavity and lineage tracking of HSCs have allowed for the identification of distinct perivascular niches.[5,6] Specifically,

a majority of HSCs have been shown to reside near sinusoids, fenestrated blood vessels, through which hematopoietic cells migrate into and out of circulation.[7,8] Few HSCs are also found near arterioles, non-fenestrated blood vessels, which are found in the outer region of the medullary cavity near the endosteum.[9] In contrast, GMPs do not appear to have a defined niche. However, during hematopoietic regeneration, GMPs form large clusters of differentiation found throughout the central marrow cavity at both the endosteum and in the central marrow cavity.[10] A large body of work has been performed using genetic ablation studies to determine HSC niche components essential for maintenance of HSCs.[11] Specifically, vascular endothelial cells (ECs), perivascular mesenchymal stromal cells (MSCs) and mature megakaryocytes (Megs), all found in close association with blood vessels, have been shown to be essential for HSC maintenance due to secretion of SCF, CXCL12, TGF-β1 and TPO (among other secreted molecules; reviewed in *Schepers et al*., 2015). This suggests that the HSC niche would be in close spatial proximity to these essential cell types and their secreted biomolecules.[8,12–16] Not surprisingly, HSCs are often found in direct contact with Megs and perivascular MSCs, but do not directly contact vascular ECs. Despite the importance of these secreted signals, evidence suggests that the biophysical properties of the BM niche can play a role in controlling hematopoietic cell fate.

Biomechanical forces are transmitted to cells by the physical architecture of the tissue, which is characterized by environmental elasticity, extra cellular matrix (ECM) composition and cell-cell adhesion.[17,18] The expression patterns of key ECM proteins have been reported to be localized to specific niche environments. Fibronectin (Fn) is distributed throughout the central marrow, in high concentrations at the endosteum, and

substantially expressed in the perivasculature space.[19,20] Fibrillar collagens, collagen (Col) I and III, are extensively confined to the endosteum, while basement membrane proteins Col IV and Laminin (Lmn) and connective tissue Col V are localized to the vasculature.[19,21–23] Interestingly, vasculature associated Megs, which have been shown to be an essential niche cell type, highly express Fn as well as Col IV and Lmn.[19,20]

Immunofluorescent localization studies of HSCs and ECM ligands suggest that HSCs reside proximally to a Meg in a Fn-, Col IV- and Lmn-rich niche within a perivascular niche. Some *in vitro* experiments show that Fn- and Lmn-coated surfaces can support HSPCs.[24] Other studies indicate that HSPCs adhere to and survive equally well on uncoated planar surfaces[25–30] or topologically modified substrates that alter dimensionality (2D vs. 3D environments).[31–35] In total, these studies suggest that HSCs adhere to distinct BM ECM niches that support HSC function. However, a comprehensive analysis of the BM niche, ECM requirements of hematopoietic stem and progenitor cells has not yet been performed.

Environmental elasticity has been shown to regulate development and homeostasis of a broad range of tissues by modulating tissue resident stem cell viability and differentiation.[36–38] Previous studies have analyzed the mechanical (or viscoelastic) properties of the BM, which revealed a wide range of mechanical environments within the central medullary cavity.[39–42] This range of mechanical environments was shown to influence the differentiation capacity of bone marrow derived MSCs.[43] Studies of hematopoietic cells have determined that the published range of matrix stiffness and the material properties of the substrate can affect differentiation, cell growth and transplantation capacity.[44–48] However, these studies have largely been performed on

hematopoietic cell lines, whole BM or unfractionated HSPC populations. Therefore, there is still a lack of direct evidence exploring how the physical properties of the BM niche microenvironment affect the true biology of HSCs or more committed progenitors, like the GMP.

In total, while there is an abundance of evidence suggesting that BM niches have distinct biophysical properties and different biomechanical environments can regulate hematopoietic cells, several key questions remain:

1. What are the mechanical properties of the distinct BM niches, in which HSCs and GMPs have been shown to reside?
2. Given the differences in ECM protein expression throughout the BM cavity, do HSCs and GMPs preferentially adhere to ECM proteins found within specific niche environments?
3. Do the biophysical properties of specific niches affect the biological function of specific hematopoietic stem and progenitor cells?
4. If biological function is affected by the material properties of the niche environment, by what mechanism do cells sense and respond to their environments?
5. Is the capacity of HSCs to be transplanted and repopulate the blood system affected by the material properties of their environment?

With these questions in mind, my research project used a rigorous quantitative approach to understand the biophysical properties of the BM and how these properties affect HSPC function. Specifically, I investigate the overarching hypothesis that the mechanical properties of a niche microenvironment affect HSC and GMP growth,

differentiation potential and adhesion, and transplantation capacity of HSCs. This hypothesis is tested and addressed in the following five sections:

1. Determine the elastic properties of specific BM niches
2. Quantify adhesion to key ECM proteins expressed in the BM
3. Investigate the effect of the biophysical properties on the growth, proliferation and differentiation of HSCs and GMPs
4. Elucidate a mechanotransduction pathway responsible for allowing HSCs and GMPs to sense and respond to their environment
5. Understand if and how the physical properties of a microenvironment affect the transplantation capacity of HSCs

The results of this investigation will have implications for our understanding of the cell extrinsic cues that control the hematopoietic system. The biophysical properties of distinct niche environments in which specific hematopoietic stem and progenitor cells reside differentially regulate cell function. Such analysis may provide new clinical target mechanisms to improve hematopoietic regeneration or control blood production throughout one's lifetime.

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# CHAPTER 2

## Determining the elastic properties of the bone marrow niche

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## Introduction

Bone marrow (BM) plays a significant role in maintaining organismal homeostasis through the regulation of the hematopoiesis. In addition to the known biomolecular control of hematopoiesis,[1] previous research has established that hematopoietic cells respond to the physical properties of their environment.[2] The central medullary cavity is composed of diverse microenvironments, in which hematopoietic cells can reside. The defining factor of the BM is its encasement by bone and osteo-lineage cells. BM is highly vascularized, containing multiple direct types of vessels, each with their own unique properties and resident endothelial cells, including sinusoids, arterioles, transition zone vessels, a central sinus and a central vein.[3] Additionally, intervening sympathetic nerves run along some arterioles within the bone marrow.

Much of the work establishing the physical properties of the BM has focused on the characterization of matrix ligand content, but the analysis of marrow tissue viscoelasticity has been limited. Previous studies have isolated, homogenized and centrifuged (to remove bone debris) the marrow before mechanical analysis and found heterogeneity in the mechanical properties of these isolated BM cells.[4–6] Others have found that BM isolated from the trabecular bone region is more viscoelastic than marrow harvested more distally, suggesting that viscosity is correlated with spatial localization throughout long bones.[7] Such analyses suggest that BM contains a diversity of viscoelastic microenvironments. However, by damaging tissue organization, these studies ignore the elastic contribution of macrostructures like blood vessels, nerve fibers and the surrounding bone, which define specific BM niches. Moreover, these studies negate extracellular matrix (ECM) contributions to the elastic properties of an

environment. Other techniques, such as intramedullary pressure or ultrasonic wave propagation, have also been used to determine how lifestyle can affect marrow content, blood flow and bone remodeling.[8–10] To our knowledge, only one study has performed mechanical analysis on isolated, intact BM.[11] Jansen and colleagues performed rheology, indentation and cavitation rheology on freshly isolated porcine femurs and determined a heterogeneity of elastic modulus (measured in kilo Pascal, kPa), ranging from 0.25 to 24.7 kPa. However, they do not report the mechanical properties of specific BM niche microenvironments.

Here, we sought to determine the elastic modulus of three distinct BM niches, the endosteum, the perivascular region and non-vascularized central marrow, where the hematopoietic stem cell (HSC) and the granulocyte/macrophage progenitor (GMP) have previously been reported to reside.

## Results

In this study, we developed an Atomic Force Microscopy (AFM) nanoindentation protocol to quantify the elasticity of the endosteal, perivascular and non-vasculature marrow niches, in which HSCs and GMPs both reside. Briefly, mice were injected with DyLightA88-Lectin to label blood vessels, and femurs were rapidly isolated, frozen in OCT. Bones were cyro-sectioned into 7 μm slices and imaged by immunofluorescence to determine the localization of blood vessels within the marrow, and bright field microscopy to determine the region of interest to be probed. Femur sections were then analyzed by AFM, throughout the diaphysis using AFM indentation of femur sections. Samples were indented with a maximum force of 8 nN using cantilevers that had spring constants ranging from .04 to 0.07 N/m, with borosilicate glass spherical tips at a resolution of 5 μm. The Hertz model was applied to the obtained force curves in order to calculate the Young's modulus, or stiffness, of the tissue.

At the endosteum, the topography of the femur sections was variable; therefore, the probing was performed in the largest area possible given the clearance of the AFM tip cantilever arm (Figure 2.1A). In the central cavity, probing was performed in 50 x 50 μm grids (Figure 2.1B). The starting position of the AFM probing tip was assessed by bright field microscopy. Bright field images of the tip location and immunofluorescent images of labeled blood vessels were then overlaid to define the exact region of the BM that was probed. These analyses were performed on 7 distinct regions from femurs isolated from 6 separate mice.

Regions within 5 μm (the minimum probing distance) of clearly identifiable vasculature, determined by DyLight488-Lectin signal, were defined as the

perivasculature. Regions within 15 μm of the inner bone surface were defined as the endosteum. All other regions were defined as non-vascularized marrow. Using these criteria and the method described above, we were able to determine the mechanical properties of specific BM niches.

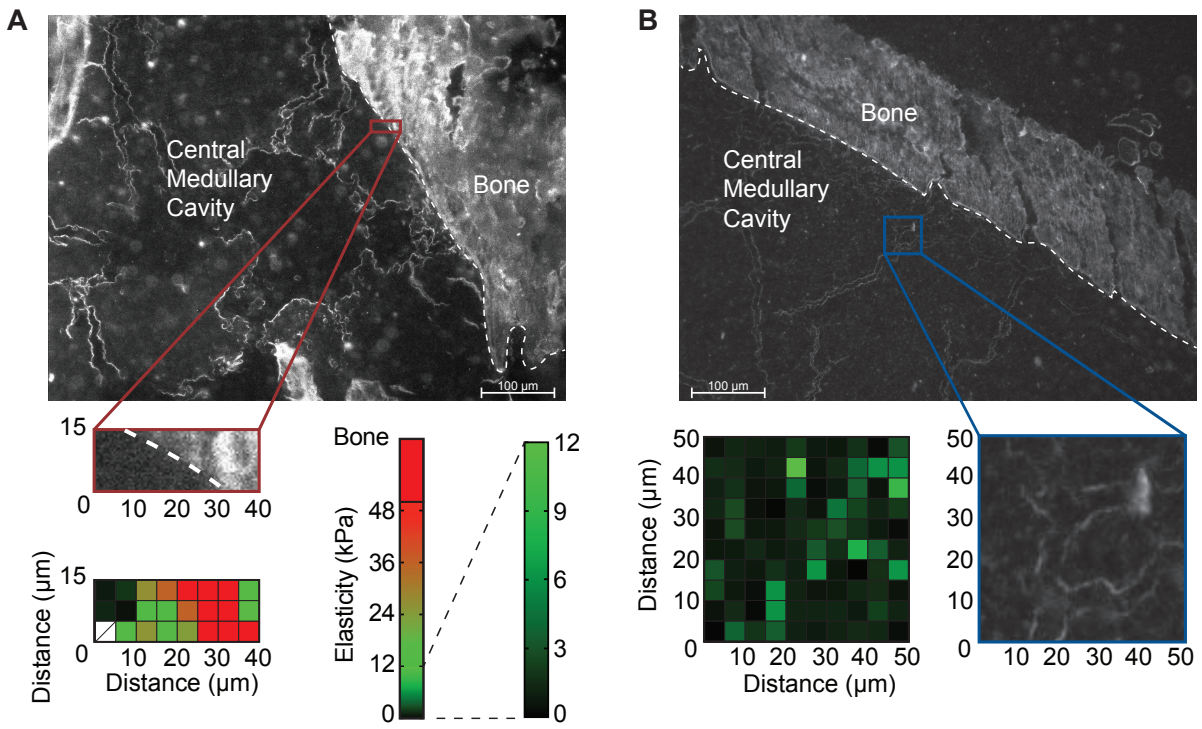


**Figure 2.1**: Atomic Force Microscopy of the Central Medullary Cavity
A) Representative overlay of Lectin-DyLight488 labeled blood vessels and bright-field images used to identify the endosteal region (red highlighted region) probed using AFM. Corresponding elasticities represented as heatmap in which red indicates all stiffness above 50kPa up to greater than 200,000 kPa. White box with a slash indicates region of poor indentation. B) AFM analyses were performed within the central medullary cavity in the blue 50 x 50 $\mu m^2$ region. Image shows overlay of Lectin and brightfield overlay to determine region probed. Heatmap shows elasticities measured.

The endosteum was measured to be on average 22.1 kPa, with indentation regions measuring as stiff as 44 kPa (Figure 2.2A). Although our measurements of the endosteum are somewhat softer than what has been reported previously, these earlier measurements were made on *ex vivo* generated osteo-lineage cell microenvironments after lengthy culture periods, which may not truly reflect the hematopoietic marrow endosteum.[12,13] Stiffness of the endosteum sharply decreased as a function of distance to the bone surface (Figure 2.2B). Due to the ease of flushing BM from long bones to isolate intact BM plugs, it is unlikely that there is a strong adhesive interaction between the bone and marrow. This suggests that although the endosteal region likely is stiff due to proximity to bone, which was measured between 2-3 Giga Pascal (GPa), it may be partially protected from this extremely stiff tissue.

Measurements of the perivasculature varied widely, ranging from 1.1 to 12.3 kPa, with a mean of 2.9 kPa (Figure 2.2A). Perivascular stiffness measurements appeared to bifurcate around 5 kPa, with measurements below 5 kPa likely representing the abundant fenestrated, softer sinusoids. Blood vessels measuring above 5 kPa likely represent less abundant arterioles that are not fenestrated and are characterized by high laminin expression (Figure 2.2C). If this were the case, sinusoids and arterioles averaged 2.2 kPa and 7.3 kPa, respectively.

Non-vascularized central marrow was measured to be on average 0.87 kPa, which is inline with previous reports.[11]

Together, these data will allow for the more accurate creation of *ex vivo* cultures to model the biophysical effects of the marrow on all BM cells.

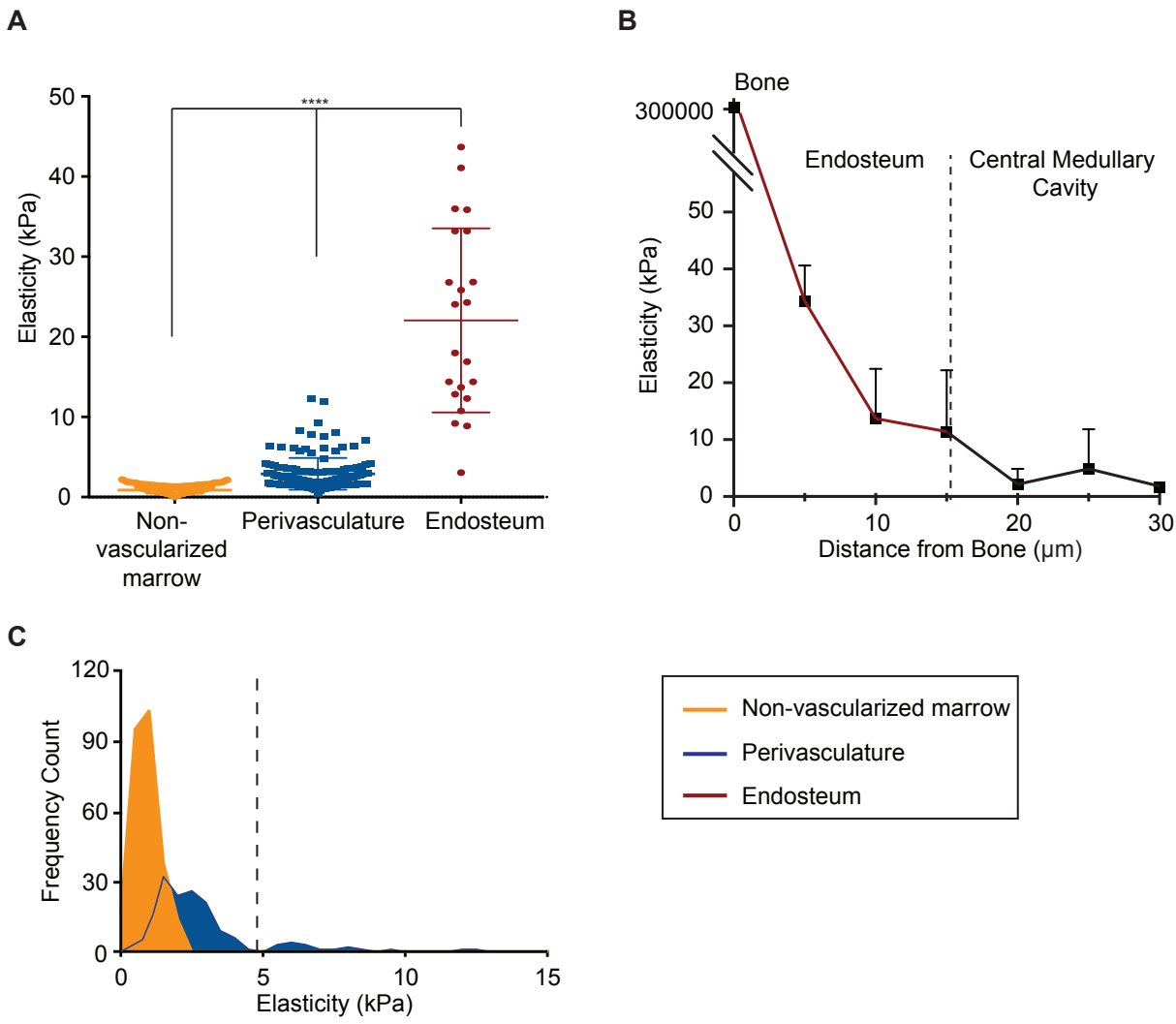


**Figure 2.2**: Mechanical Properties of Specific Bone Marrow Niches
A) Scatterplot of individual stiffness measurement values from 7 regions measured by AFM from 6 femurs isolated from individual mice. Perivasculature defined as indentations within a region of 5 μm from lectin488-labeled blood vessels (n=148). Endosteum was defined as all non-perivascular measurements within 15 μm of the inner bone surface (n=22). Non-vascularized marrow was defined as all other measurements within the medullary cavity (n=267). B) Combined analyses of all non-perivascular measurements displayed as a function of distance from the bone. Bone was determined to be 2-3 gPa. Each distance is shown as an average ± S.D. from 8 measurements from 2 biological repeats of the endosteal region. C) Frequency plot of stiffness values from all 7 regions separated into non-vascularized and perivasculature BM using immunofluorescent images of Lectin-DyLight488 labeled vasculature. Perivascular measurements appear to bifurcate at 5 kPa. All data (A-C) are represented as mean ± S.D; **** $p \leq 0.0001$.

## Discussion

To the best of our knowledge, we are the first group to use IF to identify the mechanical properties of distinct niches within the BM. While our analysis of blood vessels was not able to determine the elastic differences between sinusoids and arterioles, we were able to define the stiffness of both non-vascularized central marrow and the endosteum. Previously reported studies using *ex vivo* culture systems, which aimed to mimic the BM, had little validation for the stiffnesses assayed. This was a result of the lack of physiological measurement of the mechanical properties of BM. Therefore, our work has broad implications for the development of future culture systems for all BM resident cells (both hematopoietic and stromal), with applications ranging from drug development to increasing transplantation efficiency, and even to basic biological questions similar to those addressed in future chapters.

Two major caveats may be raised with our approach. Firstly, our sections were 7 μm thick, thereby affecting region identification due to out of plane vasculature. Additionally, due to the thinness of our sections, our measurements may be artificially high due to compressive effects within the tissue itself. In fact, two groups have reported that indention distance positively correlates with stiffness.[14,15] This may explain why our non-vascularized central marrow was stiffer than previously reported.[11,13] Secondly, our samples were previously frozen for sectioning and then thawed for AFM analysis. Previous analyses of frozen and thawed sections are inconclusive, as one group has showed an effect on the mechanical properties of the tissue, while another group has not.[16,17] These limitations aside, the range of our measurements from less than 0.1kPa to 44kPa were similar to those of the only other group to measure intact marrow.[11]

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# CHAPTER 3

## Quantifying adhesion of HSCs and GMPs to ECM proteins

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## Introduction

Bone marrow (BM) is a complex tissue composed of many different cell types and macrostructures, such as blood vessels and nerve fibers, all of which are encapsulated by bone. The space between cells is filled by the extracellular matrix (ECM), which provides a structural framework for tissue organization. Structural ECM proteins (collagens, laminins and fibronectin) provide anchorage sites for cells and play a critical role in mobilization. It is clear that ECM components are important for the maintenance of hematopoiesis, as demonstrated by the altered effects of knocking out ECM proteins tenascin and collagen (Col) X in mice, while knockouts of many other ECMs are embryonically lethal.[1–4]

ECM proteins are unevenly distributed throughout the BM cavity, being localized to specific niches. The most ubiquitous ECM protein, fibronectin (Fn), has been shown to be expressed in high concentrations at the endosteum, within the central marrow cavity and around vasculature associated megakaryocytes (Megs) and mesenchymal stromal cells (MSCs).[5–7] Fibrillar collagens (Col), Col I and Col III, are primarily found at the endosteum, being secreted by osteo-lineage cells.[5,8] Col IV, Col V and Laminin (Lmn) are found lining the vasculature, in large part being secreted by vascular endothelial cells (ECs).[5,9,10] Vascular associated Megs also secrete Lmn and Col IV into perivascular marrow.[7] Differences in ECM composition may contribute to the retention and maintenance of specific hematopoietic cells within specific niche microenvironments. Despite the known *in vivo* spatial localization of ECM proteins, and hematopoietic stem cells (HSCs) and granulocyte/macrophage progenitors (GMPs),

insights into the niche-specific ECM ligand to which HSCs and GMPs adhere remain lacking.[5,11,12]

Here, we determine to which BM niche ECM proteins HSCs and GMPs adhere. This has broad implications for the growing body of evidence demonstrating that HSCs and likely also hematopoietic progenitors have defined niches within the BM.[13] Extensive work has been done using genetic knockout mouse models to determine the complex and important role of both secreted biomolecules (*e.g.*, growth factors, cytokines, interferons) and resident cells (*e.g.*, MSCs, osteo-lineage cells, ECs, Megs, etc.) within these BM niches, with regards to controlling hematopoiesis in normal and disease conditions.[14] Our work suggests that not only the secreted biomolecular milieu, but also the physical properties of the niche, may play a role in the retention of hematopoietic stem and progenitors within specific BM niches.

## Results

To determine to which of these ECM proteins HSCs and GMPs adhere, cells were sorted by flow cytometry onto 96-well plates coated with various concentrations of ECM ligands. Cells were allowed to adhere for 3 hrs before mechanical dissociation and washing. The remaining adherent cells were fixed, stained and counted. Not surprisingly, both HSCs and GMPs strongly adhere to Fn, and increasing Fn concentration resulted in significantly greater adhesion (Figure 3.1A). Strikingly, HSCs only adhered to Fn, while GMPs bound most strongly to Col I in a concentration-dependent manner (Figure 3.1B).

We next wanted to determine if this adhesion would be comparable at all time points. Similar specific adhesion patterns were observed as early as 30 mins after plating of HSCs and GMPs (Figure 3.1C, E). However, after 30 mins, GMPs adhered more strongly to Fn than Col I. In contrast, by 12 hrs, both HSCs and GMPs were equally bound to all coated ECM proteins, likely due to cell secretion of their own ECM proteins (Figure 3.1D, F). In total, this suggests that both HSCs and GMPs adhere most strongly to Fn after initial contact with the ECM. This is unsurprising, as hematopoietic cells have been widely reported to express integrins $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ which bind Fn.[15–19] Over time, adhesion to Fn increases in HSCs and GMPs. GMPs bind most strongly to Col I after 3 hrs, which suggests that GMPs are able to significantly upregulate Col I adhesion molecules (like integrin $\alpha_2\beta_1$), while HSCs are not.

Taken together, these results indicate that HSCs preferentially bind to the most ubiquitous BM ECM protein, Fn, and thus likely reside in an Fn-rich microenvironment.

GMPs are also capable of binding Fn, but also bind to the endosteal specific ECM, Col I.

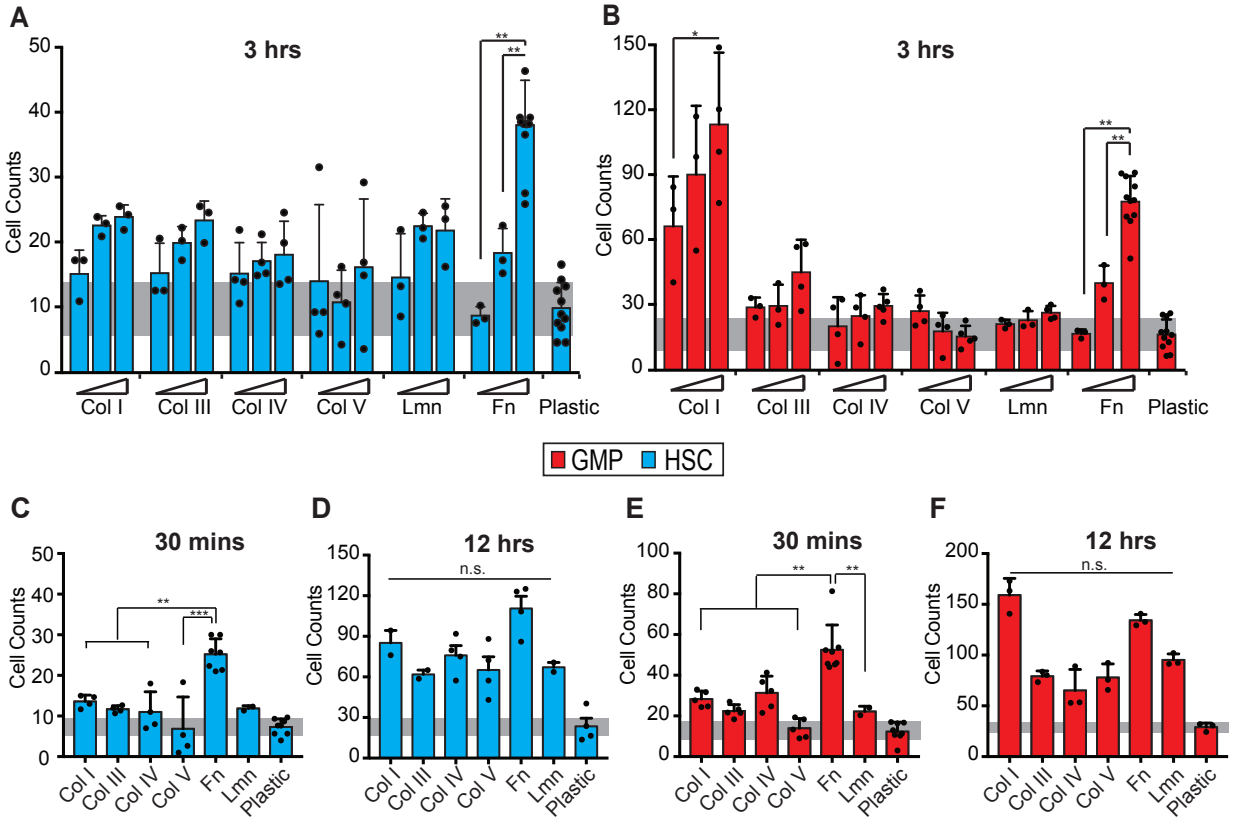


**Figure 3.1**: Time Course of Adhesion to ECM Proteins
A) Adhesion of HSCs or B) GMPs to 0.1, 1.0 or 10.0 µg/ml of ECM protein coated tissue culture plastic after 3 hrs. (C, D) Adhesion of HSCs or (E, F) GMPs to 10 µg/ml ECM for 0.5 hrs or 12 hrs. All data represented as mean ± S.D. unless otherwise indicated. Dots represent independent biological repeats. Grey bars are plastic control reference; * $p < 0.05$, ** $p < 0.01$. Collagen (Col), Laminin (Lmn), Fibronectin (Fn).

To determine if HSC and GMP adhesion to Fn was specific, we coated increasing concentrations of Col IV atop of Fn. This led to decreased adhesion of both HSCs and GMPs to Fn after 3 hrs (Figure 3.2A, B). This suggested that adhesion to Fn is specific. Moreover, Col IV found at the vascular wall may physically block adhesion to Fn, expressed by Megs and MSCs, thereby restricting HSC binding to specific perivascular niche cells.

We next wanted to determine how HSCs and GMPs adhere to ECM ligands. Integrin adhesion to ECM proteins allows a direct, mechanical link for adherent cells to transduce the physical properties of the matrix to the cell. Hematopoietic cells have been widely reported to express integrin $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_3$,[15–19] which bind Fn through an Arg-Gly-Asp (RGD) peptide motif or CS1 domain. Blocking antibodies against integrin $\alpha_4\beta_1$ leads to a mobilization of hematopoietic stem and progenitor cells (HSPCs) in both mice and primates and deletion of integrin $\alpha_4\beta_1$ resulted in the accumulation of HSPCs in the peripheral blood.[20,21] Linear RGD peptides have been shown to block integrin interaction with Fn; therefore, we next wanted to determine if HSCs and GMPs bind to fibronectin through integrin interaction with the RGD peptide sequence of Fn.[22,23] Addition of increasing concentrations of RGD peptide at the start of the adhesion assays resulted in a significant decrease of both HSC and GMP binding to Fn (Figure 3.2C, D). However, RGD peptide addition did not completely ablate cell adhesion, suggesting that HSCs and GMPs could also bind to the CS-1 domain of Fn.[24] Together, these results demonstrate that HSCs and GMPs bind to Fn through integrin engagement.

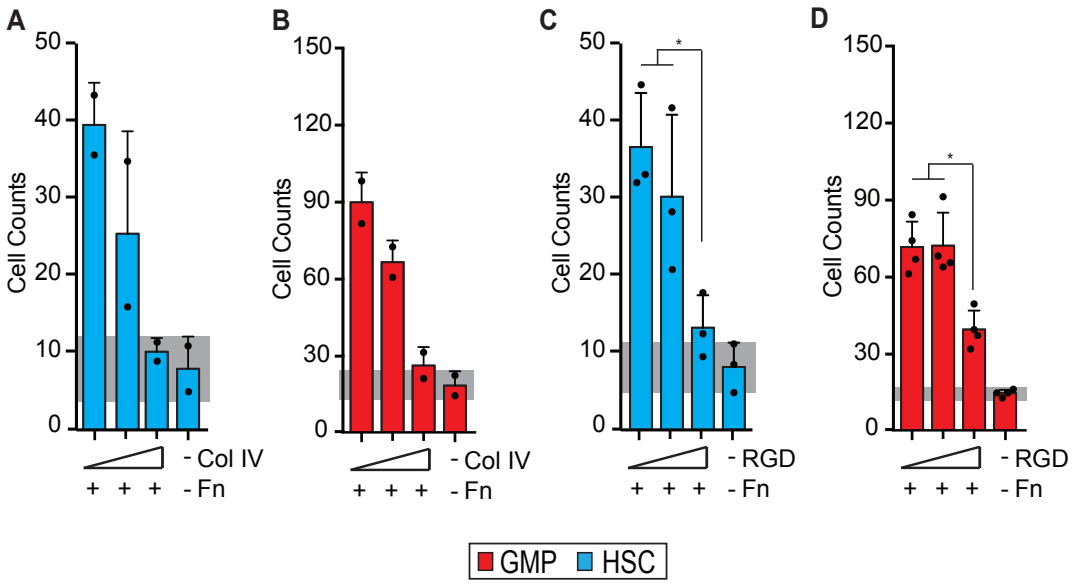


**Figure 3.2**: Probing the Adhesion of HSCs and GMPs to Fibronectin
A) Tissue culture plastic was coated with 10 µg/ml of Fn before coating with 0.1, 1.0 or 10.0 µg/ml. HSCs or B) GMPs were allowed to adhere for 3 hrs before dissociation. C) HSCs or D) GMPs were allowed to adhere for 3 hrs to 10 µg/ml of Fn coated plastic with the addition of 1, 10 or 100 µg/ml RGD peptide.

GMPs are highly proliferative at steady state, and were observed to have greater adhesion at all time points analyzed, compared to HSCs. Conversely, HSCs are largely quiescent at steady state, and overall, show limited binding activity. To determine whether adhesion is influenced by the activation state of the cells, we next isolated the more activated $CD34^{high}$ HSCs (aHSC) from the more quiescent $CD34^{low}$ HSCs (qHSC),[25] and performed 3 hr adhesion assays on Fn-coated plastic. Strikingly, qHSCs were found to be significantly less adherent to Fn than aHSCs and unfractionated HSCs (Figure 3.3A). We also used scanning electron microscopy (SEM) on qHSCs, aHSCs and GMPs sorted onto Fn-coated glass and cultured for 3 hrs (Figure 3.3B). As expected, the majority of GMPs were observed to flatten and elongate into a 'fried egg-like' morphology consistent with increased surface contact and cell adhesion. Conversely, all HSCs retained a rounded cell shape consistent with weak adhesion and a lack of cell spreading. Interestingly, cell projections from the membrane to the Fn-coated surface were observed on aHSCs, but not on qHSCs, and these filopodia-like adhesion projections may explain the increased adhesion of aHSCs to Fn compared to qHSCs.

These results suggest that adhesion directly correlates with activation state, with GMPs and activated HSCs binding more strongly to Fn than quiescent HSCs. This could be as a way to quickly extravagate into the circulation to fulfill hematopoietic demands. Additionally, integrin activation has been shown to serve as a growth signal by modulating growth factor signaling.[26] Moreover, cytokines and growth factors can affect adhesion of HSPCs by activating integrins $\alpha_4\beta_1$ and $\alpha_5\beta_1$.[27] Altogether, these

findings suggest that different hematopoietic stem and progenitor cells may favor distinct BM niches due to their preferential binding to specific ECM proteins.

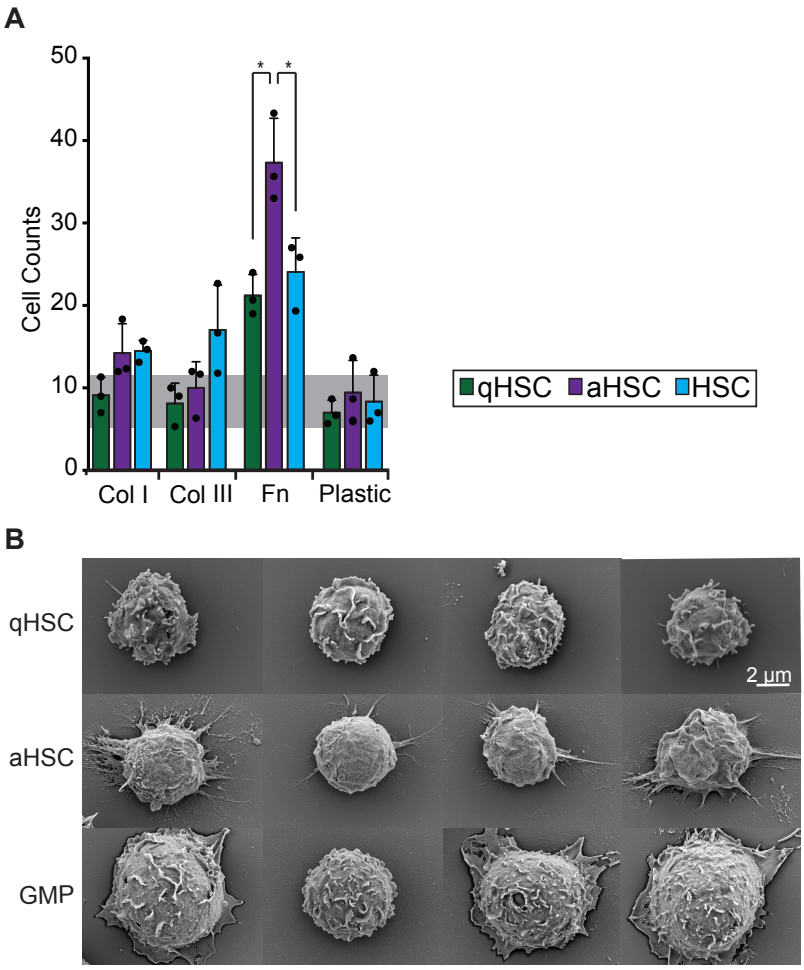


**Figure 3.3:** Activation State Correlates with Adhesion
A) Adhesion of quiescent HSCs (qHSCs; $CD34^{low}$), active HSCs (aHSC; $CD34^{high}$) and unfractionated HSCs to 10.0 µg/ml ECM proteins for 3 hrs. B) Cells imaged by Scanning Electron Microscopy (SEM) after 3 hrs adhesion to 10.0 µg/ml of Fn coated glass.

## Discussion

Given the complexity and diversity of potential niches in close spatial proximity within the central medullary cavity, it would stand to reason that retention and maintenance of hematopoietic cells within specific niches would be a result of both the secretion of biomolecules and ECM ligands. In order to sense and respond to their niche, cells must first adhere to their environment. A majority of HSCs localize within 10 μm of a sinusoid, but do not directly contact the Col IV-rich and laminin-rich vascular basement membrane.[28,29] Fn is secreted near sinusoids by Megs, as well as by perivascular MSCs when activated by thrombin secreted by Megs.[5,7,30,31] Genetic ablation studies have shown that Megs and perivascular MSCs are essential HSC niche cells, which control HSC maintenance and function via secretion of various soluble biomolecules, specifically Cxcl12 and Scf.[14] However, Megs and perivascular MSCs are also in large excess compared to HSC numbers, indicating that not all of them act as an HSC niche cell. Our data indicate that HSCs do not bind ECM proteins secreted by vascular endothelial cells, and thus are restricted to binding to Fn secreted by Megs and perivascular MSCs. We can therefore speculate that a gradient of Fn mixed with other vascular ECM ligands dictate the function of specific Megs and perivascular MSCs, with the ones having the purest Fn coating likely acting as HSC niche cells. Dynamic changes in Fn and other vascular ECM ligands could also contribute to a changing niche landscape for HSCs.

Strikingly, we also found that metabolically activated HSCs ($CD34^{high}$ HSCs, also called MPP1) adhere much better to Fn than quiescent $CD34^{low}$ HSCs.[25] These results are consistent with the preferential localization of quiescent $Ki67^{neg}$ HSCs to the

periarteriolar niche, compared to activated Ki67$^{pos}$ HSCs to the Fn-rich perisinusoidal niche.[32,13] Increased adhesion through integrins correlates with cytokine receptor signaling. This suggests that as HSPCs adhere, they become more receptive to biomolecular signaling (or visa-versa) and thus become more activated cells. Therefore, it is likely that the periarteriolar niche might contain enough Fn to retain qHSCs, but not enough to encourage significant binding and cell activation. Moreover, adhesion is essential for cell motility. We can assume that the higher adhesion of activated HSCs would promote migration in and out of the BM cavity through the sinusoid blood vessel network, while the less adherent nature of quiescent HSCs may instead favor their retention in the periarteriolar niche.

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# CHAPTER 4

## Effect of BM niche properties on HSC and GMP proliferation and differentiation potential

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## Introduction

A large body of evidence has shown that the material properties of a tissue can regulate stem cell fate, thereby affecting homeostasis.[1] This has been observed in a variety of stem cells, including muscle,[2] embryonic[3] and mesenchymal.[4] The extracellular matrix (ECM) environment can affect not only stem cell adhesion (as we have shown), but also cell growth and viability.[5] Additionally, the viscoelastic properties of the microenvironment have been shown to direct lineage fate specification during differentiation.[6]

More recent studies have shown that hematopoietic cells respond to ECM stiffness and that the material properties of the substrate can influence fate decisions, including proliferation and differentiation.[7–11] However, these studies have used whole bone marrow (BM), transformed hematopoietic cell lines or unfractionated pools of hematopoietic stem and progenitor cells (HSPCs). There is a lack of evidence to explain how hematopoietic stem cells (HSCs) and specific hematopoietic progenitors respond to the material properties of their BM niche environment.

We have previously described the elastic properties of three distinct hematopoietic niches within the BM – the endosteum, the perivasculature and non-vascularized central marrow. Additionally, we have described how the HSC and the lineage committed myeloid progenitor, the granulocyte/macrophage progenitor (GMP), adhere to ECM ligands found within these three niches. We therefore aimed to understand how the material properties of these three BM niche microenvironments might affect the biology of HSCs and GMPs. It stands to reason that not only the biomolecular, but also the biophysical properties of the niche would affect regulation of

the fate decisions of hematopoietic cells. Such an analysis may help to explain the localization of hematopoietic stem and progenitor cells *in vivo* depending on the hematopoietic needs of the organism.

## Results

While hematopoietic cells have been previously shown to be affected by changes in the physical properties of the milieu in which they are cultured,[23-34] the effects of specific BM-niche matrix elasticities and ECM ligands on HSCs and GMPs proliferation and differentiation potential remain largely unknown. Therefore, we created polyacrylamide hydrogels (PA gels) whose elastic modulus was adjusted to be mimetic of non-vascularized marrow (0.4kPa), the perivasculature (4kPa), or the endosteum (60kPa). These gels were then functionalized with either fibronectin (Fn) or collagen (Col) I, the two ECM proteins to which HSCs and GMPs adhere. Using these *ex vivo* environments mimetic of *in vivo* niches, we sought to address how the physical properties of specific BM niches affect the biology of HSCs and GMPs. More specifically, we sought to understand if matrix elasticity or ECM ligand adhesion could affect proliferation and differentiation.

Proliferation was measured by culturing 1000 HSCs or GMPs on PA gels functionalized with either Fn or Col I, and by enumerating the total numbers of cells after 3 and 6 days of culture in a media containing all the cytokines promoting growth and myeloid differentiation. For HSCs cultured on Fn-coated PA gels (Fn-PA gels), expansion directly correlated with elasticity; with significantly more cells found on the stiffer 60kPA gels at day 6 compared to either 4kPa or 0.4kPa gels (Figure 4.1A). In striking contrast, expansion was inversely correlated with elasticity for GMPs cultured on Fn-PA gels, with more cells found at day 6 on the softest 0.4kPa gel compared to either the 4kPa or 60kPa gels (Figure 4.1B). Interestingly, ligand composition did not

affect these growth trends, with no differences observed between Col I- and Fn-PA gels for HSC and GMP expansion rates at day 6 (Figure 4.1C, D).

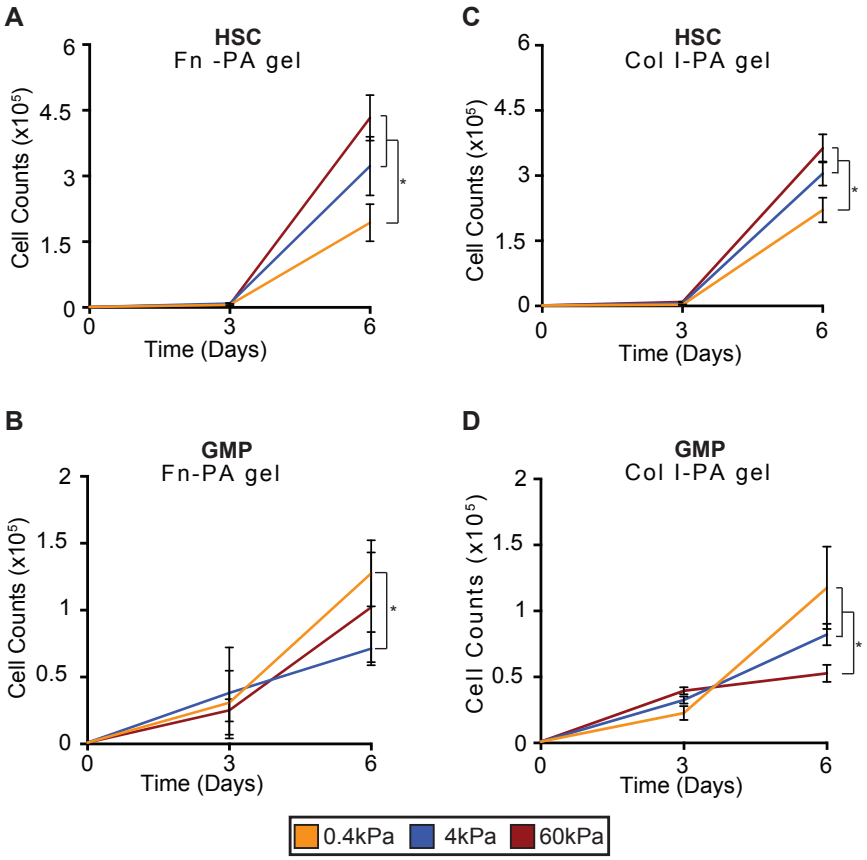


**Figure 4.1**: Population Growth due to Elasticity and ECM
A) Population counts after culturing 100 HSCs on PA gels coated with Fn or C) Col I after 3 and 6 days. B) Cell counts after 1000 GMPs cultured on Fn or D) Col I for 3 and 6 days. All data are means ± S.D.; * $p < 0.05$

At day 3, neither ligand composition, nor matrix elasticity impacted the growth of either HSCs or GMPs. This was confirmed by CFSE dilution assays performed on HSCs grown on Fn-coated gels for 3 days, which showed no significant differences in the division rates regardless of the gel stiffness (Figure 4.2A). Taken together with cell count analysis, these results suggest that environmental elasticity affects differentiating progeny rather than early stem and progenitors. Our results do not suggest that this effect is due to either proliferation or cell death, both of which may be able to explain cell count differences after day 3 in both HSCs and GMPs.

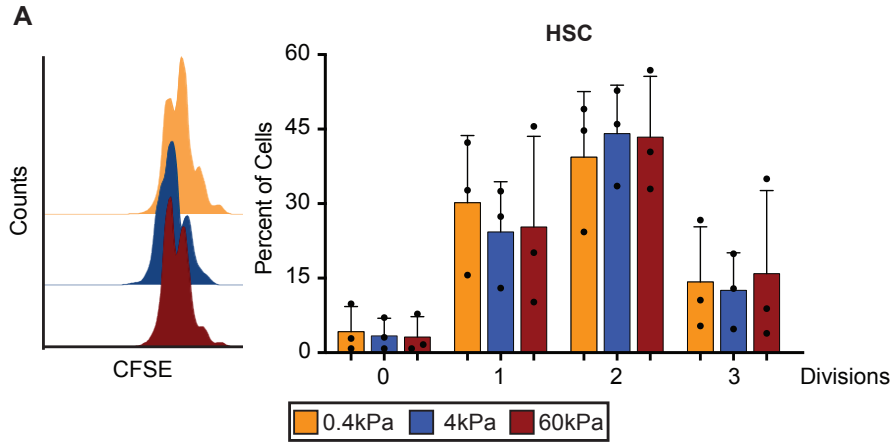


**Figure 4.2**: Proliferation Analysis of HSCs by CFSE
A) CFSE dilution assay of 1000 HSCs after 3 days on Fn-PA gels. Representative FACS plot of CFSE dilution shown on left and quantification of the numbers of cell divisions shown on right. Histogram dots represent independent biological repeats. All data are means ± S.D.

Differentiation was then analyzed by performing colony forming unit (CFU) assays and by plating cells harvested at various times from Fn-PA gels in methylcellulose to score the number and type of myeloid colonies formed after 8 days. In both cases, 1/5 of 1000 HSCs grown for 3 days or 1000 GMPs grown for 1 day were plated in methylcellulose, while the remaining 1/25 of each population were used for cell counting and the proliferation analyses described above. CFU-GM arise from immature progenitors able to produce both granulocytes and macrophages. The most committed progenitors were able to differentiate into single lineage granulocyte (CFU-G) and macrophage (CFU-M) colonies (examples of colony morphology shown in Figure 4.3A). Of note, megakaryocytic and erythrocyte colonies were not produced from HSCs in these culture conditions, most likely due to the late harvest and 3-day culture before plating in methylcellulose.

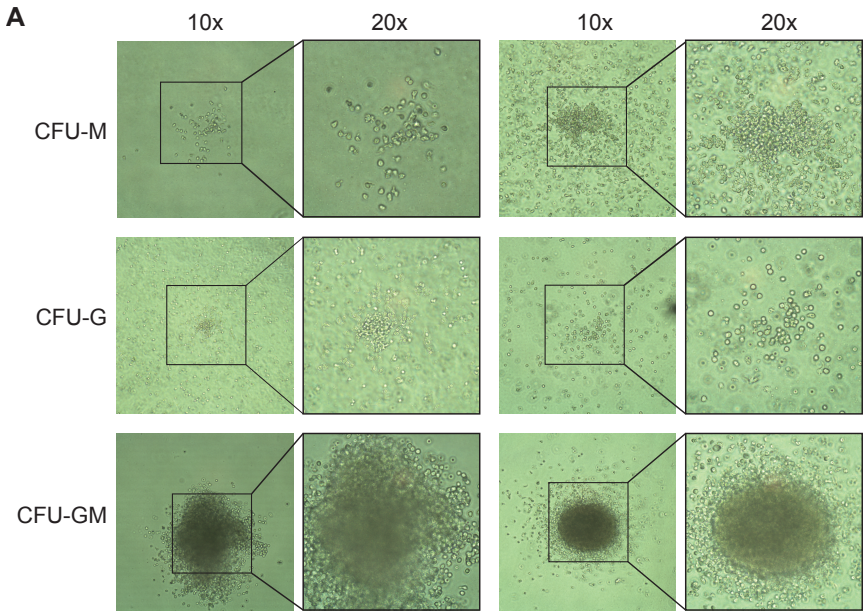


**Figure 4.3**: Methylcellulose Colony Morphology
A) Representative bright-field images of typical macrophage (CFU-M), granulocyte (CFU-G) and mix granulocyte/macrophage (CFU-GM) colonies.

Remarkably, for both HSCs and GMPs, granulocytic lineage specification was significantly diminished with increased elasticity, as shown by the loss of CFU-G formation on stiffer 4kPa and 60kPa Fn-Pa gels (Figure 4.4A, B). In contrast, increased matrix elasticity strongly correlated with macrophage differentiation, with significantly elevated CFU-M formation on stiffer 4kPa and 60kPa Fn-PA gels. Similar trends in lineage specification were observed when HSCs were harvested after 6 days and GMPs after 2 days growth on Fn-PA gels before plating in methylcellulose (data not shown). Interestingly, culture on Col I-PA gels mirrored the changes in lineage differentiation observed on Fn-PA gels, with enhanced macrophage differentiation and decreased granulocytic specification with increasing stiffness (Figure 4.4C, D). In contrast, no changes in CFU-GM colony formation were observed regardless of elasticity or ECM coating, suggesting that all conditions were equally able to maintain the growth of these most immature cells. Taken together, these results demonstrate that the physical properties of specific BM niches directly impact on HSC and GMP fate decisions. They show that HSC expansion and macrophage differentiation are promoted by stiff environments, while GMP expansion and granulocyte production are favored in soft environments. Moreover, they indicate that elasticity rather than ECM composition is the main driver of HSC and GMP proliferation and differentiation.

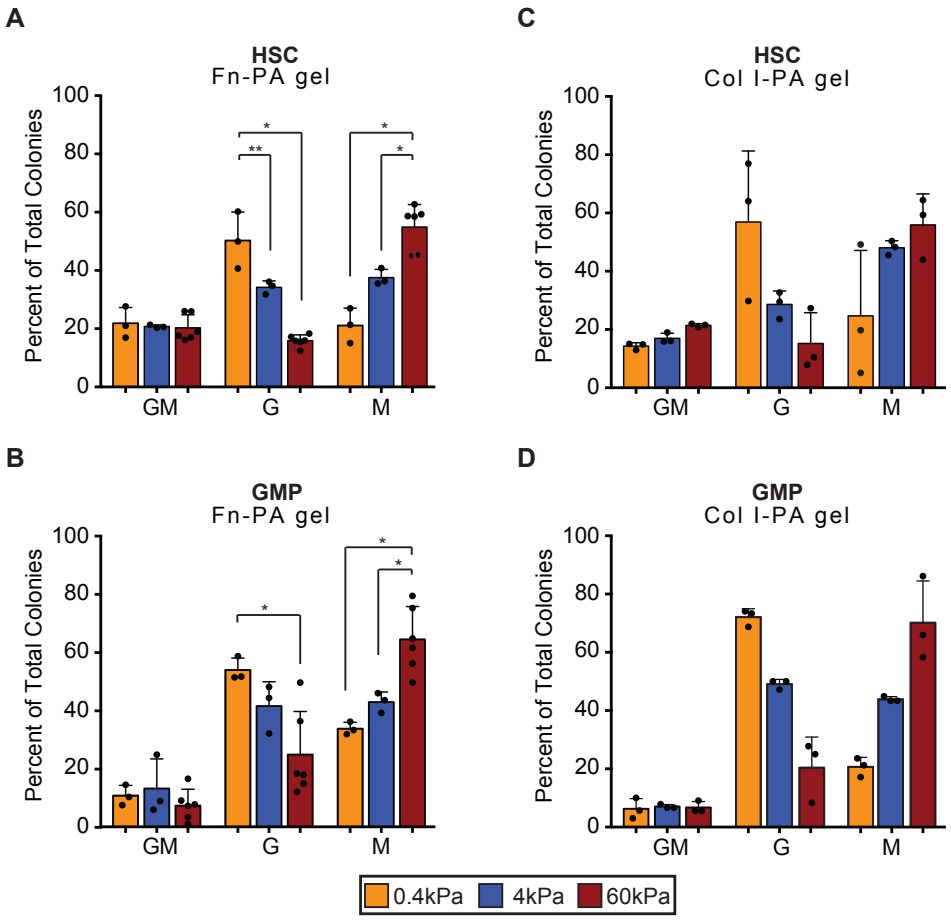


**Figure 4.3**: Differentiation Capacity after culture on PA Gels
A) Methylcellulose colony forming unit assay of GM (granulocyte/macrophage), G (granulocyte) or M (macrophage) colonies of 1000 HSCs cultured on Fn-coated PA gels or C) Col I PA gels for 3 days. B) Methylcellulose colony forming analysis after culturing 100 GMPs on Fn coated PA gels or D) Col I PA coated gels for 1 day.

## Discussion

Marrow elasticity is critical in regulating the proliferation of HSCs and GMPs that is consistent with their biology in regenerating settings. We found that a stiff environment similar to the endosteum promotes HSC proliferation, while inhibiting GMP expansion. Conversely, a soft environment similar to non-vascularized central marrow promotes GMP proliferation, while limiting HSC expansion. These results are consistent with the behavior of transplanted HSCs, which localize closer to the endosteal surface compared to progenitors[12] and first expand at the endosteum.[13,14] Meanwhile, regenerating GMP clusters develop throughout the marrow cavity.[15]

Marrow elasticity is also critical in dictating the differentiation capacity of HSCs and GMPs, in a manner very consistent between both cell types. We find that HSCs and GMPs grown on soft, central marrow-like environments preferentially give rise to granulocytes. Our lab has previously reported that GMPs form clusters of differentiation throughout the central marrow cavity during hematopoietic regeneration.[15] Of note, during regeneration, the BM cavity appears to be de-cellularized and the architecture of blood vessels is damaged, which is likely indicative of a softer environment than during homeostasis. Consistently, GMP clusters were found to produce a burst of granulocytes, which is likely favored by compliant physical environments.

In contrast, HSCs and GMPs grown on stiff, endosteum-like environments are directed towards macrophage lineage specification. Indeed, macrophages in the marrow cavity, which are called osteomacs, are primarily located along the inner bone surface.[16] While we did not directly test differentiation towards this lineage fate, endosteal environmental cues, such as stiffness, likely drive HSCs and GMPs towards

osteoclast differentiation, which is another population of macrophage-derived cells exclusively localized at the inner bone surface.[17] It is also striking that longstanding primary BM macrophages isolation procedures always involve 6 to 8 day culture on stiff plastic, suggesting that matrix elasticity may indeed play a much more direct role than previously appreciated.[18]

Monocytes are soft, pliable cells, and granulocytes are thought to be similarly pliable cells, due to their granulated cytoplasm. Granulocytes become softer as they differentiate allowing for better egress from the marrow cavity through the vascular endothelial sinusoidal wall.[19,20] Conversely, macrophages are stiff cells, resulting from large actomyosin cytoskeletons needed for migration and phagocytosis.[21] This suggests that marrow elasticity can drive hematopoietic lineage fate differentiation towards mature cells that match the mechanical properties of their environment.

Live *in vivo* imaging approaches have also shown that HSCs and early progenitor cells are not static in their niche and display significant oscillatory movement in the marrow cavity.[22–24] Therefore, it is likely that stem and progenitor cells do not have a single defined niche. HSPCs may respond to hematopoietic demands by migrating to specific microenvironments that provide both biomolecular and biophysical cues to directly tailor proliferation and differentiation specification to the needs of the organism.

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# CHAPTER 5

## Mechanotransduction of matrix elasticity

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## Introduction

Mechanical forces are generated at the tissue and cell level through cell-cell and cell-extracellular matrix interactions. Cells are able to sense and transmit these mechanical cues from the extracellular environment to the nucleus, inducing gene expression changes.[1] These mechanical cues can be transmitted to the cell via biochemical signals, like TGF-β, which are released from the extracellular matrix (ECM) milieu by cell-generated mechanical tension.[2] Additionally, there is a direct physical link between the biomechanics of the extracellular environment and intracellular signaling initiated by integrin binding to the ECM.[3] Integrin adhesion progresses through various well characterized stages from initial attachment, to the recruitment of adhesion-associated protein complexes, and to the formation of focal adhesions, which in turn are physically bound to actin bundles.[4]

This cascade of physical interactions has been widely reported to alter cellular activity. In response to ECM-integrin engagement and focal adhesion formation, the focal adhesion kinase (FAK) becomes phospho-activated.[5] FAK has been reported to be a key downstream signaling hub affecting a broad range of cellular processes in hematopoietic cells, including hematopoietic differentiation,[6,7] growth[8] and chemoattraction,[9,10] and has been directly shown to affect the activation state of hematopoietic stem and progenitor cells (HSPCs).[11] Moreover, changes in the biomechanics of the extracellular environment can lead to changes in intracellular tension resulting from actomyosin contractility. Indeed, actomyosin contractility and cortical tension increase with ECM stiffness, which in turn leads to cells applying higher tension to ECM-integrin adhesions in a variety of cell types.[12,13] It has been previously

reported that actomyosin contraction plays a role in the cellular activity of HSPC.[14–16] This mechanism can also act as a positive re-enforcing feedback mechanism.[17,18]

Although this pathway has been suggested to play a role in the mechanotransduction of biophysical cues of the environment in hematopoietic cells, there is not a clear understanding of this pathway's role in the biology of the hematopoietic stem cell (HSC) and specific progenitors.

## Results

We next sought to understand how ECM elasticity affects the proliferation and differentiation capacity of HSCs and granulocyte/macrophage progenitors (GMPs). Actomyosin contraction and subsequent membrane rigidity have been shown to increase with increasing extracellular matrix elasticity, as cells apply tension to ECM ligand adhesions.[13] It was previously reported that inhibition of myosin contractility by blebbistatin (blebb) reduced the effect of matrix cues on HSPC lineage specification; in those cases, mixed Lin-/Sca-1+/c-Kit+ mouse bone marrow (BM) cells and human CD34+ cells were used.[14–16] Addition of 10 μM blebb to PA-gel GMP cultures significantly reduced macrophage differentiation (CFU-M) on both 4kPa and 60kPa PA-gels (Figure 5.1A). Although not significant, granulocyte production (CFU-G) and total colony formation potential also appeared to increase with elasticity and blebb addition (Figure S5.1A, B). These results indicate that intracellular tension via actomyosin contraction is necessary for macrophage lineage commitment following matrix engagement, and might prevent granulocytic differentiation, at least from committed myeloid progenitors.

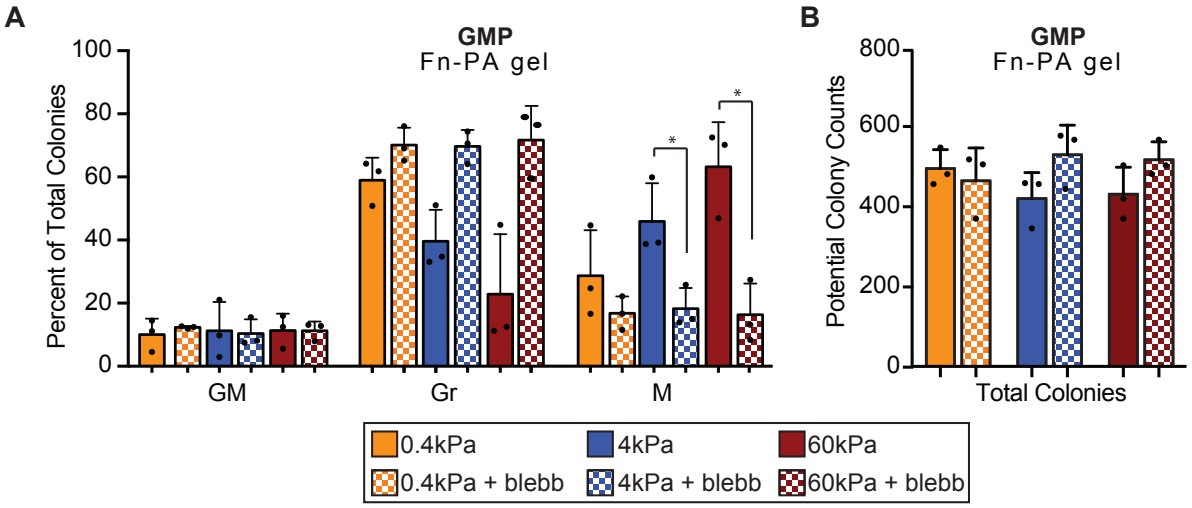


**Figure 5.1**: Actomyosin contraction affects GMP differentiation
A) Differentiation of GMPs cultured on Fn-coated PA gels ± 10 μM blebbistatin (blebb) for 1 day and plated in methylcellulose for colony forming unit assays and scored after 8 days. Colonies were scored for morphology. B) Total colonies scored. All data are means ± S.D.; dots represent independent biological repeats; * $p < 0.05$.

To test whether FAK activation was affected by matrix elasticity, we cultured HSCs and GMPs on Fn-PA gels for 18 hrs, and measured the level of phospho-activation of FAK at Try-397 ($pFAK^{397}$) by immunofluorescence. $pFAK^{397}$ increased with gel stiffness in both HSCs and GMPs (Figure 5.2A, B). In contrast, total filamentous actin (F-actin), analyzed by phallodin staining, showed no significant differences in either HSCs or GMPs across all gel stiffnesses (data not shown).

Next, we probed the role of FAK in HSC and GMP lineage fate specification, by adding 25 μM FAK inihibitor-14 to HSCs cultured on Fn-PA gels for 3 days and GMPs cultured for 1 day, before plating for 8 days in methylcellulose for CFU analysis. Macrophage differentiation (CFU-M) from both HSCs and GMPs was significantly inhibited on the stiffest 60kPa gels with the addition of FAK inihibitor-14 (Figure 5.3A, B). Granulocyte production (CFU-G) from GMPs was also significantly increased on the 60kPa gel with the addition of FAK inihibitor-14, and was similarly trending for HSCs treated with FAK inhibitor. In either case, total colony formation potential was not affected by FAK inhibition (Figure 5.3C, D). Taken together, these data demonstrate that HSCs and GMPs respond to matrix stiffness through an actomyosin contraction and FAK activation mechanotransduction pathway, which promotes macrophage differentiation while blocking granulocytic differentiation.

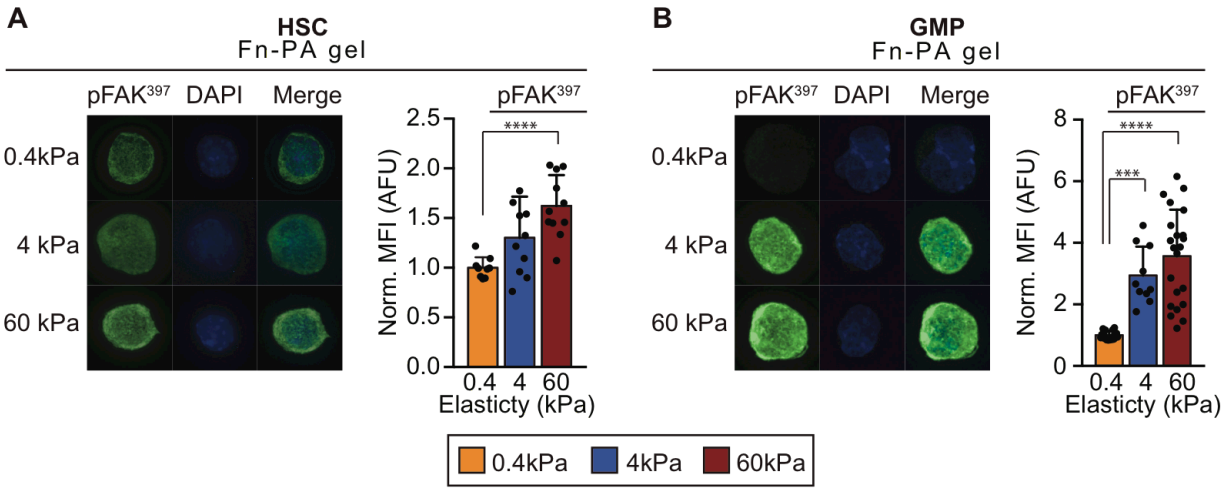


**Figure 5.2**: Focal Adhesion Kinase Activation is Affected by Matrix Elasticity
A) Immunofluorescence imaging for pFAK$^{397}$ in HSCs and B) GMPs cultured on Fn-PA gels for 18 hrs. Representative images are shown on top and quantification of mean fluorescent intensity (MFI) of individual cells on the bottom (n = 3). Results are normalized to the average MFI in cells grown on the 0.4kPa PA gels (set to 1). All data are means ± S.D; *** $p \leq 0.001$, **** $p \leq 0.0001$.

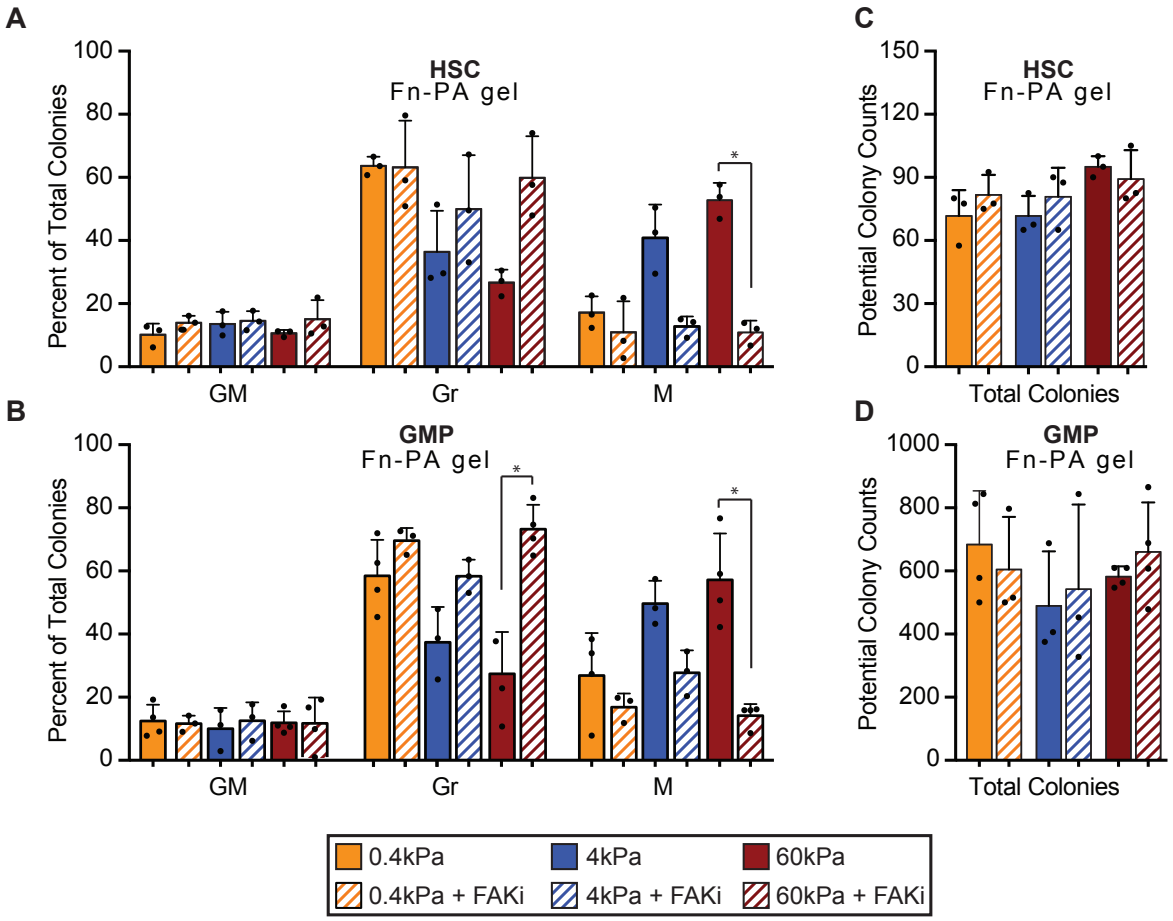


**Figure 5.3**: Mechanotransduction Controls Lineage Specification
A) Differentiation of (A, C) HSCs and (B, D) GMPs cultured on Fn-PA gels with or without (±) FAK inhibitor-14 (FAKi) for 3 days and 1 day, respectively, and plated in methylcellulose for CFU assays. Colonies were (A, B) scored for morphology and (C, D) counted after 8 days. All data are means ± S.D; dots represent independent biological repeats; * $p \leq 0.05$.

## Discussion

The maintenance of immature hematopoietic cells was previously shown to be affected by elasticity and ECM environment.[14,15] Our results indicate no functional change in immature colony formation (CFU-GM) after culture on PA gels or inhibition of matrix engagement via inhibitors. This suggests that matrix engagement (or lack thereof) cannot maintain hematopoietic cell immaturity in *ex vivo* culture.

Our data demonstrates that the biophysical properties of the BM can drive hematopoietic lineage fate differentiation in HSCs and more committed progenitors. It remained to be seen by which mechanism extracellular cues were transmitted to HSC and GMPs. Previous work has observed that mechanotransduction in HSPCs occurred via actomyosin contraction.[14,16] Here, we established a potential integrin-FAK-actomyosin mechanotransduction pathway that enables HSCs and GMPs to respond to the physical cues of their microenvironment. Inhibition of actomyosin contraction by blebbistatin or inhibition of focal adhesion by FAK inhibitor-14 prevented matrix engagement sensing and resulted in increased granulocytic differentiation. Taken together, these data suggest that macrophage production is dependent upon matrix engagement. While our results have determined that FAK and actomyosin contraction play a role in differentiation, future research could provide a more detailed analysis of hematopoietic mechanotransduction.

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# CHAPTER 6

## Matrix elasticity affects HSC engraftment capacity

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## Introduction

Hematopoietic stem cells (HSCs) are defined by their ability to self-renew and reconstitute all compartments of the blood.[1] Indeed, transplantation capacity has remained paramount for the identification and function of HSCs.[2] Transplantation engraftment is dictated by HSC activation status, proliferation state and homing ability.[3–5] Studies using genetic ablation analyses have been performed focusing on the milieu of growth factors and cytokines secreted by supporting niche cells, which maintain HSCs in a quiescent, or low activation and proliferative state.[6] These quiescent HSCs have been widely reported to have higher transplantation capacity than phenotypically similar, yet more activated and proliferative HSCs. The ability of HSCs to home to the bone marrow (BM) has been shown to be dependent on the expression of various adhesion receptors, like integrin $\alpha_4\beta_1$[5,7], and homing receptors, like Cxcr4, the receptor for the main HSC chemoattractant and retention molecule Cxcl12 (also called stromal cell-derived factor 1, SDF-1).[4,8]

A large body of work has attempted to culture HSCs *ex vivo*, focusing on biomolecular signaling, with a few studies observing higher transplantation efficiency in specific conditions.[9] A limited number of studies have aimed to create *ex vivo* culture systems mimetic of the physical properties of BM niches to address HSC transplantation capacity in response to mechanical cues.[10] These studies are intriguing, as some have shown higher transplantation capacity due to extra cellular matrix (ECM) composition and elasticity.[11] However, the direct effect of the physical properties of an environment on the ability of HSCs to be transplanted and reconstitute the blood system remains unclear, due to the use of unfractionated stem and progenitor populations in

previous studies. Using fibronectin-coated polyacrylamide gels (Fn-PA gels) mimetic of the central marrow, vasculature and endosteum, we sought to understand how the physical properties of specific BM niches affect HSC transplantation efficiency.

## Results

To address how physical properties could affect HSC engraftment capacity, we performed transplantation experiments following *ex vivo* culture of 1500 HSCs on fibronectin-coated polyacrylamide gels (Fn-PA gels), Fn-coated plastic or plastic alone for 3 days. All cells were harvested from the gels and transplanted into lethally irradiated congenic mice. 500 freshly isolated HSCs were transplanted in parallel as reference for standard engraftment without *ex vivo* culture. Strikingly, at every time point, a significantly lower total peripheral blood (PB, representative PB analysis, Figure 6.1A) chimerism was observed from HSCs cultured on soft 0.4kPa and 4kPa Fn-PA gels compared to HSCs cultured on the stiffest-60kPa Fn-PA gel (Figure 6.1B). Multilineage engraftment potential (*i.e.*, donor cell production of both myeloid and lymphoid lineages) was observed in 2 out of 5 (40%) mice (0.4kPa Fn-PA gel), 3 out of 5 (60%) mice (4kPa Fn-PA gel) and 5 out of 5 (100%) mice (60kPa Fn-PA gel). Plastic is an exceedingly stiff material (2-3 GPa).[12] Interestingly, no significant differences in total PB chimerism (Figure 6.1B) and donor HSC cell number (Figure 6.1C) were observed between HSCs cultured on Fn-coated plastic, uncoated plastic and the stiffest 60kPa Fn-coated PA gel. This suggests that stiffness, and not ECM coating, directly contributes to the maintenance of higher multilineage HSC engraftment potential.

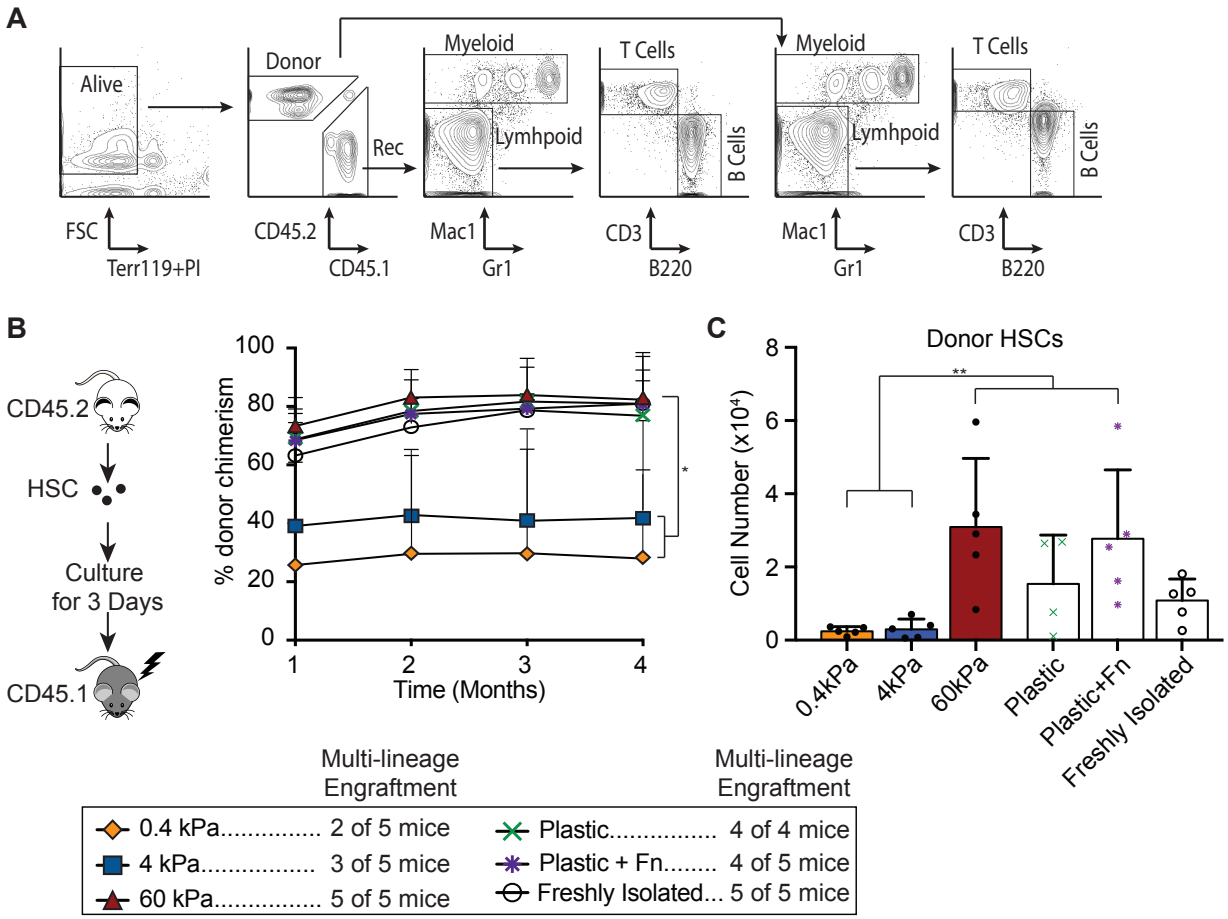


**Figure 6.1:** HSC Transplantation is Dependent on Matrix Elasticity
A) Gating strategy used to assess donor cell chimerism in peripheral blood by flow cytometry. B) 1000 HSCs were isolated from CD45.2 donor mice and cultured on a Fn-coated PA gel for 3 days, before all cells were harvested and transplanted into a single lethally irradiated CD45.1 recipient mouse. Mice were bled monthly to determine the percent of CD45.2 donor chimerism. Mice that maintain both myeloid and lymphoid chimerism above 0.5% and multilineage engraftment at 4 months post-transplantation are indicated on the right. (C) Total donor HSC cell number in bone marrow was analyzed after 4 months. Dots represent individual mice. Dots represent individual mice. All data represented as mean ± S.D.; * $p < 0.05$, ** $p < 0.01$.

Similar results showing better long-term multilineage engraftment and total donor HSC cell number with transplantation of HSCs after 3 days of culture from the stiffest Fn-PA gels were obtained in a second independent experiment (Figure 6.2A). After 4 month, multilineage engraftment potential was observed in 5 of 5 mice transplanted with cells cultured on the stiffest 60kPa Fn-PA gel. Conversely, 0 of 5 and 1 of 5 mice maintained multilineage engraftment from cells transplanted from 0.4 kPa and 4 kPa Fn-PA gels, respectively (Figure 6.2B).

To exclude the possibility that the higher engraftment from HSCs grown on stiff gels was due to more cells being transplanted, we also re-isolated 1,000 LSK-like cells from HSCs expanded for 3 days on either 0.4kPa, 4kPA or 60kPA Fn-PA gels and transplanted them into lethally irradiated recipients (Figure 6.2C). Cells isolated from the stiffest 60kPa Fn-PA gel also maintained better PB chimerism, with 5 out of 5 (100%) mice continuing to produce both myeloid and lymphoid lineage cells 4 months after engraftment. Additionally, LSK-like cells transplanted from the stiffness gel showed a significantly higher total donor HSC cell number after 4 months (Figure 6.2D). In contrast, cells isolated from the soft 0.4kPa and 4kPa gels did not maintain PB chimerism and only 1 out of 5 (20%) mice from the 4kPa transplanted group produced low levels of both myeloid and lymphoid PB blood cells after 4 months.

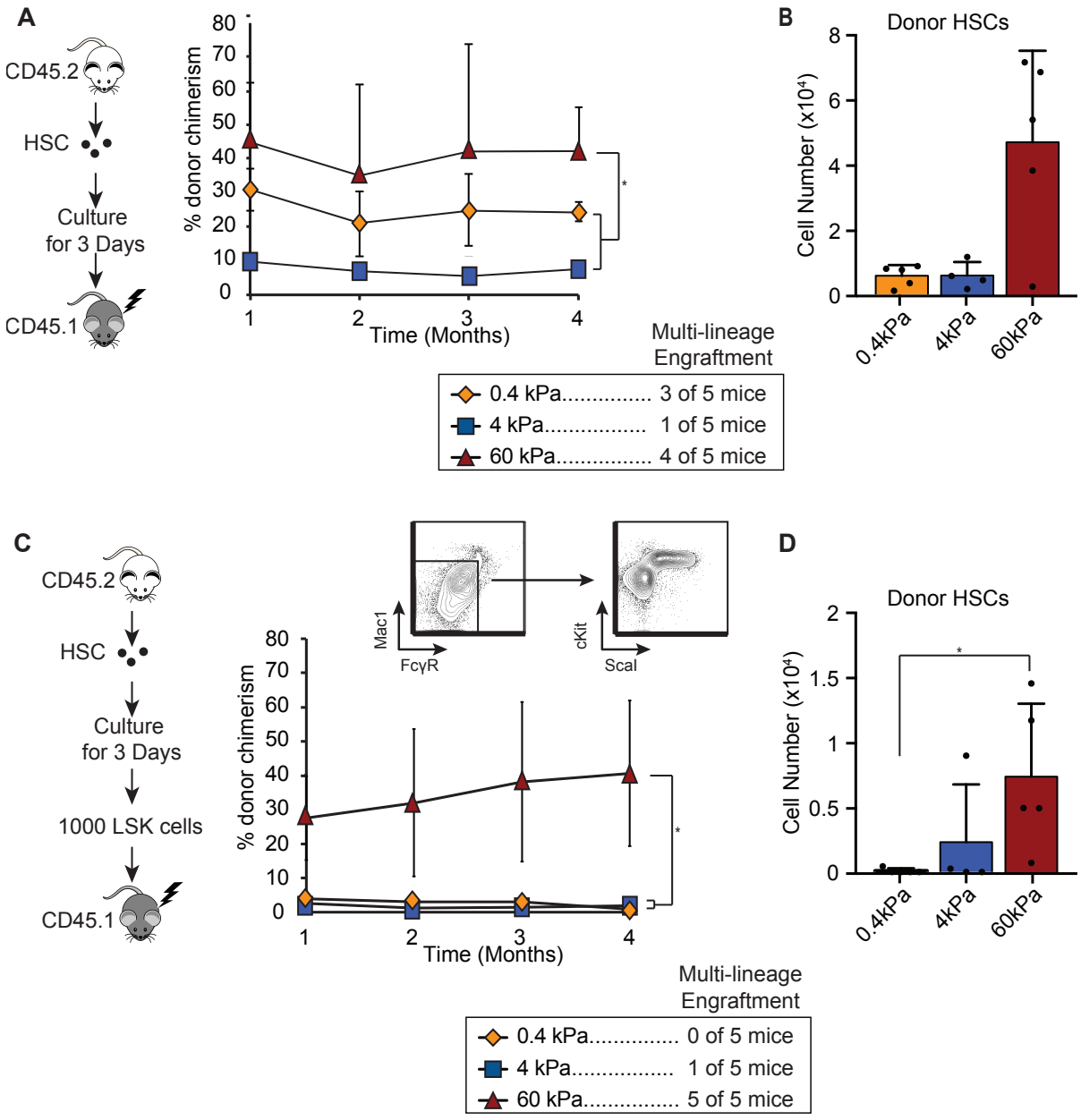


**Figure 6.2:** Stiffness Increases Transplantation Efficiency
A) 1000 HSCs were isolated from CD45.2 donor mice and cultured on a Fn-coated PA gel for 3 days before all cells were harvested and transplanted into a single lethally irradiated CD45.1 recipient mouse. Mice were bled monthly to determine the percent of CD45.2 donor chimerism. Mice that maintain both myeloid and lymphoid chimerism above 0.5% and multilineage engraftment at 4 months post-transplantation are indicated on the right. (B) Total donor HSC cell number in bone marrow was analyzed after 4 months. Dots represent individual mice. (C) 1000 HSCs were isolated from CD45.2 donor mice and cultured on Fn-coated PA gels for 3 days, harvested and pooled from several gels to re-isolate LSK-like ($FcγR^-/Mac1^-/Sca\text{-}1^+/c\text{-}Kit^+$) cells that were transplanted into lethally irradiated CD45.1 recipient mice (100 LSK-like cells per

mouse). Mice were bled monthly to determine the percent of CD45.2 donor chimerism. Mice that maintain both myeloid and lymphoid chimerism above 0.5% and multilineage engraftment at 4 months post-transplantation are indicated on the right. (D) Total donor HSC cell number remaining in bone marrow 4 months post transplantation. Dots represent individual mice. All data represented as mean ± S.D.; * $p < 0.05$.

To understand how softer environments decrease the engraftment of expanded HSCs, we next measured by flow cytometry the expression levels of various homing receptors expressed by HSCs cultured on Fn-PA gels for 18 hrs. CXCR4 expression significantly increased with elasticity (Figure 6.3A). No differences were seen between the stiffest 60kPa gel and supra-physiological stiff plastic, whereas freshly isolated HSCs had less CXCR4 expression than HSCs cultured on stiff 60kPa Fn-PA gel. Integrin $\alpha_4$ also showed increased expression on HSCs cultured on stiff 60kPa Fn-PA gel (Figure 6.3B), while $\alpha_5$ expression, which has not been directly linked to HSPC homing,[13–15] remained unchanged (Figure 6.3C). Together, these data reveal that culture on a soft *ex vivo* environment is detrimental to HSC engraftment potential, likely due to decreased expression of homing receptors on HSC surface. In contrast, CXCR4 and integrin $\alpha_4$ expression increased on HSCs cultured on stiff microenvironments, demonstrating the importance of matrix elasticity in maintaining HSC function and engraftment capability.

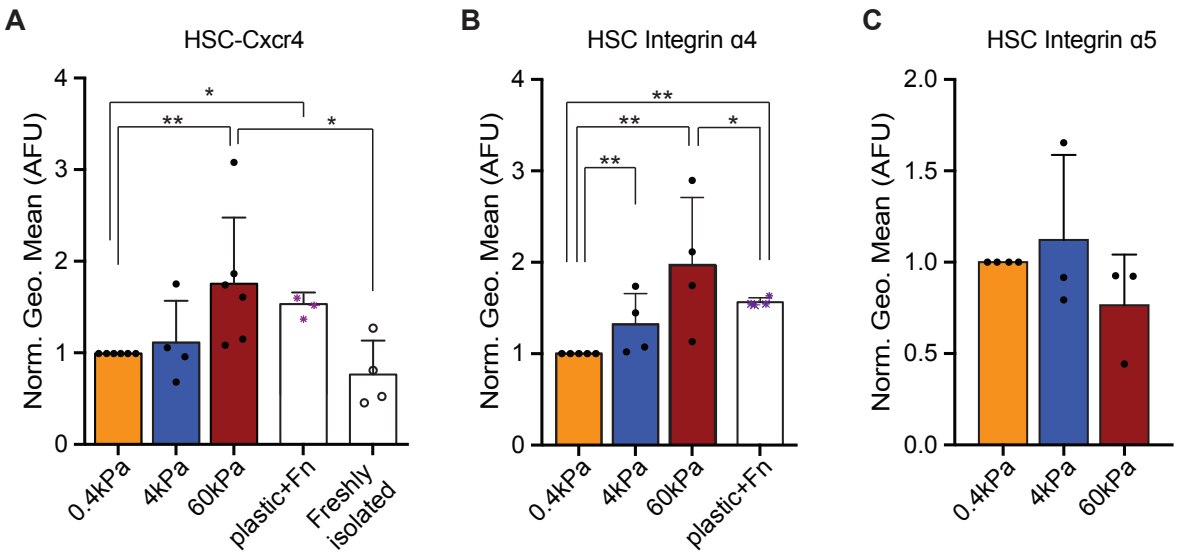


**Figure 6.3:** Elasticity Affects Homing Receptor Expression
(C-E) HSCs either cultured on Fn-PA gels or Fn-coated plastic for 18 hrs, or freshly isolated were analyzed by flow cytometry for (B) CXCR4 (C) integrin $\alpha_4$ and (D) integrin $\alpha_5$ expression levels. Dots represent biological replicates. Results are expressed as geometric mean of arbitrary fluorescent units (Geo. Mean (AFU)) normalized to the average of the 0.4kPa PA gels (set to 1). All data are means ± S.D; * $p < 0.05$, ** $p < 0.01$.

## Discussion

Our results demonstrate that culture of HSCs on soft environments decreased their engrafting ability compared to stiffer environments, likely as a consequence of decreased expression of BM homing receptors such as CXCR4 and integrin $\alpha_4$. In contrast, HSCs cultured on stiff, endosteum-like environment showed similar engraftment capacity to cells grown on supra-physiological stiff plastic. These results confirm that metabolically activated and migratory HSCs, mostly found in soft, pliable niches are not as engrafting as quiescent HSCs, mostly located in stiffer microenvironments.[3] These data help explain why HSCs isolated from the endosteum have been found to have a superior transplantation capacity and homing efficiency compared to HSCs isolated from the central marrow.[16] Moreover, this provides an explanation for why plastic expanded HSCs are still able to engraft despite being induced to differentiate over the culture period.

These data raise interesting questions about the targets of mechanosensing pathways in HSCs. A positive feedback mechanism between pFAK activation, integrin expression and CXCR4 level has been previously described in a variety of hematopoietic cells.[17–20] Our results show that this feedback loop is likely also active in HSCs, and may directly contribute to the homing capability and engraftment potential of HSCs found in various niches with distinct matrix elasticity. These findings may also help explain why pre-HSCs found in the developing embryo do not engraft in adult BM, due to a lack of CXCR4 expression.[1] This is likely a result of the pliable nature of developing fetuses, which may only start establishing stiffer environments as the fetal liver develops.[21] These speculations support the need for analyses of the biophysical

properties of all the niches in which HSCs traffic over their lifetime, to gain a more complete understanding of the spectrum of cell extrinsic cues governing their function. Taken together, our results demonstrate that distinct marrow niches affect hematopoiesis through specific biophysical mechanisms that play an important role in controlling stem and progenitor fate. Such biophysical regulation of hematopoiesis offers a new mechanism to tailor blood production and improve blood regeneration.

In the clinic, HSCs have been extensively used in human medicine for the treatment of various hematological and immune diseases via BM transplantation. Despite the success of these treatments, the limited number of HSCs suitable for transplantation poses a major obstacle.[22] Our data directly demonstrate that culturing HSCs on soft environments is deleterious for subsequent transplantation. Stiff environments would likely yield better results in future studies using 2D and 3D culture systems and niche reconstruction approaches to expand engrafting HSC *ex vivo.*[10]

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# CHAPTER 7

## Conclusions and future directions

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Although a significant amount of work has been performed to determine the impact of the soluble biomolecular factors and cellular components of the bone marrow (BM) on hematopoietic cells, understanding how the biophysical microenvironments of the BM affect hematopoiesis remains less clear.[1] This thesis aimed to understand how specific extracellular matrix microenvironments within the BM affect the function of the hematopoietic stem cell (HSC) and a myeloid-committed progenitor, the granulocyte/macrophage progenitor (GMP). This project aimed to test the overarching hypothesis that the mechanical properties of a niche microenvironment affect HSC and GMP growth, differentiation potential and adhesion, and transplantation capacity of HSCs. This hypothesis was addressed by answering the following questions:

1. What are the mechanical properties of the BM niche microenvironments?
2. How do HSCs and GMPs adhere to the niche?
3. Do the biophysical properties of specific niches affect the fate decisions of HSCs and GMPs?
4. What mechanisms control how hematopoietic stem and progenitor cells sense and respond to their environment?
5. Do the physical properties of the microenvironment affect the maintenance of HSCs and their ability to repopulate the entire blood system?

The results and future directions of each aim/question are summarized below and in Figure 7.1.

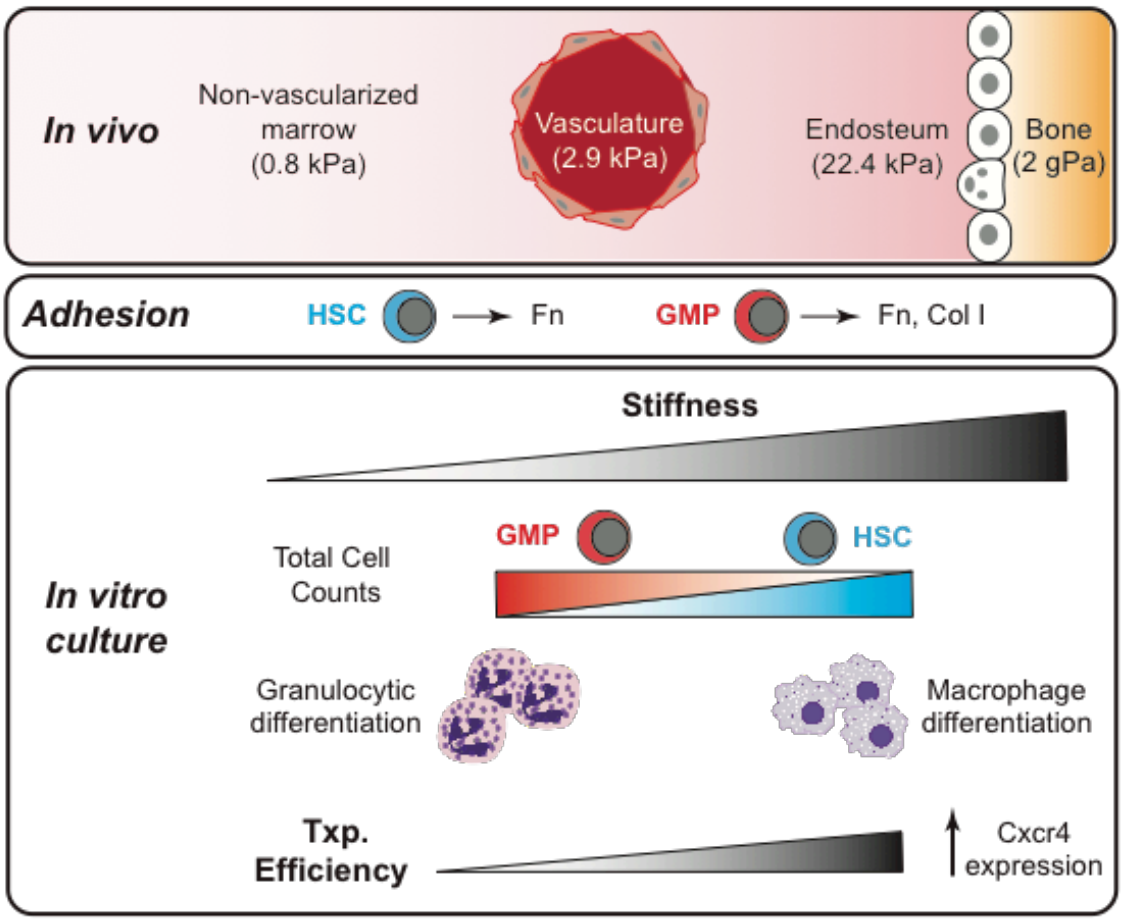


**Figure 7.1**: Biophysical Regulation of HSCs and GMPs
Model of the mechanical environments of BM microenvironments, adhesion of HSCs and GMPs to ECM ligands, and the effects of elasticity on HSC and GMPs fate

**Determining the elastic properties of the BM niche**

The work described here provided the first mechanical analysis of specific BM niches. Previous work has mainly been performed on isolated, filtered and homogenized BM cells.[2–5] Such techniques are limited due to a loss of spatial information of BM macrostructures like blood vessels, nerve fibers and the inner bone surface. Only one other study has performed viscoelastic analysis on intact bone marrow, but again, ignored the mechanical contribution due to macrostructures.[6] Our analysis is the first to determine the elasticity of the endosteum at 22.1 kPa, the vasculature at 2.9 kPa and non-vascularized central marrow at 0.87 kPa.

Although we have described the elasticity of specific niches, more work remains to get a complete mechanical understanding of BM microenvironments. Labeling blood vessels with DyLightA88-Lectin enabled us to determine the elasticity of the BM vasculature, which seemed to bifurcate around 5 kPa. Soft blood vessels ranging from 1.1 kPa to ~5 kPa likely represent fenestrated sinusoids, while stiff vessels ranging from 5 kPa to 12.3 kPa likely represent laminin-rich arterioles where laminin is more highly expressed. Typically, blood vessels can be distinguished based on anatomical position, size, morphology, and continuity of the basal lamina.[7] Our thin, 7 μm-thick sections would possibly make vascular identification a technical hurdle. However, it may still be feasible to determine the exact elasticity of sinusoids and arterioles using retro-orbital injection of labeled Scf and laminin antibodies. Sinusoids are marked by low laminin expression, and sinusoid-associated stromal cells express high Scf. Conversely, arteriolar stromal cells express low Scf and high laminin.[8] Such analysis would better refine *ex vivo* culture systems for different stem cell populations within the BM (e.g.

quiescent Ki67$^{neg}$ HSCs have been reported to reside near arterioles or perisinusoidal LepR$^{+}$,Nes-GFP$^{dim}$, PDGFR$^{-}$ mesenchymal stromal cells).

Our study of the mechanical properties of BM niches was exclusively performed within the diaphysis of femurs. HSCs reside within all bones that contain marrow. Therefore it remains to be determined whether the endosteum, the vasculature and the non-vascularized marrow niches within all bones maintain similar mechanical properties. Are there differences in niche elasticity within the epiphysis vs. diaphysis of a femur? Are niches similar in the sternum and calvarium, as load-bearing long bones like the femur?

While the technique described above was used to perform atomic force microscopy (AFM) on specific BM niches, this same technique may be able to be applied to a broad range of tissues, specifically other highly vascularized tissues like the lung, brain, spleen and liver. Moreover, the use of fluorescently labeled cells or antibodies may further our ability to measure the microenvironmental changes in elasticity within any tissue.

**Quantifying adhesion to ECM proteins**

While the localization of extracellular matrix (ECM) proteins in the BM had been reported, it remained unknown to which of these HSCs and GMPs adhere.[9–12] We found that HSCs preferentially bind to only fibronectin (Fn) acutely. This adhesion preference to Fn may explain the localization of HSCs to sinusoids, which have high concentrations of Fn due to secretion by sinusoid-associated megakaryocytes (Megs) and mesenchymal stromal cells (MSCs).[7,12] GMPs bind to Fn as well, but have higher affinity

toward collagen (Col) I. The adherent nature of GMPs to both Fn and Col I may explain the lack of specific GMPs and wide distribution of GMPs throughout the BM and endosteum.[13]

Here, we have focused on the adhesion of HSCs and GMPs to a limited number of ECM ligands found within the BM. It is known that other ECM ligands, including osteopontin, osteonectin, hyaluronic acid, fibulin, vitronectin, thrombospondins and other collagens, are also expressed in the BM.[14] The localization of these ECM ligands is not well characterized. It is likely that HSCs and GMPs also adhere to some of these ECM ligands and that the expression of these ECM ligands is niche specific. Studies further charactering the localization of and adhesion to these ECM ligands may add further evidence to the physical properties of niches within the BM and may help define hematopoietic cell specific niches. For example, Megs and perivascular MSCs are in large excess compared to HSC numbers, indicating that not all of them can act as niche cells. Therefore, we speculate that the definitive HSC niche is a perisinusoidal niche near Megs and MSCs, which highly express Fn and other potentially important ECM ligands.

**Effect of elasticity on HSC and GMP proliferation and differentiation potential**

Marrow elasticity is critical in regulating the proliferation rates of HSCs and GMPs. We demonstrate that a stiff environment promotes HSC proliferation and inhibits GMP proliferation. Conversely, a soft environment promotes GMP expansion. These results appear to be consistent with hematopoietic biology during active, regeneration conditions. After transplantation, the HSC needs to re-establish the entire hematopoietic

system and has been shown to localize closer to the endosteal region.[15] Conversely, our lab has previously shown that GMPs form clusters of differentiation throughout the central marrow cavity, during regeneration.[13]

We have also demonstrated that elasticity directs hematopoietic lineage fate specification. Soft, central marrow-like environments promoted granulocytic differentiation, and stiff endosteal-like environments promoted macrophage differentiation. These findings appear to match the innate biology of the hematopoietic system. The GMP clusters that form throughout the soft, central marrow cavity during regeneration initially produce a burst of granulocytic differentiation before returning to control hematopoiesis.[13] Conversely, standard culture conditions of HSCs or GMPs grown on stiff plastic result in an outgrowth of macrophages.[16] Additionally, macrophages within the BM cavity, called osteomacs, are primarily found at the endosteum.[17] We demonstrate that regardless of culture condition, there was no benefit to the maintenance of a more immature stem-cell like population.

While this work has revealed that matrix elasticity may control the function of both HSCs and GMPs, many questions remain. Our culture systems used 3 day cultures, which may explain why we did not see megakaryocytic or erythrocytic lineage fate specification. It has been reported that soft environments promote megakaryocytic or erythrocytic differentiation; however, such analysis has not been performed on HSCs.[18,19] Furthermore, short-term cultures of a pool of unfractionated stem and progenitor cells have shown that ECM ligand engagement can also direct lineage fate.[19,20] Our longer-term cultures saw no differences in cell proliferation or

differentiation capacity between different ECM ligand coatings. Would ECM ligand engagement have an effect on lineage specification in short-term cultures of HSCs?

We know that the biophysical properties of the BM exist in gradients of ECM ligand composition and elasticity. Recent advances in HSC clonal tracking have revealed that HSCs are a heterogeneous population, such that HSCs appear to be biased toward specific lineage differentiaton.[21–23] It is likely that this heterogeneity is a result of niche localization controlling the fate of the resident HSC through both biomolecular and biophysical cues. With the recent advances in barcoding,[21–23] *in vivo* real-time microscopy[15,24] and HSC specific fluorescent markers,[7,25] it may be possible in the near future to functionally phenotype HSC fate specification due to localization within the BM and the known viscoelastic and ECM ligand composition of that niche.

**Mechanotransduction of matrix elasticity**

We had determined that matrix elasticity had a significant impact on the proliferation and lineage differentiation potential of both HSCs and GMPs. Yet, the mechanism of mechanotransduction was unknown. Previous work had determined that ECM stiffness correlated with higher cellular tension due to increased actomyosin contractility.[5,26] Additionally, it had been reported that the differentiation potential of an unfractionated pool of hematopoietic stem and progenitor cells was affected by actomyosin inhibition using blebbistatin.[19,27] Therefore, we next aimed to determine if HSC and GMP lineage specification due to matrix elasticity was dependent on actomyosin contraction. Consistent with previous reports, macrophage production from GMPs was dependent on intracellular tension following matrix engagement. A loss of

actomyosin production increased granulocytic differentiation. We have not determined if inhibition of actomyosin contraction by blebbistatin would result in a loss of macrophage production from HSCs, but we suggest that this result is highly likely.

Following these results, we next aimed to probe this pathway upstream of actomyosin contraction. Choi and colleagues determined that inhibition of ROCK, a downstream effector of RhoA, also played an essential role in hematopoietic stem and progenitor cell differentiation capacity.[19] Therefore, we sought to determine how focal adhesion activation affected HSC and GMP lineage fate specification. We found that the phospho-activation of FAK increased with stiffness in both HSCs and GMPs. Inhibition of FAK caused similar effects to actomyosin inhibition, such that FAK inhibition resulted in increased granulocytic differentiation and a loss of macrophage differentiation. Taken together, these data suggest that HSCs and GMPs respond to matrix elasticity via a mechanotransduction pathway dependent on integrin engagement, FAK activation and actomyosin contraction.

While the integrin engagement, focal adhesion and actomyosin contraction pathway have been widely reported, other potential mechanisms of mechanotransduction may help to explain how hematopoietic cells sense and respond to the physical properties of their environment. We initially looked at the Hippo pathway co-transcriptional activators YAP and TAZ. Analysis of MSCs reveals that nuclear localization of YAP/TAZ and formation of F-actin are proportional to matrix elasticity.[28] My own analysis of published microarray data revealed that expression of *Yap* and *Taz* is higher in HSCs, and it decreases in progenitors and mature cells. This suggests that expression the Hippo pathway may be a potential mechanism for

mechanotransduction.[29] We found no expression of YAP or TAZ in adult hematopoietic cells (data not shown). This is not entirely surprising, as a recent study demonstrated no measurable effects of *Yap* overexpression on steady state hematopoiesis or HSC/P engraftment upon transplantation.[30]

Another mechanotransduction pathway of interest is the TGF-β signaling pathway, which plays key roles in hematopoiesis. TGF-β is regulated by cell-generated mechanical tension, which releases active TGF-β from stiff ECM.[31] TGF-β is secreted by Megs and is a known regulator of HSC quiescence.[1] This would suggest that in stiffer environments, HSCs may generate more mechanical tension, thereby releasing more TGF-β in the active form, promoting quiescence. Quiescent HSCs are thought to localize to a periarteriolar niche, which is likely stiffer than a perisinusoidal niche, where activated HSCs are found to reside.[8] It is possible that the biomechanics of the periarteriolar niche enforce quiescence through TGF-β mechanotransduction. Additionally, sympathetic nerve fibers, which run adjacent to arterioles, are sheathed by non-myelinating Schwann cells, which have been shown to activate latent TGF-β.[32] There are a number of other potential mechanotransduction pathways that are likely activated in hematopoiesis, including Jak/Stat signaling transduction and other Rho family GTPases like cdc42 and Rac1. Detailed analyses of these mechanotransduction pathways would greatly benefit our understanding of the biophysical regulation of hematopoiesis.

**Matrix Elasticity Affects HSC Engraftment Capacity**

HSCs are defined as cells that are capable of regenerating the complete hematopoietic system following transplantation.[33] The ability of HSCs to engraft is a result of activation status, proliferation state, and the expression of adhesion and homing receptors.[34–36] We sought to understand how HSCs engraftment capacity was affected by the elasticity of *ex vivo* culture and determined that stiffness directly contributed to the maintenance of multilineage engraftment potential. Due to significant decreases after only one month following transplantation from culturing in soft vs. stiff environments, we analyzed the expression levels of various homing receptors. CXCR4, which is the receptor for the main HSC chemoattractant and retention molecule CXCl12, was significantly lower after culture on more compliant gels. Additionally, integrin $\alpha_4\beta_1$, which has been shown to be necessary for the retention of HSCs within the BM, was decreased on soft gels. These results confirm that activated HSCs, which are found in a softer perisinusoidal niche, have less engraftment potential compared to quiescent HCSs, found within a periarteriolar niche.[8,34] Additionally, our data is consistent with previous work demonstrating that HSCs isolated from the endosteum have greater transplantation potential and homing capacity compared to HSCs isolated from central marrow.[37]

Several questions remain to further understand how elasticity affects homing efficiency. Once an HSC is in circulation, there is a multi-step process of rolling, adhesion and transmigration as a cell moves from circulation into the BM. Rolling and adhesion are mediated by E- and P-selectin, and integrin, namely $\alpha_4\beta_1$.[38] It remains to be seen if elasticity affects selectin expression and thus the initial rolling and adhesion

of HSCs to the vascular wall. Once in the BM, an HSC must migrate towards its niche, which is dependent on integrin adhesion and the chemokine receptor Cxcr4. We have demonstrated that elasticity correlates with increased integrin $\alpha_4\beta_1$ and Cxcr4 expression, but have not determined if migration capacity is affected.

As stated above, engraftment is a result of activation status, proliferation state, and the expression of adhesion and homing receptors. In total, our data suggests that soft environments cause homing deficiencies. The early proliferation of HSCs showed no difference regardless of ECM ligand or ECM elasticity. Additionally, all of our methylcellulose analysis shows no significant difference in colony formation of the most immature CFU-GM colonies regardless of elasticity. It remains to be seen if cells isolated from environments of different elasticities could have the same capacity to rebuild the hematopoiesis system, not withstanding the initial homing to the BM. Would negating homing deficiencies via intrafemoral injection of HSCs eliminate the transplantation differences seen when HSCs were isolated from cultures of different elasticities? We would predict that due to lower Cxcr4 and integrin $\alpha_4\beta_1$ expression HSCs isolated from soft environments might still have lower transplantation efficiency compared to those HSCs isolated from stiff environments.

The work performed in this thesis may have broad implications for the clinical transplantation of HSCs, which are potentially curative for a wide range of patients. Much work has been performed to increase both the safety and efficacy of BM transplantation. Our data suggests that short-term culture on stiff environments may be advantageous for the homing of HSCs to the BM.

**Future Directions:**

Alterations in the BM niche are commonly observed in blood malignancies and directly contribute to the aberrant function of transformed HSCs with disease-initiating leukemic stem cells (LSC) properties.[1,39] It has also become clear that leukemic hematopoiesis directly remodels the BM niche into a self-reinforcing malignant BM niche that supports disease development at the expense of normal hematopoiesis. Using the *Scl-tTA::TRE-BCR/ABL* (BA) mouse model of human chronic myelogenous leukemia (CML), our laboratory has recently shown that leukemic cells stimulate MSCs to proliferate and adopt an abnormal differentiation program. This results in the overproduction of functionally altered osteoblastic lineage cells, which accumulate in the BM cavity as inflammatory myelofibrotic cells.[39,40] The fibrotic remodeling of the BM microenvironment is characterized by abnormal deposition of Col I and III, which directly alters the physical properties of the BM niche (Figure 7.2A). Performing adhesion analysis as previously described, BA LSCs revealed greater adhesion to both Col I and III, than wild-type (WT) HSCs. In fact, BA LSCs were twice as adherent to Col I than WT (Figure 7.2B). In contrast, BA GMPs showed no change in adhesion compared to WT GMPs (Figure 7.2C). This suggests that the transformed leukemic cells have adapted to bind these fibrotic regions, while healthy WT HSCs have not.

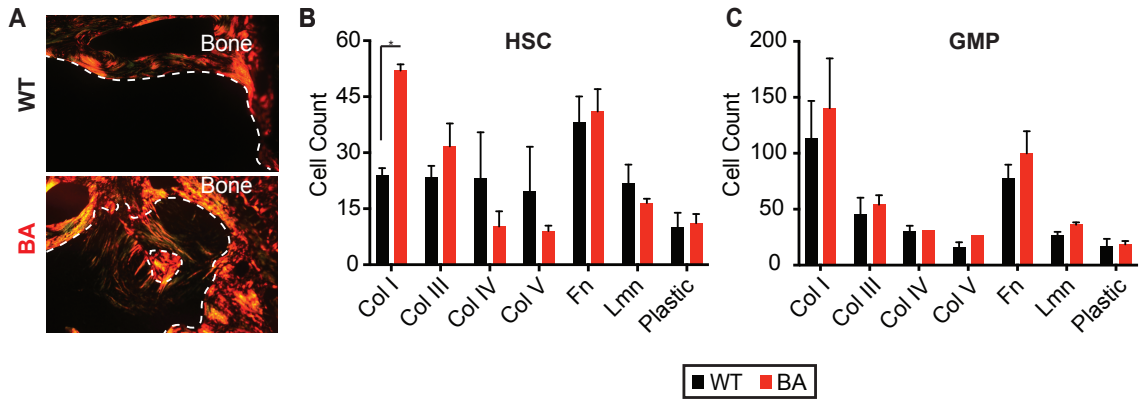


**Figure 7.2:** Adhesion of Leukemic HSCs and GMPs to ECM Ligands
A) Polarized light microscopy of sternum sections stained by picrosirius red (PSR) staining from wildtype (WT) or *Scl-tTA::TRE-BCR/ABL* (BA) mice. Collagen bundles appear in green, red and yellow. B) Adhesion of HSCs or C) GMPs to 10.0 µg/ml of ECM protein coated tissue culture plastic after 3 hrs.

The ability of LSCs to outcompete normal HSCs has been widely reported.[40–42] Currently, the only known curative therapy for specific types of leukemia is high dose chemotherapy followed by allogeneic HSC transplantation.[43] However, LSCs are often able to re-engraft following transplantation, leading to disease relapse.[44,45] As we have described earlier, stiffer environments lead to greater engraftment of WT HSCs following transplantation. It is likely that fibrosis not only affects the ECM ligand concentration and localization, but also increases the stiffness of BM niches. Therefore, BA LSCs residing in a stiffer, fibrotic niche may have increases in homing receptor expression, thus outcompeting engraftment following chemotherapy and transplantation. Understanding how the physical properties of the fibrotic, leukemic niche affect how the niche control hematopoiesis could have direct translational applications. It may be possible to manipulate normal or transformed HSC fate by directly changing the biophysical properties of the BM niche, or by altering HSC mechanosensing activity using small molecule inhibitors. Understanding how the biomechanics of the leukemic BM niche contribute to the clonal dominance of transformed HSCs could identify potential targets to disfavor LSC re-engraftment, thus paving the way for the development of truly curative treatments.

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# CHAPTER 8

## Materials and methods

**Mice**

Wild-type C57Bl/6-CD45.2 mice, 8- to 12-weeks old, were used for all primary cell isolation and as donor cells for transplantation experiments. Scl/Tal-1-tTA and TRE-BCR/ABL mice were interbred in presence of 20 mg/l doxycycline (Sigma-Aldrich) in their drinking water. Bones were isolated from 8- to 12-weeks old mice.Wild-type C57Bl/6-CD45.1 mice (BoyJ), 9- to 13-weeks old, were used as recipient mice for cell transplantations. Female and male mice were used both as donor and recipients. Recipient mice were lethally irradiated (11 Gy delivered in a split dose 3hrs apart) using a $Cs^{137}$ source (J. L. Shepard) and transplanted via retro-orbital injection with freshly isolated HSCs and HSC-derived cells harvested from PA-gel cultures together with 300,000 Sca-1-depleted helper CD45.1 bone marrow cells (in 100 µl PBS). Transplanted mice were treated with antibiotics (10^6 U/L polymyxin B sulfate and 1.1 g/L neomycin sulfate) for 4 weeks post-transplantation and analyzed for donor-chimerism by monthly retro-orbital bleeding. Blood was collected into tubes containing 4 ml of ACK (150 mM $NH_4Cl$ and 10 mM $KHCO_3$) and 10 mM EDTA and analyzed for donor chimerism and multilineage reconstitution by flow cytometry analyses as previously reported [1]. No specific randomization or blinding protocol was used, and all experiments were performed in accordance with UCSF IACUC approved protocols.

**Atomic Force Microscopy (AFM) measurements of femur sections.**

Mice were injected with 100 µL of 1:2 DyLight488 Labeled Lycopersicon Esculentum Lectin (Vector Laboratories):PBS 15 min prior to euthanizing. Femurs were harvested, snap frozen in OCT (Tissue-Tek) and stored at -80°C until sectioning.

Frozen samples were cryosectioned (7 μm) onto adhesive tape windows using a Cryostat equipped with a tungsten blade and the CryoJane tape transfer system (Leica Microsystems). Tape windows were then mounted section side up onto a standard microscopy slide and keep on dry ice until use. Samples were rapidly thawed and imaged for bright field and immunofluorescence before being immersed in PBS with protease inhibitors (Roche, Complete Mini protease inhibitor cocktail) for the duration of the AFM measurements. All AFM indentations were performed using an MFP3D-BIO inverted optical AFM (Asylum Research) mounted on a Nikon TE2000-U inverted fluorescent microscope as previously reported [2–4]. Silicon nitride cantilevers with spring constant of 0.04 to 0.06N/m with borosilicate glass spherical tip of 5μm diameter (Novascan Tech) were used. The cantilever was calibrated using the thermal oscillation method prior to each experiment. Samples were indented at 1 μm/s loading rate, with a maximum force of 2 nN. AFM force maps were typically obtained as a 10 x 10 raster series of indentations utilizing the FMAP function of the IGOR PRO build supplied by Asylum Research, for a total of 100 data point per area of interest measured every 5 μm (50 x 50 $\mu m^2$ total area). Measurements at the endosteum we performed in variable sized regions due to topography of sections. The Hertz model was used to determine the elastic properties of the tissue (E1). Tissue samples were assumed to be incompressible and a Poisson's ratio of 0.5 was used in the calculation of the Young's elastic modulus.

**Flow cytometry**

Hematopoietic stem and progenitor cells were isolated or analyzed as previously described [5]. BM cells were isolated by crushing legs, arms, pelvis and sternum in staining medium consisting of HBSS supplemented with 2% heat-inactivated FBS (Sigma-Aldrich). Erythrocytes were lysed with ACK buffer and contaminating bone debris and dead cells were removed by centrifugation on a Ficoll gradient (Histopaque 1119, Sigma-Aldrich). BM cells were first enriched for c-Kit+ cells using c-Kit microbeads (Miltenyi Biotech) and either an automated AutoMACS cell separator (Miltenyi Biotech) or manual MACS Separation LS Columns (Miltenyi Biotech). C-Kit-enriched BM cells were then stained with purified, unconjugated lineage antibodies (CD3 from BioLegend; CD4, CD5, CD8, B220, Ter119, Mac-1, and Gr-1 from eBioscience) followed by goat anti-rat-PE-Cy5 (Invitrogen) and subsequently blocked with purified rat IgG (Sigma-Aldrich). Cells were then stained with c-Kit-APC-eFluor780 (eBioscience), Sca-1-PB (BioLegend), CD48-A647 (BioLegend), CD34-FITC (eBioscience), CD150-PE (BioLegend), and Flk2-bio (eBioscience) followed by SA-PeCy7 (eBioscience). For peripheral blood chimerism analyses, cells were stained with Mac-1-PE-Cy7 (eBioscience), Gr-1-PB (eBioscience), B220-APC-Cy7 (eBioscience), CD3-APC (eBioscience), and Ter-119-PE-Cy5 (eBioscience), CD45.2-FITC (eBioscience) and CD45.1-PE-Cy7 (eBioscience). All staining and washing steps were performed in HBSS + 2% FBS, and stained cell were finally re-suspended in the same staining media containing 1 μg/mL dose of propidium iodate (PI). Cells were sorted on a fluorescence-activated cell sorting (FACS) ARIAII and/or analyzed on an LSRII (Becton Dickinson) upon PI exclusion of dead cells. Each population was double sorted to ensure maximum

purity. For analyses of cells cultured on PA gels, cells were first detached from the ECM ligands by 5 min incubation with Accutase (Gibco) or 0.25% Trypsin (Thermo Fischer) followed by two washes with staining medium to collect every cell. Cells were then incubated with CXCR4-BrilliantViolet-605 (Biolegend), CD49d-PE (Biolegend) or CD49e-PE (Biolegend) before analyses on an LSRII upon PI exclusion of dead cells. CFSE (Thermo Fischer) analyses were performed per manufacturer's suggestion. Briefly, isolated HSCs or GMPs were incubated in 1 mL of warm 1:1000 CFSE in PBS for 20 min at 37 °C before washing with 5 mL of staining medium. The staining reaction was stopped by a 10 min incubation with warm culture media (described below) at 37 °C before transferring CFSE-labeled cells to the PA-gels.

**Cell Culture and Adhesion Assay.**

All cultures were performed at 37 °C in a 5% $CO_2$ water jacket incubator (Thermo Scientific). Cells were grown in StemPro34 medium (Invitrogen) supplemented with penicillin (50 U/ml)/streptomycin (50 µg/mL), L-glutamine (2mM), SCF (10 ng/ml), Flt3L (10 ng/ml), IL-11 (10 ng/ml), IL-3 (10 ng/ml), GM-CSF (20 ng/ml), Epo (10 U/ml) and Tpo (0.1 mg/ml) (Peprotech). Cultures on PA gels (described below) were performed in 300 µl of media, with half of the medium replaced after 3 days of culture. For inhibitor studies, cells were isolated in 150 µl of medium, plated onto PA gels and allowed to adhere for 1 hour before another 150 µl of medium containing 2x the final concentration of each inhibitor or vehicle was added. 10 µM Blebbistatin (Abcam) and FAK inhibitor-14 (Tocris) were used at the final concentration of 10 µM and 25 µM, respectively, and were both prepared in DMSO. For adhesion assays, 96 well plates were pre-coated

overnight at 37°C with 0.1, 1.0 or 10.0 µg/ml per well of each ECM protein (re-suspended in 100 µl of 100 mM HEPES/100 mM NaCl, pH 7.2) and washed once with PBS. ECM proteins used were: Collagen I (Col I, mouse, Corning); Collagen III (Col III, human, Corning); Collagen IV (Col IV, mouse, Corning); Collagen V (Col V, human, Corning); Fibronectin (Fn, bovine, Sigma-Aldrich); Laminin (Lmn, mouse, Corning). For combined Fn and Col IV adhesion experiments, 10.0 µg/ml Fn was coated first overnight at 37°C, washed once with PBS, and increasing amounts of Col IV (0.1, 1.0 or 10.0 µg/ml) were then added for 12 hours at 37°C and finally washed once with PBS. HSCs or GMPs (250 cells in 100 µl of culture medium) were then added to each well, allowed to adhere for 0.5, 3 or 12 hours before mechanical dissociation by gentle vortexing. Non-adherent cells were washed with PBS, and adherent cells were fixed with 4% PFA and stained with 1:1 Trypan blue in PBS to allow counting using a 20x objective tissue culture bright field microscope. RGD adhesion analysis was similarly performed with the addition of 1, 10 and 100 µM RGD peptide (Sigma) together with 10.0 µg/ml of Fn.

**Polyacrylamide Gels, Growth Analysis and Methylcellulose Culture.**

Polyacrylamide hydrogels (PA-gels) were prepared as previously described (Lakins *et al.*, 2012; Przybyla *et al.*, 2016). Briefly, gels were cast on 18-mm glass circular coverslips using previously described recipes calibrated for 0.4, 4 and 60kPa stiffness gels. Gels were coated with 300 µl of either Col I or Fn (50 µg /ml in of 100 mM HEPES/100 mM NaCl, pH 7.2) overnight at 37 °C. 1000 HSCs or GMPs were directly sorted onto gels and cultured, as described above for cell growth and methylcellulose

assays. For growth analyses, cells were harvested after 3 and 6 days of culture on PA-gels using 0.25% Trypsin with EDTA, and live cells were manually counted using 1:1 Trypan blue in PBS with hemocytometer. For methylcellulose colony-forming unit (CFU) assays, GMPs were collected after 1 day and HSCs after 3 days of culture on PA-gels using 0.25% Trypsin with EDTA. A fraction of GMP-derived (1/25th or 1/50th) and HSC-derived (1/5th) cells were plated directed into 3 cm dishes containing 1 ml of methylcellulose (Stem Cell Technologies, M3231) supplement with IL-3 (10 ng/mL), GM-CSF (20 ng/mL), SCF (10 ng/mL), IL-11 (10 ng/mL), Flt-3L (10 ng/mL), Tpo (100 ng/mL) and Epo (4 U/mL). Colonies were visually scored for numbers and types of colonies (CFU-GM, CFU-G and CFU-M) after 8 days of culture.

**Scanning Electron Microscopy.**

Glass slides were incubated with 10 µg/mL of Fn diluted in 100 mM NaCl/100 mM Hepes, pH 7.2 overnight at 37°C. Sorted cells were resuspended in 150 µL of culture medium, pipetted onto Fn-coated slides and cultured for 3 hours at 37°C. Cell were then fixed in 4% PFA in PBS for 30 min, washed once with PBS, re-fixed in 0.1 M sodium cacodylate/1% glutaraldehyde, pH 7.5, for 2 hours, washed once in 0.1 M sodium cacodylate, then dehydrated in a series of EtOH baths (30, 50, 70, 90, 100%) as previously described [9,10]. Samples were dried using a critical-point dryer, followed by 8 nm sputter coating with either gold or gold/platinum prior to image acquisition on a Zeiss Ultra55 FE-SEM in the San Francisco State University Electron Microscopy Facility. A minimum of 15 cells were imaged per population.

**Statistics**

All experiments were repeated as indicated, with n indicating the numbers of independent biological repeats. For all culture experiments, measurements were performed in triplicate for each independent biological repeat, except where indicated. For all imaging experiment, representative examples of at least two independent biological repeats are shown. Data are expressed as means ± S.D. or ± S.E.M. where appropriate. Statistical analyses were performed using Prism 5.0 software (GraphPad). Pairwise statistical significance was evaluated by nonparametric t-test (Mann-Whitney) for all histograms, unless otherwise noted. Mice for treatment and transplantation were randomized, and no blinding protocol was used. No statistical method was used to predetermine sample size. $P \leq 0.05$ was considered statistically significant.

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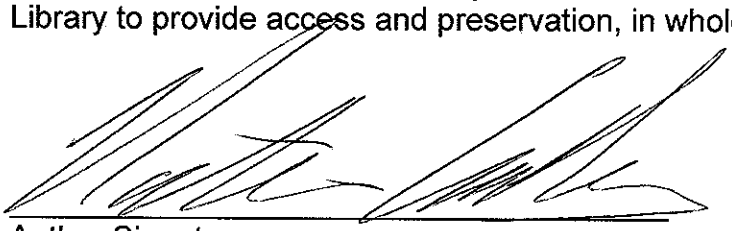

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