

ELUCIDATING CELL FATE REGULATION OF THE BONE MARROW VASCULAR
NICHE THROUGH DEVELOPMENT OF ENGINEERED EXTRACELLULAR MATRICES

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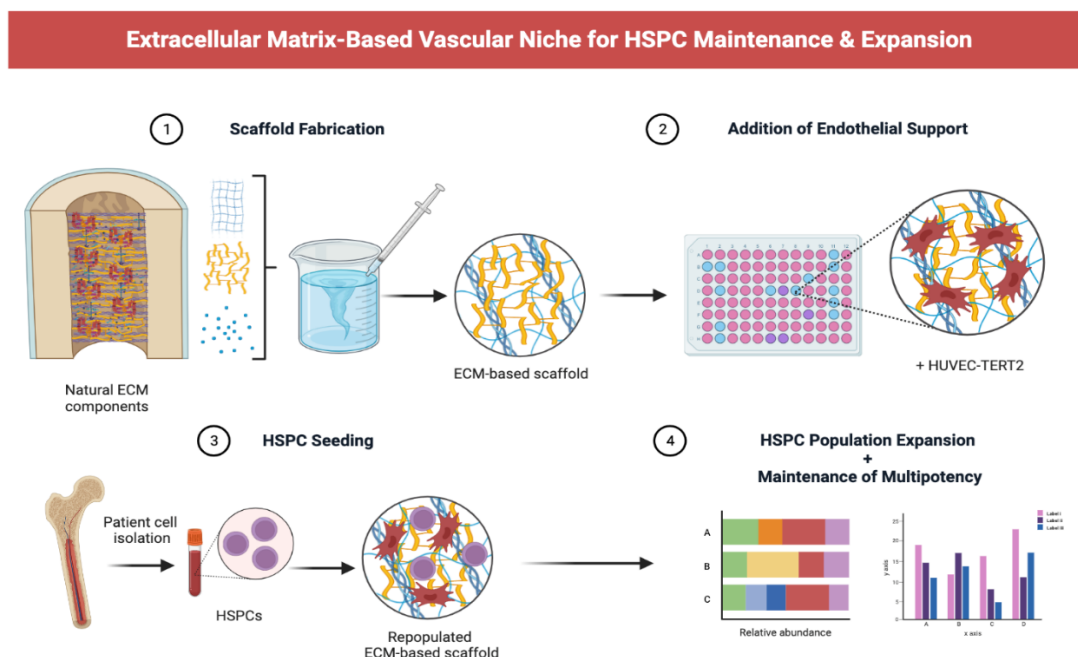
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ABSTRACT

Haematopoietic stem and progenitor cells (HSPCs) are crucial to the curative treatment of a variety of leukaemias and autoimmune diseases. However, their rapid differentiation and exhaustion within 72 hours *ex vivo* poses a barrier to efficient treatment. Here, we have developed a culture method that mimics that of the *in vivo* haematopoietic vascular niche in order to extend viability and maintain multipotency of HSPCs. The two-factor approach combined the paracrine support of endothelial cells as well as the structural and chemical support of an engineered two-dimensional scaffold comprised of several extracellular matrix (ECM) components. HSPCs that were co-cultured on ECM-mimicking scaffolds maintained higher viability in culture for up to seven days in comparison to solo-culture counterparts, and functional tests revealed an improvement in colony formation potential. Continued development of the culture system and understanding of the molecular pathways at play can lead to improved patient outcomes after transplantation.

Graphical Abstract



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LIST OF ABBREVIATIONS

AML	Acute myeloid leukaemia
C	Collagen type I
CD34	Cluster of differentiation 34 protein
CD38	Cyclic ADP ribose hydrolase
CD45	Protein tyrosine phosphatase, receptor type, C
CD49f	Integrin $\alpha 6$
CXCR4	Chemokine receptor 4
CXCL12	CXC motif chemokine ligand 12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
E	Elastin
EDTA	Ethylenediaminetetraacetic acid
ECM	Extracellular matrix
GAG	Glycosaminoglycan
mHA	Hydroxyapatite powder, 10 μm
nHA	Hydroxyapatite nanopowder (<200 nm)
HSC	Haematopoietic stem cell
HSPC	Haematopoietic stem and progenitor cell
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PECAM1	Platelet endothelial cell adhesion molecule
PEF	PBS, EDTA, FBS
PS	Polystyrene
ROS	Reactive oxygen species
SCF	Stem cell factor
SFRP1	Secreted frizzles-related protein 1
VEGF1	Vascular endothelial growth factor
2D	Two-dimensional
3D	Three-dimensional
7-AAD	7-aminoactinomycin D

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CHAPTER 1

Introduction

CHAPTER 1: INTRODUCTION

1.1 The Study of Haematopoietic Stem Cells: An Overview

In order for the human body to function, haematopoietic stem and progenitor cells (HSPCs) must maintain blood production and facilitate immune responses. This typically occurs through the process of hematopoiesis, where native stem cell populations are maintained in a quiescent state while some occasionally enter the cell cycle to continue to replenish blood cell supply¹. However, when this process is impaired in various disorders or diseases, for example those that deplete stem cell populations or restrict multi-lineage differentiation, immediate intervention is required². The most efficient curative treatment for hematological dysfunction is bone marrow transplant, consisting of both autologous transplants with cells sourced from the patient themselves and allogenic transplants with cells sourced from a donor³. Despite the existence of this treatment, several barriers remain to expanding transplant access, such as poor cell survival, insufficient expansion from certain cell sources like umbilical cord blood⁴, or differences in engraftment and homing potential of cells into the patient's native bone marrow once transplanted⁵. To understand how to improve HSPC treatments, it is vital to first understand the fundamentals of the cells themselves.

1.1.1 The Haematopoietic Hierarchy

Haematopoietic stem cells were first discovered by Till & McCulloch in 1961 using a combination of irradiation and transplantation studies with an *in vivo* spleen colony forming unit (CFU) assays to determine the function of the transplanted bone marrow aspirate. This foundational research found that only a very small fraction of their cell sample was capable of colony growth and repopulation, and that all progenitor and mature cells must have been derived from this singular

subset of cells⁶. As research progressed, the use of cell surface markers gave rise to what is now seen as a classical hierarchal model of haematopoiesis. This hierarchy is one that is able to be studied at each stage using a variety of techniques. While the use of phenotypic identifiers can identify the compartment to which a cell belongs, these analyses must be coupled with assays that can determine if the cell has maintained function. The most primitive and rare population of cells are the HSCs themselves, which have the capacity to differentiate into any mature haematopoietic cell. As the cells continue to differentiate, their fates become increasingly restricted from multipotent to oligopotential and eventually unipotent progenitors (Figure 1). Most mature haematopoietic cells, such as erythrocytes, can be identified morphologically. However, the function of precursors, lineage-committed progenitors, and even multipotent progenitors can be demonstrated in a CFU assay. The determining experiment in confirming HSC identity is a transplantation and repopulation assay to determine if the cells are of a long-term (LT)-HSC identity or short term (ST)-HSC identity. LT-HSCs will be able to perpetually repopulate the haematopoietic compartment and maintain their population in a state of quiescence⁷. Similarly, ST-HSCs can also rapidly repopulate the haematopoietic compartment after irradiation, but will differentiate into multipotent progenitors that are not capable of continuous self-renewal and thus will be exhausted⁸. It is important to note that this hierarchal model is not static – that is, even cells of a specific compartment can show variations in fate. Recent developments in single-cell sequencing have demonstrated that the phenotypically compartmentalized cells possess a great deal of heterogeneity in their fate decisions⁹. Even those that share a compartment, such as granulocyte and macrophage progenitors, can differ in chromatin accessibility that can skew their lineage towards a myeloblast or monoblast precursor fate¹⁰. Despite having an understanding of these processes, the ability to expand HSPC populations *ex vivo* while maintaining multipotency and restricting differentiation has remained elusive¹¹.

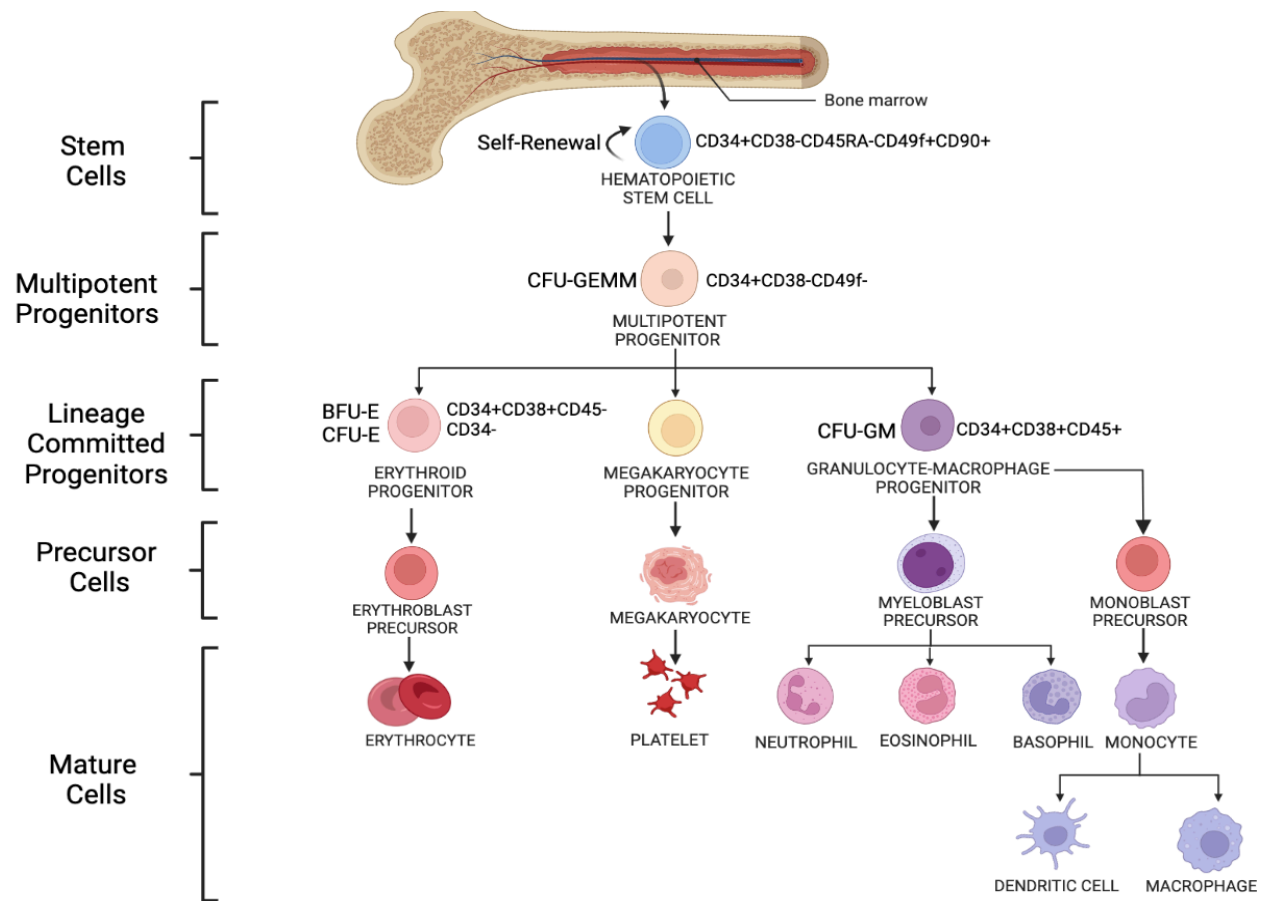


Figure 1. The hierarchy of haematopoietic stem cell differentiation to mature cells. Small populations of self-renewing HSPCs generate multipotent progenitors capable of repopulating each mature blood cell type¹⁸⁴. Created with BioRender.com.

Development of culture systems designed to maintain and expand HSPCs for various translational applications has remained at the forefront of the field. While these strategies are constantly evolving, the structure of investigations has remained the same.

1.1.2 Structuring HSPC Research

When examining common strategies used in the experimental design of studies focused on HSPC behaviour, a hierarchy of four distinct but necessary steps emerges. The first includes effective expansion of the desired cell population, in this case HSPCs. Previous studies have demonstrated that by purifying the respective sample to isolate CD34+ cells, whether from cord blood, peripheral

mobilized blood, or bone marrow aspirates, colony growth of erythroid, granulocyte, and macrophage lineages as demonstrated using a CFU assay is significantly improved¹². This indicates that isolating the CD34⁺ cells within a sample can enhance stem and progenitor expansion, further improving the efficiency of resulting transplantation. Typical cell expansion techniques involve cytokine cocktails, and while a singular unified composition has yet to be approved for clinical translation, the cytokines with the most frequent use include stem cell factor (SCF), thrombopoietin (TPO), and Flt-3 ligand¹³. A cell cycle study found that SCF preferentially acts during the G₀/G₁-phase of cell division, and can synergize with other present factors such as interleukin-6 or interleukin-3 to induce mitosis¹⁴. TPO has demonstrated its role in the maintenance of quiescence in the HSPC population, with previous studies reporting that knockout models showed a 150-fold HSC reduction as well as accelerating cell cycle kinetics which exhausts the stem and progenitor fraction of the population¹⁵. Flt-3 ligand has been found to both synergize with other cytokines to enhance proliferation of HSPCs *in vitro*¹⁶, and also enhance motility and homing potential *in vivo*¹⁷.

Aside from cytokines, several other techniques have been used in an attempt to expand HSPC populations. Numerous molecular and genetic approaches to cell expansion have been attempted in previous studies, including Notch signaling activation¹⁸, the introduction of copper chelators¹⁹, purine derivatives²⁰, and epigenetic factors such as nicotinamide²¹. These approaches are highly specific, and the rapid development of their formulations via clinical trials have shown promise. However, one crucial element missing is a thoughtful consideration for other aspects of the haematopoietic microenvironment or ‘niche’, including both cellular and non-cellular aspects of the native niche *in vivo*. The bone marrow is highly complex, and contains numerous components such as diverse support cells, precise levels of oxygenation, and specific biophysical properties of the associated extracellular matrix (ECM). While bone marrow oxygenation varies from 1-6%,

previous studies have shown success in significantly expanding HSPC populations in hypoxic conditions ranging from 3% to 10% oxygenation in the presence of cytokines when compared to standard 20% O₂ incubation methods¹³. HSPC interactions with ECM components, for example collagen films, have demonstrated success in increasing cell viability in culture²². Rather than overlooking these aspects, it is important to piece these findings together in order to develop the most robust niche possible for *in vitro* cell expansion.

The second step in the hierarchy of HSPC study includes the development of the system in order to maintain multipotent stem cell and progenitor populations, which are the cell types required for the intervention. While surface markers can effectively act as indicators of the general population dynamic, for example through flow cytometry, only a functional assay such as the CFU can definitively determine whether a cell population can continue to expand and give rise to committed progenitors of all lineages. The mature cell types developed from HSPCs include lymphocytes, monocytes, granulocytes, erythrocytes, and platelets³. This process can be tailored to skew lineages in a particular pathway, such as the use of erythropoietin to stimulate erythroid regeneration and survival²³, or simply performed to maintain a broad multipotent population. It is critical for translational purposes that all lineages are represented and can effectively repopulate a recipient with each cell type.

The third and fourth steps focus on *in vivo* approaches in order to confirm cell function and survival after transplant, as well as assess potential immune rejection risks. These are late-stage studies that are performed to confirm previous *in vitro* results and take the next step towards clinical translation and widespread use in disease treatment. While many targets of bone marrow transplantation include leukaemias and other hematological cancers, their applications range broadly and can be a curative intervention for various genetic, immune, metabolic, or neural disorders and diseases²⁴.

Improving transplant efficiency has wide reaching implications on human health and can serve to expand this treatment to a broader range of conditions.

1.1.3 Towards Developing an ex vivo HSPC Culture Model

When considering the complexity of the HSPC environment *in vivo*, there are a variety of other cell types present to facilitate mobilization, differentiation, and quiescence. With this understanding, co-culture models have been extensively employed in an attempt to provide cytokines and cell-cell interactions which can influence HSPC fate. The most commonly used support cells in HSPC co-culture conditions include endothelial cells (ECs) and mesenchymal stromal cells. However, the vascular niche of the native bone marrow is of particular importance given its multifaceted role in HSPC regulation. Bone marrow endothelial cells (BMECs) have been demonstrated to release SCF to maintain an adequate long-term repopulating HSC population²⁵. In addition, they have also been found to secrete pleiotrophin, a growth factor responsible for regulating maintenance and self-renewal of native HSPCs²⁶. One of the most contentious aspects of EC and HSPC co-culture is the source of the ECs themselves, as early studies focused on using BMECs in order to maintain fidelity to the native bone marrow. These studies laid the foundation for further co-culture studies by confirming that BMECs are supportive of HSPC expansion and maintenance of multipotency during *in vitro* culture due to their supply of cytokines such as interleukin-6 or granulocyte macrophage colony-stimulating factor²⁷. Despite the successful use of the HSPC-BMEC model, alternative sources of ECs have successfully been used in co-culture models to enhance HSPC population expansion. For example, a study by Davis *et al.* found that co-culture of bone marrow-derived HSPCs with porcine brain microvascular ECs could also successfully expand the HSPC population and maintained differentiation potential²⁸. Similar to other studies, the model depended on direct cell-cell contact and was also supplemented with

cytokines such as SCF to facilitate further expansion. The particular ability of this cell line to expand the most primitive subset of multipotent cells, generally defined as CD34⁺/CD38⁻, shows promise in the continued expansion of EC sources that can be used to study HSPCs.

ECs and HSPCs are closely linked throughout development, as primordial stem cells originate from ECs in the developing dorsal aorta²⁹. Through the production of stromal derived factor-1 (SDF1), ECs are thought to drive HSC colonization of fetal bone marrow³⁰. This link is continued during adulthood, where HSPCs localize to the vasculature for mobilization. In cases of haematopoietic regeneration after myelosuppression, it is known that HSPC repopulation of the bone marrow cannot occur without revascularization, further cementing their synergy *in vivo*³¹. Their interactions are reflective of their complementary adhesion molecules and binding partners, for example VLA-1 on HSPCs and VCAM-1 on local sinusoidal ECs³². Endothelial plasticity allowing for a mesenchymal transition to produce fibroblasts and osteoblasts also facilitates embryonic development, and plays a key role in marrow regeneration³³. As examining this relationship *in vivo* is extremely difficult due to the encasing of bone marrow in calcified bone, continued development of *in vitro* models to elucidate the mechanisms of these interactions is required.

1.1.4 HUVECs in HSPC Culture Techniques

As umbilical cord blood becomes more frequently used in bone marrow transplantation, the use of human umbilical cord endothelial cells (HUVECs) seems a natural source of ECs for co-culture in order to further expand the primitive stem cell population of the respective sample. Their shared developmental origins with HSPCs as well as their expression of quiescence-maintaining cytokines such as angiopoietin-1 make them an excellent choice in examining niche behaviour³⁴. Interestingly, the use of primary HUVEC samples is often not employed due to serum being necessary in culture. Alternatives, such as the E4ORF1-transduced HUVEC line or the HUVEC-

TERT2 immortalized cell line, have been generated in order to maintain angiogenic properties of the cells while eliminating the need for sera altogether³⁵. These changes allow for co-cultures to be performed in a way that preserves the multipotency of HSPCs without sacrificing the contributions of normally functioning HUVECs.

Thus far, there has been success using the HUVEC-HSPC co-culture model. One study using a HUVEC and HSPC co-culture system saw a 17-fold increase in cell expansion *in vitro* as well as significantly improved colony formation versus traditional cytokine-supplemented culture³⁶. All colony types were represented, and genetic analysis saw 75 changes in gene expression of co-cultured HSPCs which remarkably had no negative impact on engraftment or homing potential. The use of HUVEC co-culture has even been employed in *in vivo* studies such as by Srivastava *et al.*, where HSPCs were expanded on HUVECs for 8 days and subsequently used in autologous bone marrow transplants of rhesus macaques³⁷. The study was successful at expanding HSPCs and maintaining repopulation and homing capabilities as demonstrated by successful engraftment after approximately 4 years. Together, these studies show promise in the use of co-culture models using HUVECs in order to expand primitive HSPC populations for use in transplantation. Fidelity to native bone marrow conditions and continued development of the model can lead to significant breakthroughs.

1.2 The Extracellular Matrix

The ECM is a vital component of structural integrity within the human body. It is a highly dynamic structure, constantly being broken down and remodeled with assistance by various proteases and other cellular elements. While its role in maintaining physical scaffolding in tissues is well understood, it also plays a critical role in cell division and differentiation processes in order to maintain homeostasis³⁸. The ECM is also vital during cellular migration, as cytoskeletal adhesion

to the matrix facilitates mobilization. All matrices are comprised of a fundamental collagen-based structure, with different collagens imparting different levels of strength to varying tissues³⁹. Other components, most notably adhesive glycoproteins and proteoglycans, bind to the collagenous scaffold structure and to soluble factors in order to facilitate a local cellular response. Together, these components maintain the extracellular environment and contribute to homeostasis.

One of the most fundamental roles of the ECM affecting the haematopoietic system lies in the binding of secreted factors, acting as an internal storehouse within the extracellular space. As a result, the ECM is responsible for the differential access of ligands to cellular receptors, thus controlling the diffusion of soluble factors across the microenvironment and influencing cell fate in a highly controlled manner⁴⁰. For example, the ECM has been found to capture fibroblast growth factor-2 (FGF-2) in brain tissue to enhance neural stem cell expansion and differentiation⁴¹. In a similar fashion, cytokines have been found to bind to various glycosaminoglycans in the basal lamina of muscle in order to regulate the behaviour of muscle satellite cells⁴². Components such as fibronectin and collagen are able to bind growth factors like vascular endothelial growth factors (VEGF) or transforming growth factor beta (TGF β), facilitating their exposure to resident stem cells. This is of particular importance to the haematopoietic system, which requires a controlled response to stimuli in order to maintain the HSPC pool. The components of the ECM vary in structure and function, but all play a vital role in tissue structure and cytokine storage.

1.2.1 Fibrous ECM Proteins

Collagens (C) are the largest family of proteins in animals, with 28 currently identified. They are classified by their three distinct α chains forming a triple helix, which can be further formed into fibrillous, network-forming, or transmembranous structures⁴³. For example, the basement membrane-abundant collagen type IV consists of six α chains that form intricate 3D structures⁴⁴, while the transmembranous collagen type XVII is embedded in the plasma membrane⁴⁵. These structures vary wildly in tensile strength depending on the Collagen type I, the most abundant form in the human body is a heterotrimer of two pro α I (I) chains and one pro α 2(I) chain⁴⁶. These then self-assemble into fibrils, forming the structural scaffold of the ECM. Collagen imparts structural integrity to tissues and works in tandem with other more flexible structural components to withstand forces acting upon them.

Elastin (E) is another major fibrous protein that exists in the ECM. Elastin is tightly associated with fibrillar collagen, and its flexibility within the matrix is limited by this bond⁴⁷. The association of elastin within the matrix allows for the structure to be able to withstand tensile and compressive stresses⁴⁸. Elastin is widely considered to be the major player in low pressure elasticity due to its stiffness being three orders of magnitude lower than that of collagen, while the stiffer collagen is able to bear high pressures⁴⁹. It is composed of several monomers of tropoelastin, which are cross-linked together at lysine residues of the hydrophilic domain of the protein by lysyl oxidase⁵⁰. Elastin is a component of the ECM that does not see a great deal of turnover or degradation throughout a human lifetime; in fact, its half-life is estimated to be approximately 70 years⁵¹.

1.2.2 Proteoglycans

Proteoglycans are a class of molecule formed by chains of glycosaminoglycans (GAGs) with a protein core. They are broadly classified into three families consisting of small leucine-rich, modular, and cell surface proteoglycans. These classifications are reflective on their binding and location; for example, small leucine-rich and modular proteoglycans are found in the extracellular matrix while others localize to the plasma membrane⁵². An exception to this structural classification is hyaluronic acid, which lacks a protein core and is able to function both in the ECM to confer structural benefits or as a soluble factor influencing cell growth and differentiation⁵³. They are extremely hydrophilic, and work with collagen and elastin to confer stretch to the ECM via inflation and collapse in response to osmolarity⁵⁴. Proteoglycans are known to associate with a wide variety of soluble factors and associated receptors, such as those for FGF associated with HSPC cycling, or TGF β associated with HSPC quiescence⁵⁵.

1.2.3 Hydroxyapatite

While not classically considered when discussing the ECM, hydroxyapatite (HA) is the largest inorganic component of the ECM and an important structural component of hard tissues. It is a structural bioceramic that makes up hard bone, and is bound within the ECM along with other minerals⁵⁶. During HA synthesis, minerals bound within the ECM interact along a collagen template allowing for controlled deposition in localized areas⁵⁷. Osteoblasts around the bone marrow periphery are often responsible for this hard bone formation and resulting HA production. HA has a high capacity for the binding and concentration of bone morphogenic protein (BMP) *in vivo*, leading to the development of osteoblasts from bone marrow stromal cells⁵⁸. Though it is strongly associated with hard bone, HA is used in bone marrow regeneration techniques in HSPC study⁵⁹.

1.2.4 HSPC-Specific ECM Interactions

The dynamic between tissue-specific ECM and their respective resident stem cells has begun to be explored, and has promising applications within the realm of bioengineering. Specific ECM components play unique roles in the maintenance and differentiation of the HSPC population within the bone marrow. For example, binding to fibronectin has been found to upregulate hematopoiesis and HSPC population expansion *in vivo*, while laminin enhances the ability of HSPCs to home in a target area in the body⁶⁰. Collagen type I has also been found to support expansion of HSCs in the bone marrow, while HA has been considered to support HSPC maintenance by producing a calcium gradient⁶¹. As expansion of HSPC populations remains elusive, it is possible that the use of certain ECM proteins in an engineered scaffold may be able to enhance their expansion *in vitro*.

The biophysical properties of the ECM are also tightly linked to HSPC fate, influencing a change towards a more proliferative or quiescent cell population. For example, while the stem cell fraction of the population is enriched during culture on elastin-coated plates, this effect disappears when the elastin is cross-linked to increase stiffness⁶². Similar impacts are seen in collagen matrices, where increasing coating concentrations from 1 ng to 100 µg per mL increased the viability of HSPCs⁶³. This also translates into functional assays, with more primitive subpopulations of the HSPC pool present in colonies produced from cells cultured on stiff coated culture plates⁶⁴. In addition to viability considerations, biophysical forces also guide HSPCs toward specific fates. For example, increasing hydrostatic pressure in vasculature where high amounts of elastin assist in withstanding such force has been shown to maintain HSC quiescence and upregulate colony forming potential, preventing asymmetrical division from depleting the most primitive subset of cells⁶⁵. These properties of the ECM are thus highly plastic, and *in vitro* culture methods using

engineered scaffolds must consider how variations in component concentration will impact the overall structure of the scaffold and in turn the fate of the cells.

HSPCs are able to interact with local ECM and surrounding cells via integrins, and selectively express different types of integrins depending on the specific phenotype of the HSPC. For example, only long-term repopulating HSCs express ITGA6, while more broadly the entire HSPC population expresses ITGA4 and ITGA5⁶⁶. Different integrins are able to exert different effects depending on the subunits present. For example, blocking integrins that contain a $\beta 2$ subunit inhibits adhesion between HSPCs and the bone marrow stroma, while blocking the VLA4 integrin induces a mobilized and proliferative stem cell phenotype⁶⁷. Another important class of receptors that mediate ECM and HSPC interactions are selectins, a type of transmembrane protein. Endothelial expression of E-selectin is upregulated in the bone marrow sinusoidal niche, and enhances HSC proliferation⁶⁸. These interactions have been utilized in *in vivo* culture models to examine ECM influence on HSPC fate. For example, primary murine HSCs demonstrate enhanced proliferation on collagen scaffolds while the matrix stiffness maintained progenitor populations⁶².

In addition to HSPCs, the role of the endothelium and its own ECM is critical when considering how to best design experiments mimicking *in vivo* conditions. This ECM is often referred to as the perivascular matrix, and it is capable of controlling growth factor signaling to regulate homeostasis in its respective tissue⁶⁹. The role of the ECM in the maintenance of stem cell populations *in vivo* is well understood, but further studies are required to determine if an engineered combinatorial matrix with the appropriate components can allow for cell survival and expansion *ex vivo*.

1.3 Human Bone Marrow Niches

In adult humans, most HSPCs are located within the bone marrow. While seemingly unremarkable, the bone marrow is a highly vascularized organ and has direct sympathetic nerve innervation. It is comprised of several diverse types of cells, such as endothelial cells, macrophages, mesenchymal stem cells (MSCs), and osteoblasts⁷⁰. The heterogeneity of the environment is further organized into distinct microenvironments, or ‘niches’¹¹. These niches are typically comprised of specific subsets of local support cells and factors such as structural proteins that make up the extracellular matrix (ECM)¹². The different cells found in the niche are responsible for the secretion of various cytokines affecting cell behaviour, and the extracellular matrix has been implicated in the retention and immobilization of these cytokines in order for them to exert their effects on local HSPCs¹³. There has been a plethora of research on the function of the osteoblastic or endosteal niche, located near the periphery of the marrow closest to the bone, and its role in maintaining HSPC quiescence and homing¹⁴. However, simply maintaining HSPC populations is not sufficient for therapeutic or research applications. The more recent target of study is the vascular niche, in which HSPCs are localized in close proximity to the bone marrow vasculature (Figure 2)¹⁵.

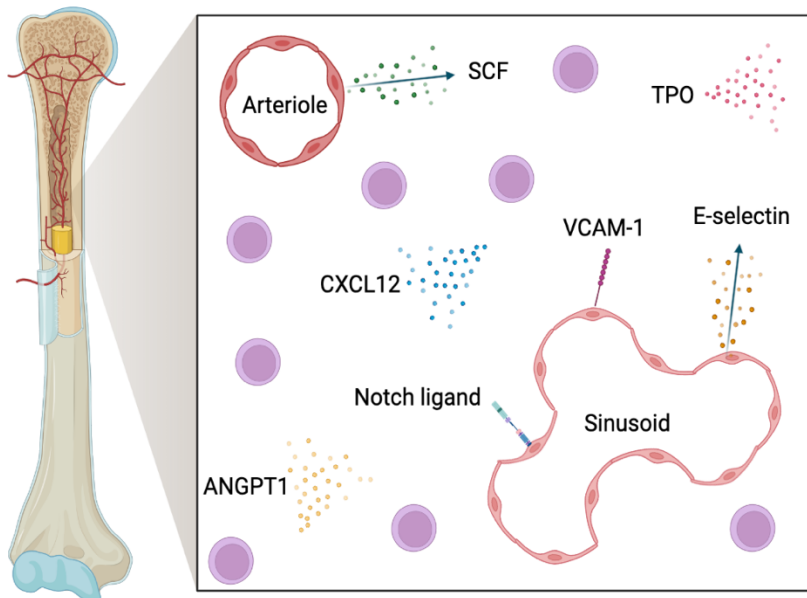


Figure 2. Model of the vascular bone marrow niche. Arteriolar and sinusoidal ECs preferentially express cytokines and receptors related to HSPC (purple) maintenance, such as stem cell factor (SCF), thrombopoietin (TPO), angiopoietin 1 (ANGPT1), E-selectin, vascular cell adhesion molecule 1 (VCAM-1), C-X-C Motif Chemokine Ligand 12 (CXCL12), and Notch ligands⁷¹. Created with BioRender.com.

This niche is comprised of various bone marrow endothelial cells (ECs) of arterial or sinusoidal identity whose interactions with HSPCs incite a more proliferative stem cell population in comparison to their endosteal counterparts¹⁶. Each unique EC phenotype differentially expresses cytokines that exert different effects¹⁷. For example, stem cell factor (SCF), which is a regulator of HSPC population maintenance, has been found to be preferentially expressed by arterial ECs, while adhesion molecules enhancing HSPC mobilization such as E-selectin have been found to be preferentially expressed by sinusoidal ECs¹⁸. This differential cytokine and ligand expression is able to exert different regulatory effects on HSPCs depending on the demands of the haematopoietic system.

In addition to the niche cells themselves, the components of the ECM are instrumental in regulating HSC function²⁰. For example, laminin has been shown *in vivo* to regulate HSPC cycling, while heparan sulfate proteoglycans such as syndecans and glypicans have been shown to immobilize and activate various HSC-related growth factors such as vascular endothelial growth factor or fibroblast growth factor^{13,21}. *In vitro* studies have primarily focused on the analysis of individual matrix proteins and their effects on cultured HSCs, but this type of experimental design does not accurately reflect the complexity of the bone marrow niches in which native HSCs are found *in vivo*. While the use of decellularized matrices have been modestly successful in the expansion of HSCs, these systems would be inefficient for widespread therapeutic use and the process of decellularization risks damaging the matrix proteins²². Engineering combinatorial scaffolds for cell growth using purified matrix proteins is a possible alternative that could be easily replicated and allow for a more holistic analysis of ECM composition²³. In order to recapitulate a matrix that more closely mimics the bone marrow microenvironment, it is necessary to analyze how matrix proteins work in unison to exert regulatory effects on associated vascular niche cells.

The bone marrow niches are relatively hypoxic environments, ranging from two to six percent oxygenation²⁵. There is evidence to suggest that ECs within the niche enhance their deposition of ECM-related proteins in hypoxic conditions, and it has been well established that vascular growth is enhanced when oxygen is low²⁶. Several genes related to HSC proliferation and differentiation expressed by ECs are transcriptionally regulated by hypoxia inducible factor 1 α (HIF1 α), suggesting that a hypoxic environment can enhance the regulatory role of endothelial cytokines on HSCs²⁵. In addition, HSCs themselves have been noted to require the presence of HIF1 α protein to maintain normal function²⁷. It is possible that the combination of endothelial ECM deposition and the release of HSC-related cytokines under hypoxic conditions is responsible for HSC expansion, but a combinatorial study recapitulating the vascular niche in this way has yet to be realized.

The concept of analyzing the entirety of the vascular niche for its contributions to HSC proliferation and differentiation is not one that has been often explored²⁸. Various investigations into the effects of endothelial cells or their cytokines alone have been completed, but have not provided critical breakthroughs in the field. For example, co- culture systems containing bone marrow-derived ECs and HSCs have revealed that direct cell-cell contact is required in order for ECs to have any effect on HSC expansion²⁹. In addition, studies utilizing conditioned media derived from bone marrow endothelial cultures have found that endothelial-derived cytokines alone are not sufficient to cause a significant difference in HSC expansion *ex vivo*³⁰. Differences have also been noted when endothelial cells derived from different tissues have been compared in their capability to expand HSCs, reflecting endothelial heterogeneity in cell surface proteins and secreted factors that give ECs their unique organ-specific identity. The need to isolate haematopoietic tissue-derived ECs provides yet another barrier to development and adoption of a uniform culture system for HSC

expansion. Use of existing EC lines for haematopoietic expansion applications would reduce associated costs and challenges of haematopoietic tissue harvesting and EC isolation, making the resulting culture system easier to implement for transplant therapeutics and further HSC study. ECs derived from haematopoietic tissues such as the bone marrow or spleen have most successfully expanded HSC populations, indicating that tissue specificity is critical in the study of HSPC behaviour *ex vivo*³¹. This has led researchers to question what the identity of the bone marrow vasculature-derived factor could be, and how it is responsible for HSC expansion. Simultaneously recapitulating multiple aspects of the vascular niche, including niche-associated cells, the extracellular matrix, and low oxygenation can aid in this process. If the underlying factors that make haematopoietic-derived ECs effective in haematopoietic expansion are identified, they can then be applied to other ECs in order to enhance HSC proliferation without the need for highly specific cell types.

1.3.1 Residents of the Vascular Niche

Overall, many studies focusing on the *in vitro* expansion of HSPCs have utilized endothelial co-culture in order to suppress the differentiation of these cells to committed progenitors or mature haematopoietic cells. Endothelial cells have previously been found to regulate the delicate balance between HSPC proliferation and differentiation into mature haematopoietic cells⁷². For example, deletion of stem cell factor and stromal cell-derived factor-1 from only the endothelium has been found to compromise the maintenance of the HSPC population within the bone marrow⁷³. This specific dynamic can also be broken down by the type of endothelium to which an HSPC is in close proximity.

Differences in arterial and sinusoidal EC phenotypes facilitates the study of the unique niches, as arterial ECs highly express stem cell antigen-1 while sinusoidal ECs almost exclusively express

vascular endothelial growth factor receptor-3. Though both structures are required for proper regulation of haematopoiesis *in vivo*, some studies have demonstrated that the arterial niche better maintains HSCs in a quiescent state⁷⁴. The arterial niche contains vessels of low permeability, maintaining the HSPCs in an environment with low reactive oxygen species. Recent research has found that this niche keeps HSPCs dormant, with population expansion taking precedence over differentiation and mobilization⁶⁰. In contrast, the high permeability of sinusoids within the sinusoidal niche activate HSPCs to differentiate and mobilize, while repopulation becomes compromised. Factors such as stem cell factor or stromal cell-derived factor 1 are produced by sinusoidal endothelial cells and act on HSPCs within the niche to promote differentiation. There is evidence to show that after irradiation, human sinusoidal ECs are able to reconstitute hematopoiesis by expressing Notch ligands and insulin-like growth factor-binding proteins⁶⁹. The differences between the two subsets of bone marrow endothelium make experimental design highly specific and has led to the discovery of several distinguishing functions for the two niches. For example, SCF deletion impairs the ability of resident HSPCs to regenerate, but this effect is only seen when the deletion occurs in arterial ECs and not sinusoidal ECs⁷⁵.

The role of the vascular niche becomes more complex when considering how blood flow is able to affect the development of HSPCs in the bone marrow. Areas with low oxygen tension and extremely low velocity blood flow enrich the HSPC population, where cells remain quiescent⁷⁶. In addition to this, apoptosis is decreased in areas with decreased blood flow. Hypoxia has been shown in numerous studies to be associated with HSPC quiescence, and some studies even suggest that HSPCs are more likely to localize and home in areas of low oxygenation. In a similar fashion, HIF signaling pathways have previously been shown to promote the expansion of ECs. Endothelial-specific HIF-1 α mutants result in decreased stem cell antigen-1-expressing endothelial cells, a

global decrease in stem cell factor, and an overall reduction in the HSPC population of the bone marrow⁷⁷.

The dynamic between the endothelium and HSPCs is critical for the process of hematopoiesis and thus is an excellent target of further study. Studies have found that deletion of VEGF1 receptor-2 within the endothelium of the bone marrow prevents bone marrow regeneration, indicating that a healthy endothelium is crucial in order to respond to signaling molecules and thus release pertinent angiocrine factors for an appropriate tissue response⁷⁸. HSPCs within the bone marrow and resident ECs are profoundly interconnected beginning during human fetal development. ECs convert into HSCs during development via the activation of a haematopoietic transcriptional program and various morphological changes⁷⁹. In fact, many bone marrow ECs and HSPCs share a large portion of expressed genes, and these similar expression profiles continue into disease states such as leukemia. Overall, the presence of a healthy endothelium is critical in the maintenance of hematopoiesis in the bone marrow.

1.4 Biocompatible Scaffold Engineering

The use of scaffolds for cell culture applications has been widely explored. However, a general consensus as to what approaches are most effective has yet to be established. In order to generate viable *in vitro* culture systems that can be utilized to repair damaged tissues or replace a specific function, cells should have a physical scaffold and additional cytokines to make the system as robust as possible. The ECM components of the scaffold should function optimally to promote viability and population expansion of the most primitive cell types in the system. The dynamic cross-talk between cell surface receptors, cytokines, and matrix components allows for dynamic ECM remodeling within the system to continue to influence cell fate. The most critical components

to the approach include a demonstrated lack of cytotoxicity, vital for *in vitro* approaches, and a lack of severe immune or inflammatory responses that may alter *in vivo* results.

1.4.1 Synthetic Scaffolds

Synthetic scaffolds use chemical components in order to better recapitulate the mechanical and physical properties of the native tissue. For example, the use of plastic filaments comprised of polylactic acid as a cell scaffold allows for physical aspects such as pores or polymer composition to be controlled and examined for their effects on HSPC differentiation⁸⁰. Similar and commonly used materials include the polyester poly-L-lactic acid (PLLA) and copolymer poly lactic-co-glycolic acid (PLGA), used for their biocompatibility and ease of manipulation⁸¹. Synthetic materials may pose a more economical approach to niche modelling in comparison to biological materials, but also come with several disadvantages. Studies have often concluded that synthetic scaffolds should be combined with biological materials such as collagen as well as cytokine supplementation to sufficiently support HSPC population expansion⁸¹. In addition, there are several concerns with low bioactivity and rejection risk when using synthetic polymers. The use of PLLA, PLGA, and their derivatives is limited *in vivo* due to their degradation by hydrolysis which can alter local pH and lead to necrosis⁸². Thus, they are not ideal for long-term human studies with translations to *in vivo* patient models.

1.4.2 Decellularized Bone Marrow

Decellularized bone marrow is a common choice for biological scaffolds. Through the process of decellularization, only the structural components of the matrix remain for cell seeding⁸³. This model is seen as particularly advantageous as it maintains the natural structures and mechanical properties found *in vivo*. However, complete decellularization and removal of immune response-

inducing compounds is impossible, meaning that a delicate balance between decellularization and maintenance of the natural structure must be sought⁸⁴. Even with the use of human cadaver samples to unify the scaffold and cell population source, decellularized marrow still poses immunogenicity and cytotoxicity risks to the culture system⁸⁵. In addition, their behaviour *in vivo* remains poorly understood, and the use of this type of scaffold without some alteration to their natural structure has not yet been achieved.

1.4.3 Biological ECM Scaffolds

Biological ECM scaffolds are comprised of natural biological materials derived from model organisms. By engineering a biological scaffold with custom components, the complexities of the native bone marrow niche can be recapitulated in order to better understand the mechanisms underlying HSPC behaviour. Their biological activity promotes cell adhesion and expansion *in vitro*, and the dynamic nature of ECM degradation and formation can be controlled by local cells within the system⁸². This is of particular importance when engineering a model niche, as resident support cells can also be seeded to alter the composition of the matrix. Collagen is one of the most commonly used biological scaffold materials due to its abundance within the niche ECM and demonstrated plasticity during fabrication⁸⁶. In particular, bovine collagens have successfully been applied in applications ranging from tissue engineering to drug delivery⁸⁷. Terminal sterilization of these components is required, but often comes with great disadvantages. For example, the use of ethylene oxide can maintain growth factor secretion from local cells but is also mutagenic and can promote an immune response when cells are seeded⁸⁸. While non-chemical techniques such as electron beam irradiation can alter the structure and mechanical properties of scaffolds, gamma irradiation is considered a fast and safe alternative that does not degrade collagen⁸⁹. It has also been found to be an effective physical treatment to increase the mechanical strength of the scaffold

without causing toxicity in culture systems⁹⁰. The use of biological scaffolds to develop *in vitro* culture models along with support cells can be a promising route to better understanding how to expand elusive HSPCs.

1.5 Hypothesis, Aims & Objectives

I hypothesize that identification of optimal vascular niche-mimicking culture conditions including an optimized extracellular matrix and endothelial support will enhance endothelial cell expression of pertinent paracrine factors to upregulate haematopoietic stem cell expansion and differentiation potential *ex vivo*.

While HSPCs remain a critical topic of study for the continued development of transplantation protocols as well as the process of drug discovery, true self-renewing stem cells are extremely rare in a given cell sample and challenging to expand *ex vivo*. The development of an *in vitro* vascular niche model can be used to elucidate the molecular pathways governing HSPC population maintenance in order to better translate findings into human therapeutics. A singular optimal culture protocol for human HSPCs has not been established, leaving a critical gap in understanding HSPC behaviour. However, current understanding of the bone marrow environment reveals that the ECs of the vascular niche play a vital role in cytokine production and release, while the native ECM of the microenvironment binds and presents these cytokines in an organized fashion in order to elicit a specific HSPC response. By recreating the interactions of the vascular microenvironment with ECM scaffolds and ECs, the mechanisms that control quiescence and differentiation can be identified.

The first aim is the development of a high-throughput co-culture system that mimics the natural vascular niche in humans. The use of biological components for scaffold fabrication and gamma

irradiation sterilization allowed for the manipulation of scaffold chemistries and fabrication of numerous multi-combinatorial 2D structures for analysis. Furthermore, the addition of HUVECs as a feeder cell population allowed for the continuous production of cytokines within the system to be bound and presented by the ECM components. Next, the establishment of a co-culture protocol for cell seeding, harvesting, and analysis were optimized in order to maintain viability of both cell populations.

The second aim focused on analyzing the effects of the niche-mimicking culture system. High-throughput functional screens were performed to examine the effects of various scaffold chemistries on HSPC behaviour and to classify the stage of differentiation of the cells. Scale-up repeats of optimal scaffolds was made in order to further understand the mechanisms behind enhanced colony formation properties. The dependence on physical cell-cell contact between HSPCs and HUVECs in the system to induce a proliferative response was examined using a HUVEC conditioned media bank. Quantification and identification of cell subpopulations coupled with functional output examined the ability of the scaffold compositions and EC support to induce a quiescent or proliferative fate in HSPCs.

CHAPTER 2

Materials & Methods

CHAPTER 2. MATERIALS & METHODS

2.1 Scaffold Fabrication

In order to examine the relationship between ECM components and HSPC behaviour, unique chemical combinations of collagen-based slurry including several other components were fabricated in a 96-well format. To form the scaffolds, collagen type I flakes derived from bovine Achilles tendon (Sigma-Aldrich) were mixed with 0.05 M acetic acid (Sigma-Aldrich) to form a slurry (Figure 3). The solution was chilled at 4°C overnight to rehydrate the flakes, and subsequently blended to homogenize the mixture. A rapid centrifuge at 200 g for 2 minutes was applied to de-gas the slurry and prevent air pockets from forming within the scaffold. To prepare the various chemical combinations of ECM components, three different concentrations of collagen type I were prepared by weight - 0.5%, 0.75%, and 1%. The other chemical components, including elastin derived from bovine neck ligament and the bioactive ceramic hydroxyapatite in both a 10 µm macropowder and 200 nm nanopowder (Sigma-Aldrich), were added directly to the collagen slurry and blended .

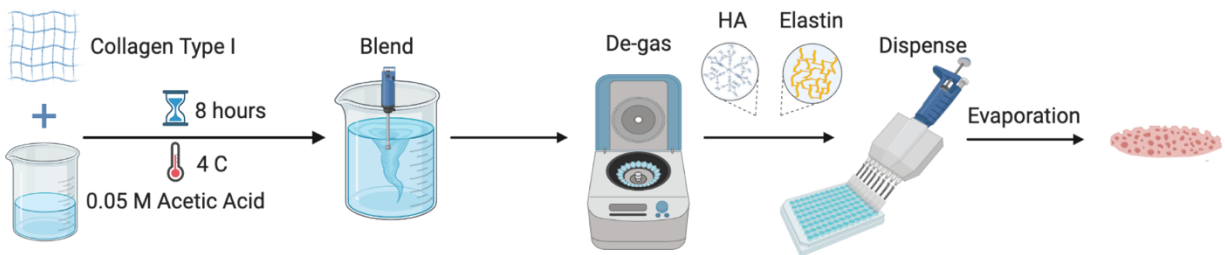


Figure 3. Schematic of scaffold fabrication process. Collagen type I and acetic acid are mixed, blended, centrifuged and dispensed into wells of a 96-well plate with various other ECM components. After evaporation occurs, a thin film of components is formed in the bottom of the well. Created with BioRender.com.

Slurries for wells containing two components were fabricated using a 1:1 ratio of collagen type I and the respective component, while slurries for wells containing three components were fabricated using a 1:0.5:0.5 ratio of collagen type I to the other two respective components. 50 μL of each unique chemical slurry composition was dispensed into each well of a flat-bottom 96-well plate (VWR), and the plates were transferred to a fume hood for 24 hours to allow for evaporation to occur, resulting in thin films of the various scaffold compositions at the bottom of each well. Scaled-up scaffolds were cast into a flat-bottom 6-well plate using 1500 μL of each slurry composition. The plates were then transferred to the University of Toronto Gamma Irradiation Services for gamma irradiation at 2.5 Mrad before cell culture.

Prior to use, sterile scaffolds were immersed in 100 μL of crosslinking solution consisting of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysuccinimide (NHS) dissolved in 100% ethanol (Sigma-Aldrich). Once submerged, the scaffolds were placed on an orbital shaker for 3 hours at 200 RPM. After 3 hours, the scaffolds were washed a minimum of 3 times with PBS and an additional 100 μL of crosslinking solution was dispensed onto the scaffolds. The plates were subjected to the orbital shaker for an additional 10 minutes, and washed again in PBS. The scaffolds were kept hydrated with PBS until cell seeding.

2.2 Cell Culture

HUVEC-TERT2 cell lines were obtained from ATCC (CRL4053). The cells were maintained in EBM™ endothelial cell growth basal medium (Lonza) supplemented with EGM™ SingleQuots™ including 0.1% hEGF, 0.1% hydrocortisone, 0.1% GA-1000, 0.4% BBE, 0.1% ascorbic acid, and 2% FBS. Patient samples of mobilized peripheral blood mononuclear cells were obtained from Dr. Mark Minden of the University Health Network. Samples were anonymous, preventing specific

analysis of age, sex, and other factors that may influence cell behaviour. The cells were maintained in phenol-free IMDM (Thermo Fisher) supplemented with 1% BSA, 1% 2 mM L-glutamine, 0.1% M β -mercaptoethanol, 1% sodium pyruvate, and 1% non-essential amino acids. Immediately before thawing, the media was supplemented with three key cytokines including 0.1% SCF, 0.1% Flt3 ligand, and 0.02% TPO. HSPCs were protected from light, and both cell types were maintained at 37°C at 5% CO₂ saturation.

2.3 HUVEC Spheroid Assay

For the preliminary assay, cell suspensions containing 1000 HUVECs were dispensed into 500 μ L of MethoCult™ H4434 (Stem Cell Technologies) in a 24-well plate and cultured continuously for 14 days. The peripheral wells were filled with PBS to maintain hydration of the methylcellulose during the time trial. The methylcellulose suspensions were imaged every 24 hours using a confocal microscope to identify spheroids within the sample. To determine the effects of cell number on spheroid formation, the experiment was repeated with four methylcellulose-based suspensions containing 5000, 10,000, 25,000, and 50,000 HUVECs respectively.

2.4 Media Viability Assay

In a flat-bottom 6-well polystyrene plate, HUVECs were seeded at a density of 100,000 cells per well and supplemented with 2 mL of media. Three distinct media conditions were tested to assess cell survival. The first two were the maintenance culture medias used for HUVECs and HSPCs detailed previously (refer to 2.2). The third condition was a mix of 1 mL of each respective media to assess if HSPCs could tolerate low amounts of sera and phenol as well as HUVEC resiliency in low-sea conditions. The samples were incubated for 72 hours, and were imaged in 24-hour intervals using a confocal microscope. After 72 hours, the samples were lifted from the wells during a 5-

minute incubation with 1 mL Trypsin-EDTA 0.25% (Thermo-Fisher) and resuspended in their respective medias. 10 μ L of the cell samples were suspended in 10 μ L of Trypan blue stain and cell counts were performed using the Countess™ automated cell counting machine (Thermo Fisher).

2.5 HUVEC Scaffold Recovery

The wells of a flat-bottom 96-well plate were coated in 10 μ L of 1.5% gelatin to provide a substrate for HUVEC adherence. Cell suspensions of 1000, 2500, 5000, and 10,000 cells were seeded into each well to explore a wide variety of plating densities within the recommended plating range. After 24 hours of incubation, a calibration curve was set with a series of 8 dilutions beginning at 20,000 cells seeded onto the plate and allowed to adhere to the bottom of the well for an additional 24 hours. After an additional 24 hours, the media was aspirated and 45 μ L of either Trypsin-EDTA 0.25% (Thermo Fisher) or Accutase (Stem Cell Technologies) was added to the wells to dissociate the HUVECs from the gelatin coating. The detachment solutions were neutralized after 5 minutes with an additional 45 μ L of media, and 10 μ L of alamarBlue cell viability reagent (Thermo Fisher) was added to each well. The plate was then incubated for approximately 1 hour and the cell suspensions were transferred to a black flat-bottom 96 well plate for the fluorescence to be recorded via a microplate reader (Synergy H4, BioTek). Fluorescence was read from the bottom of the plate at an excitation and emission wavelength of 560/590, respectively. The number of cells contained in each well after scaffold culture was deduced from the fluorescence values by adjusting the values to remove background fluorescence recorded by a control column of media only, plotting a standard curve using the series of dilutions with known cell numbers, and using the equation of the line to calculate the number of cells per well.

2.6 Endothelial morphology imaging

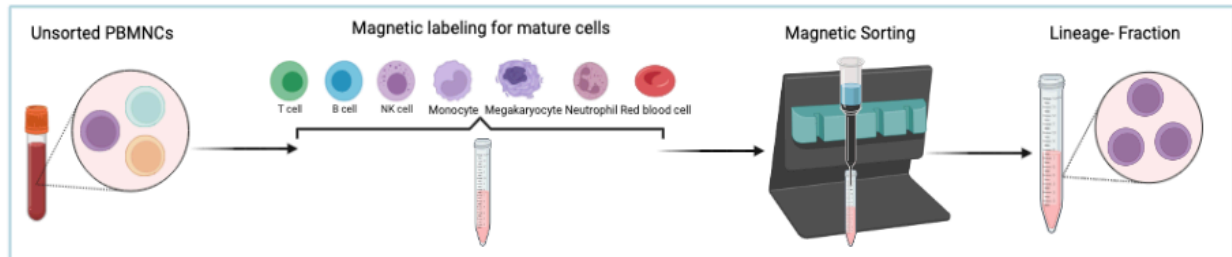
Glass coverslips were coated with 100 μ L of collagen at concentrations of 1%, 0.75%, 0.5%, and 0.25%. Gelatin was used as a control adherence substrate. After 48 hours of incubation, cells were fixed with 4% formaldehyde and permeabilized with 0.5% Triton-X 100 (Thermo Fisher) for 15 minutes. The cells were then treated with fluorescein phalloidin conjugate (Thermo Fisher) for 20 minutes, washed 3 times in PBS, and mounted onto a glass slide using ProLongTM Diamond Antifade Mountant (Thermo Fisher) containing DAPI. Both 1:100 and 1:250 concentrations of phalloidin were compared in order to determine the optimal concentration for imaging. The slides were covered in foil and left to cure for 24 hours, and subsequently imaged using a fluorescent microscope.

2.7 Co-Culture Scaffold Screening

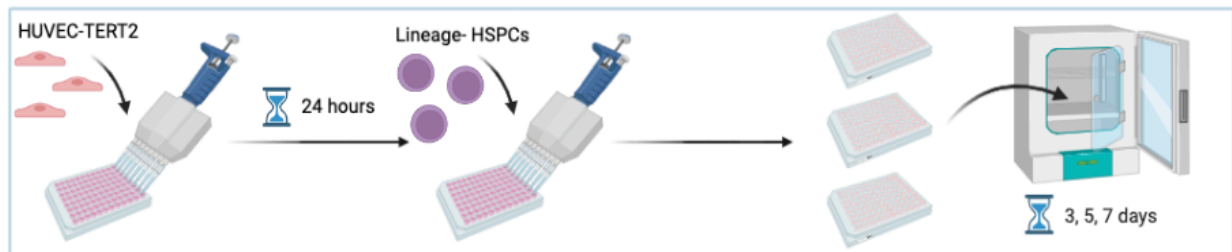
To screen pre-fabricated scaffolds for their effects on HSPC survival and multipotency, co-cultures were performed in a high-throughput 96-well format (Figure 4). This was compared to polystyrene controls of HSPCs and alternate co-culture control containing HSPCs and HUVECs. Each scaffold condition contained 2 co-culture and solo HSPC samples in order to further investigate the effects of endothelial support in the system. Each set of experiments takes 22 days to completion. Irradiated scaffolds were washed with 100 μ L of incomplete HSPC media in order to temper potential pH alterations resulting from the acetic acid-based scaffolds. The plates were washed 3 times with 15-minute incubation periods between each wash. HUVECs were subsequently lifted from a T75 flask using Trypsin-EDTA, re-suspended in HSPC complete media, and plated onto scaffolds at a density of 2,500 cells per well. This density was chosen due to preliminary experiments showing optimal growth between 2,500 and 5,000 HUVECs in a 96-well format. The

HUVECs were incubated for 1 day in order to allow them to adhere to the scaffold before the addition of suspension cells.

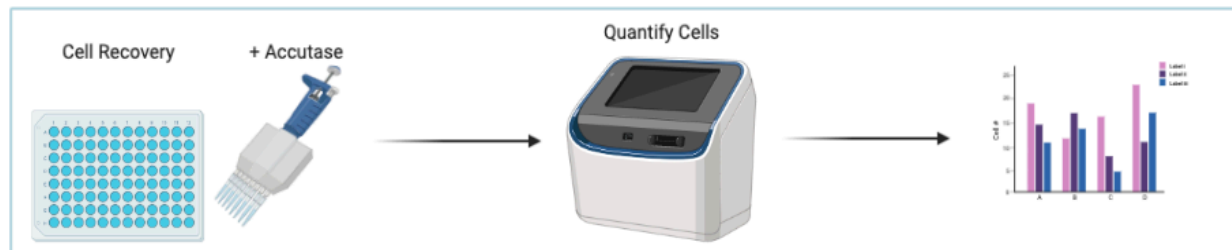
1 Lineage depletion



2 Seeding and incubation



3 Recovery and analysis



4 CFU Assay

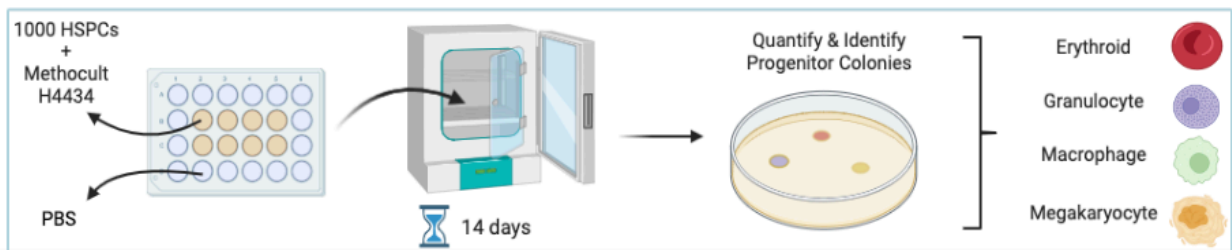


Figure 4. Schematic of co-culture protocol. On day 1, HUVECs are seeded onto scaffolds. On day 2, lineage depletion on patient samples of mobilized peripheral blood mononuclear cells is used to isolate uncommitted stem and progenitor cells which are plated onto HUVECs and incubated for 3, 5, or 7 days while day 0 CFUs are plated. After the respective incubation period, HSPCs are counted and plated into CFUs. After 14 days of CFU incubation, colonies are quantified and identified.

24 hours later, patient samples of peripheral blood mononuclear cells (Princess Margaret Hospital, Toronto) were thawed and processed using a lineage cell depletion protocol to isolate HSPCs (Miltenyi Biotech). The samples were treated with magnetic labels targeting factors present in mature haematopoietic cells and then run through an LS column on a MidiMACS™ separator where the effluent containing unlabeled HSPCs is collected. After removal from the separator, the remaining labeled mononuclear cells were collected and stored for alternative experiments. The unlabeled cells, representative of the lineage negative (Lin-) component of the sample, were then counted and plated onto scaffolds containing an adherent HUVEC monolayer at a density of 10,000 cells per well. 2 day 0 CFU replicates with 1000 live cells were also plated to serve as a baseline for colony formation potential. HSPC incomplete media without cytokines was removed from the wells before HSPCs in complete media were added. The co-culture was incubated for 3, 5, and 7 days respectively to assess if survival of HSPCs persisted at extended time points.

After the respective incubation period, cell samples from each well were plated into a CFU assay. Briefly, non-tissue culture coated 24-well polystyrene plates were coated with Pluronic™ F-127 (Thermo Fisher) in order to avoid cell adhesion to the plate. 500 µL of MethoCult™ H4434 (Stem Cell Technologies) was dispensed into the innermost 8 wells of the plate. This particular methylcellulose-based medium contains SCF, interleukin-3, erythropoietin, and granulocyte macrophage colony-stimulating factor in order to facilitate the growth of human haematopoietic progenitor colonies. Following this, 10 µL of cell suspension was removed from each scaffold and dispensed directly into each well containing the methylcellulose medium. The remaining 16 perimeter wells were filled with PBS to maintain hydration through incubation. The plates were incubated for 14 days to allow for sufficient colony growth before identification and quantification.

2.8 CFU Processing and Imaging

CFU output for each scaffold condition was assessed on day 14 of incubation. Using a light microscope, each well was assessed in order to quantify and identify the colonies present. Using a 4X objective, each colony was counted to give an overall colony output for the condition. Further to this, using 10X and 20X objectives, the colonies were identified as either burst forming unit-erythroid (BFU-E), colony forming unit-erythroid (CFU-E), colony forming unit-granulocyte, macrophage (CFU-GM), and colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM). Erythroid-based colonies such as CFU-E and BFU-E are easily identifiable by their distinct red colour, with the main identifiable difference being the size of the colony as CFU-E contains less than 200 cells while BFU-E contains more than 200 cells. CFU-GM and CFU-GEMM are identifiable by their composition, with CFU-GM containing spherical granulocyte progenitors and longer, spread macrophage progenitors as opposed to CFU-GEMM containing the aforementioned progenitors as well as the easily identifiable red erythroid progenitors⁹¹. Each type of colony was quantified for each scaffold chemistry, and images of representative colonies were captured using a confocal microscope.

To image the colony formation output from each scaffold chemistry, 20 μ L of MTT solution was added to each well and incubated for 1 hour to maximize colony visualization. Using an Epson Perfection V370 scanner, each plate was scanned to document the number and size of colonies present for each condition. Following imaging, the methylcellulose suspensions were washed 3 times with PBS, collected, and centrifuged at 300 g for 5 minutes in order to separate the cell pellet from suspension. These pellets were then frozen for use in later gene or protein expression analysis.

2.9 HUVEC Genetic Expression Analysis

Approximately 500,000 HUVEC-TERT2 cells were cultured over 72 hours on two scaffold chemistries, 1% C and [1% C + 0.5% E + 0.5% HA], as well as a polystyrene control in a 6-well format. Two replicates were plated for each condition. After 72 hours, Accutase was used to dissociate cells from the scaffolds and TRIzol™ (Thermo Fisher) was used to lyse the cells. RNA was extracted using chloroform and centrifugation to collect the aqueous phase of the suspension and isopropyl alcohol was used to precipitate the RNA. After washing with 75% ethanol, the RNA pellet was re-suspended in 40 µL of RNase-free water. RNA concentration was assessed using a spectrophotometer.

300 ng of RNA from each condition was used to reverse transcribe cDNA in a polymerase chain reaction (PCR). This was done on a 2720 thermal cycler (Applied Biosystems) with the addition of dNTPs (New England Biolabs), Oligo dT (Invitrogen), RNase inhibitor (Thermo Fisher), random hexamers (Invitrogen) and M-MuLV reverse transcriptase and buffer (New England Biolabs). 2 µL of the transcribed cDNA was combined with master mix, RNase-free water, and 1 µL of the probes of interest: VEGF1, PECAM1, and SFRP1 using HPRT1 as a housekeeping gene. RT-qPCR was performed using a Rotor-Gene Q cycler (Qiagen) for 60 cycles. Using the cycle threshold (C_T) values, fold change in expression was calculated to determine the role of the scaffold condition on relative genetic expression.

2.10 Statistical Analysis

Fluorescent and confocal images were processed using ImageJ 1.53u software. Data were analyzed using GraphPad Prism® 9.0 software (GraphPad Software Inc.) and reported as mean $\pm$ standard

deviation (SD) unless otherwise stated. Multiple two-tailed t-tests were conducted to determine statistical significance. P-values representative of statistical significance are denoted by asterisks: $<0.05^*$, $<0.01^{**}$, and $<0.001^{***}$.

CHAPTER 3

Results

CHAPTER 3. RESULTS

3.1 Scaffold fabrication

In fabricating scaffolds, an important consideration is including as many component combinations as possible while also being able to accommodate space for biological replicates. The use of a 96-well plate is an excellent strategy to accommodate these component combinations. The scaffold design shown in figure 5 accommodated 18 collagen type I-based scaffolds with the addition of E, nHA, and mHA. In addition, one polystyrene column was left available for seeding a series of dilutions necessary for fluorescence-based assays. Two columns were also left empty to account for control samples. Gamma irradiation sterilization was selected in order to minimize damage to the structural proteins contained in the scaffold that may occur with other chemical methods. The resulting scaffolds were thin and translucent, and adhered to the entire surface area of the flat-bottom well.

	1	2	3	4	5	6	7	8	9	10
A	Dilution	0.5C	0.5C	0.5C	0.75C	0.75C	0.75C	1C	1C	1C
B			+ nHA	+ E		+ nHA	+ E		+ nHA	+ E
C	↓			+ nHA			+ nHA			+ nHA
D										
E		0.5C	0.5C	0.5C	0.75C	0.75C	0.75C	1C	1C	1C
F		+ E	+ mHA	+ E	+ E	+ mHA	+ E	+ E	+ mHA	+ E
G				+ mHA			+ mHA			+ mHA
H										

Figure 5. 96-well plate map of ECM-based scaffolds fabricated for HSPC co-culture experiments. A total of 18 unique 2D scaffold chemistries were fabricated for analysis (excl. single component E/nHA/mHA for media supplementation). All components additional to C were added at a concentration of 0.5%.

3.2 HUVEC Spheroid Assay

CFU assays are critical to understanding HSPC function, but the development of a co-culture protocol introduces adherent cells that may become embedded in the methylcellulose-based media. Since endothelial cells can form cell aggregates or spheroids in viscous medias, such as Matrigel, a spheroid assay was necessary in order to determine if the formation of spheroids would interfere with the growth and identification of subsequent HSPC-based CFU assays. This assay informs best practices for co-culture models as the presence of spheroids would require cell sorting after co-culture in order to improve colony output from HSPCs.

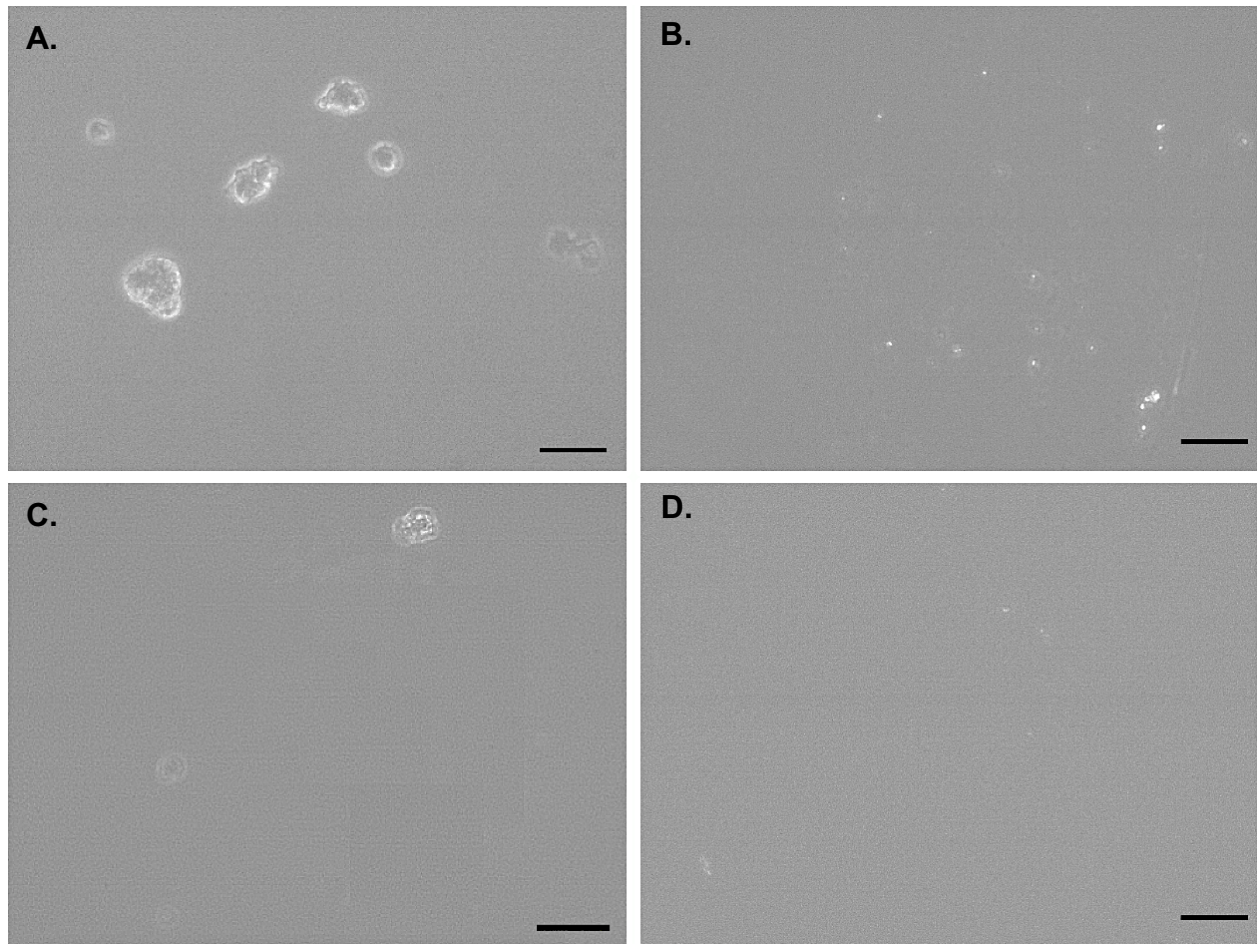


Figure 6. 500 HUVEC aggregate formation in methylcellulose. Formation was analyzed for a 1:1 ratio of cells to μL of methylcellulose for 48 hours (A), 144 hours (B), 216 hours (C), and 288 hours (D). Scale: 70 μm

The first spheroid assay assessed the behaviour of 500 HUVECs suspended in MethoCult™ H4434, the medium to be used for subsequent CFU assays. It found that cells tended to aggregate together in the methylcellulose medium immediately after seeding, but no large spheroids surpassing 100 µm were detected. While cell aggregates were more prominent at the earlier stages of the assay, they decreased throughout the trials until only small aggregates <10 µm and individual cells were detectable (Figure 6). This indicates that 500 or less HUVECs will not form large spheroids in methylcellulose that may impact CFU identification.

The second spheroid assay assessed if the formation of spheroids was dependent on cell density within the medium. Very few large aggregates were visible across all cell densities in the early stages of the assay, but at later stages a majority of identifiable cells were either individual or made up of extremely small aggregates <10 µm (Figure 7). The small aggregates were diffuse throughout the sample and did not center around a specific location of the well. The detection of aggregates was dependent on the number of cells in the sample, informing the decision of limiting HUVEC plating densities in the co-culture system to under 10,000 cells per well.

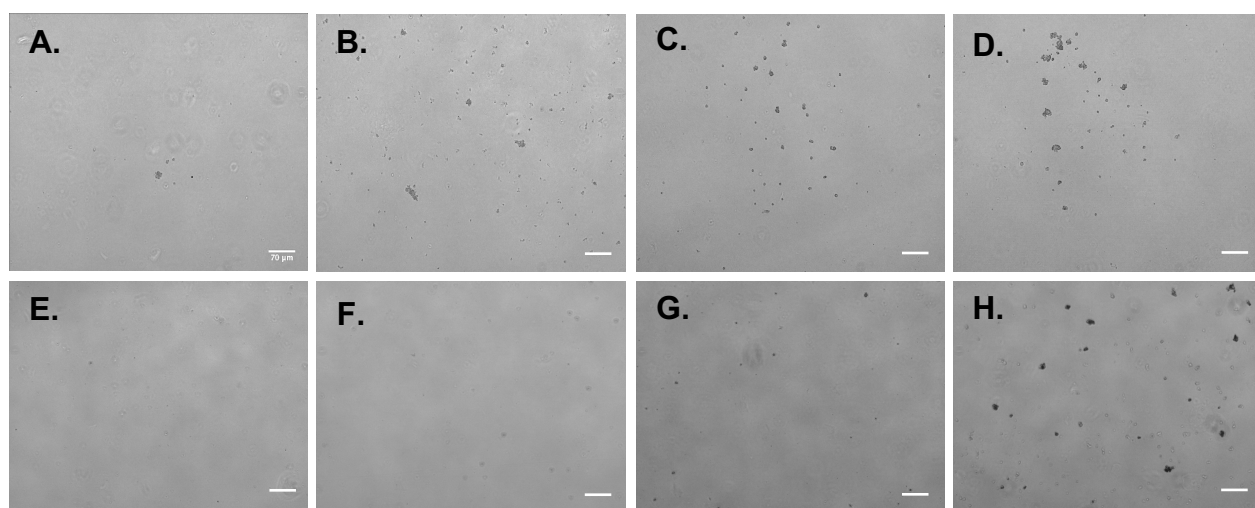


Figure 7. HUVEC aggregate formation in methylcellulose. Dose dependency of aggregate formation was analyzed at 144 hours of culture for 5000 (A), 10,000 (B), 25,000 (C), and 50,000 (D) cells, and at 480 hours of culture for 5000 (E), 10,000 (F), 25,000 (G), and 50,000 (H) cells. Scale: 70 µm.

In order to assess the survival of immortalized HUVEC-TERT2 cells within the limits of an HSPC-tailored system, including requirements for sera-free and phenol-free media, a media viability assay was performed. After 3 days of culture, the EBM control sample demonstrated a 45.6% increase in live cell population. In assessing HUVEC-TERT2 survival in various medias, HSPC complete media performed remarkably well, with an 87.6% increase in the HUVEC population over 72 hours (Figure 8D). The condition containing both media types displayed the least amount of cell proliferation, with only a marginal HUVEC population increase of 10.8% (Figure 8F). Imaging analysis confirmed that HUVECs cultured in HSPC complete media were able to achieve confluency and displayed similar adhesion to the plate as those cultured in EBM. Due to the specialized requirements that HSPC require to prevent differentiation, HSPC complete media was selected for use with co-culture scaffold models.

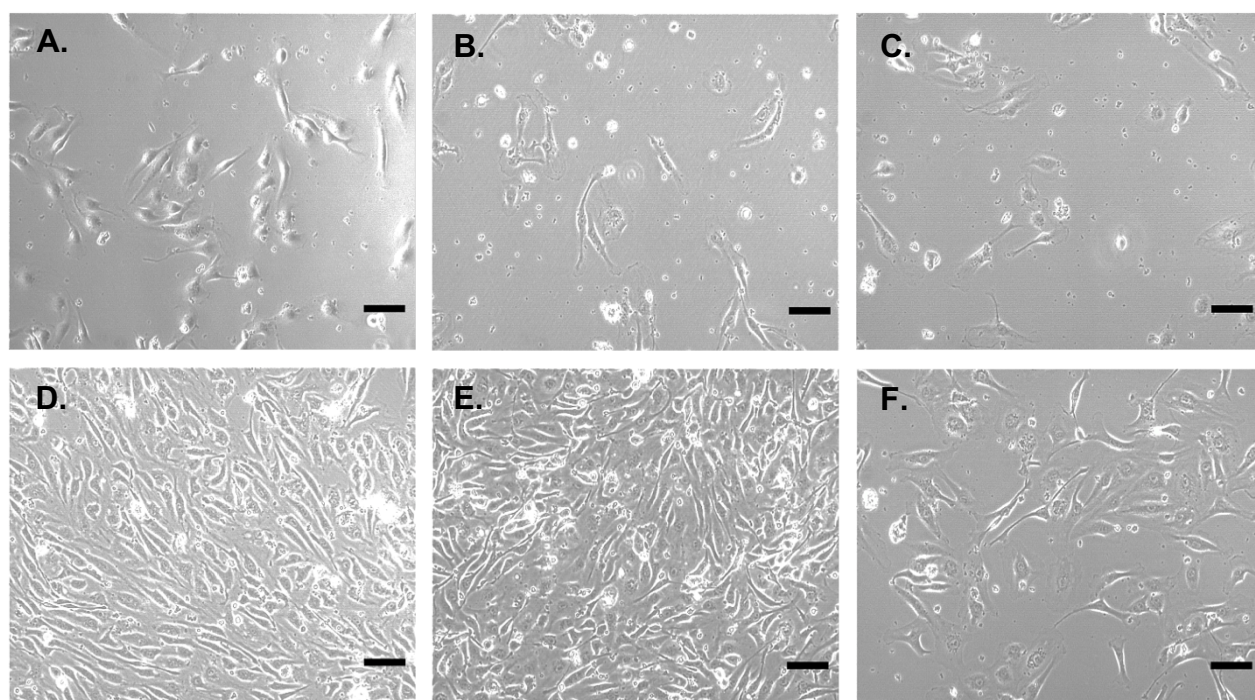


Figure 8. HUVEC-TERT2 cells after 24 hours of incubation in HSPC complete media (A), EBM (B), and 1 mL of each media (C) and 72 hours of incubation, respectively (D, E, F). Scale: 100 μm

3.4 HUVEC Scaffold Recovery

To optimize co-culture cell plating density, a series of experiments analyzing HUVEC population growth and recovery were completed. These experiments were vital in determining whether the adherent cells can be lifted from scaffolds to a sufficient degree for analysis, as well as finding an ideal cell number to prevent over-confluency of the adherent monolayer while also having a sufficient cell population to exert an effect on the HSPCs. The reagents Accutase and Trypsin-EDTA were employed to lift cells from the plate to determine as well as how different cell densities behaved during growth and recovery. Literature provides inconsistent optimal plating densities for HUVECs, with ranges from 1000 cells to up to 3000 cells per cm^2 ⁹². A range of numbers from 1000 to 10,000 cells was applied to investigate an optimal co-culture plating density.

Accutase performed significantly better in recovering cells at densities of 1000 and 10,000 cells, though there was no significant difference between Trypsin and Accutase at densities of 2500 and 5000 cells (Figure 9A). Across all samples, Accutase recovered 12.3% more cells than were originally plated while the population of cells recovered with Trypsin decreased by 1.1% relative to the original plating density. When examining cell populations, densities of under 2500 cells exhibited 100% recovery, with samples surpassing that of their original plating density. In contrast, densities over 5000 cells exhibited a 25% decrease in recovered cells relative to the original plating density (Figure 9B). Accutase was chosen as the dissociation reagent for use during co-culture due to its superior performance in cell recovery. Similarly, a plating density of 2500 HUVECs per well was selected for use in co-culture experiments due to its complete recovery and enhanced cell growth.

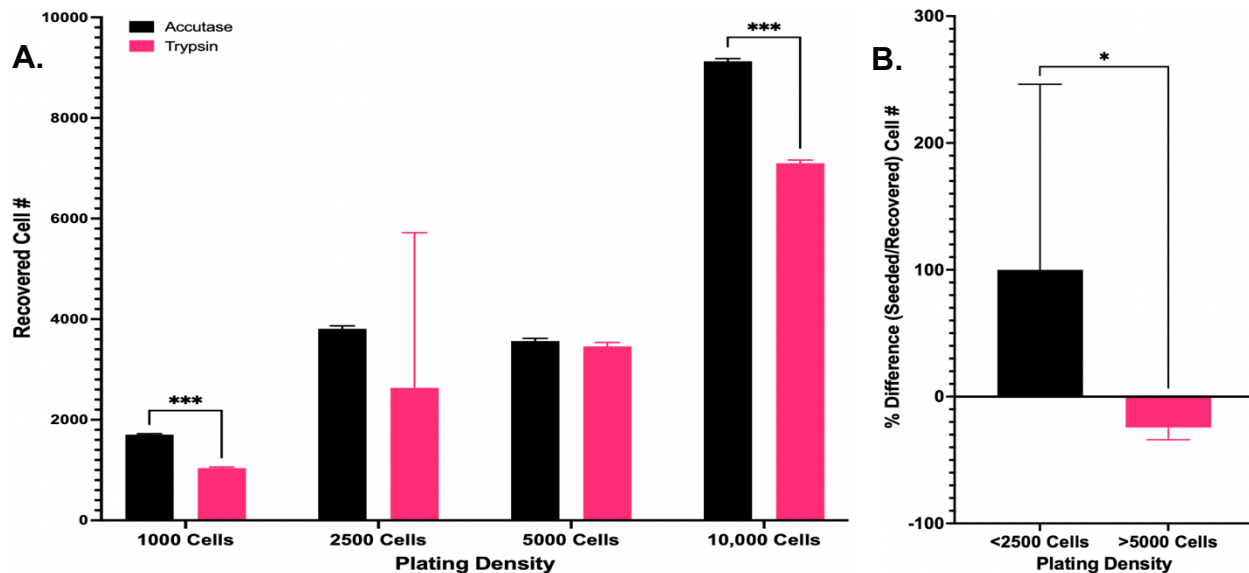


Figure 9. Optimization of plating and recovery of adherent HUVECs. Varying cell concentrations were plated and incubated for 72 hours. After incubation, the cells were recovered with either Accutase and Trypsin-EDTA dissociation reagents and assessed for viability using alamarBlue to detect metabolic activity. A. Number of recovered cells at 4 plating densities using both Accutase and Trypsin-EDTA as dissociation reagents B. Percent difference in seeded and recovered cell number at densities beneath 2500 cells and above 5000 cells. . Data reported as mean $\pm$ SD, n=2, t-test two-tailed, (*) p<0.05, (**) p<0.01, (***) p<0.001.

3.5 Endothelial morphology imaging

Fluorescence microscopy can be a powerful tool to deduce morphological changes in HUVECs on collagen-based scaffolds. Detection of stress-induced endothelial-to-mesenchymal transition can be performed through F-actin staining in order to reveal the cytoskeletal structure of adherent HUVECs. In order to determine if imaging can be completed on the scaffolds without removing the adherent cells and altering their morphology, it was vital to examine if the cytoskeletal structure of the HUVECs could be visualized through collagen. On a gelatin coated coverslip, the F-actin structure of the cytoskeleton is clearly visualized along with cell nuclei (Figure 10A). However, as the concentration of collagen increases from 0.25 to 1%, the number of cells visualized within the same field of view decreases (Figure 10B-E). In addition, areas of indiscriminate fluorescence

continued to appear as collagen concentration increased, as the cytoskeletal structures of the cells contained in the 1% collagen sample were nearly unidentifiable. Unfortunately, this increase in background fluorescence and decreased cell number led to the conclusion that fluorescent cytoskeletal imaging of HUVECs adhered to the scaffolds would not be possible.

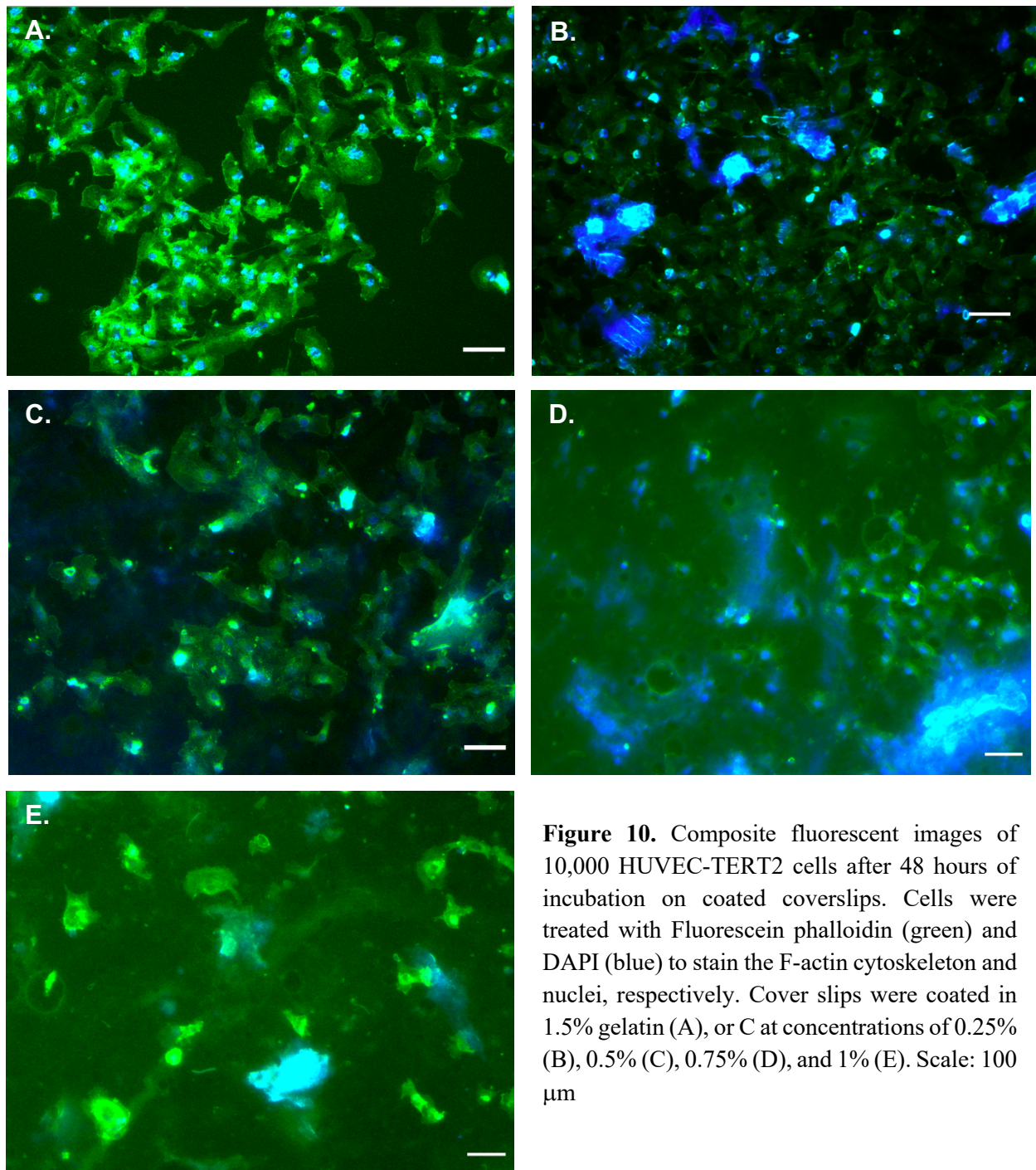


Figure 10. Composite fluorescent images of 10,000 HUVEC-TERT2 cells after 48 hours of incubation on coated coverslips. Cells were treated with Fluorescein phalloidin (green) and DAPI (blue) to stain the F-actin cytoskeleton and nuclei, respectively. Cover slips were coated in 1.5% gelatin (A), or C at concentrations of 0.25% (B), 0.5% (C), 0.75% (D), and 1% (E). Scale: 100 μm

3.6 Co-Culture Scaffold Screening

Before beginning the screening process, a proof-of-concept experiment was performed to confirm the results of preliminary experiments and subsequent protocol development. Previously fabricated scaffolds all based in 1% C were utilized to confirm the efficacy of the system before moving towards scaffold screening. After lineage depletion of a patient sample of 10^8 PBMNCs, 645,000 lineage-negative cells remained. 2 replicates were plated per scaffold condition, and all HSPCs were plated with HUVEC-TERT2 support to confirm the viability of the co-culture system. After 48 hours, cell numbers were recorded for each condition (Figure 11).

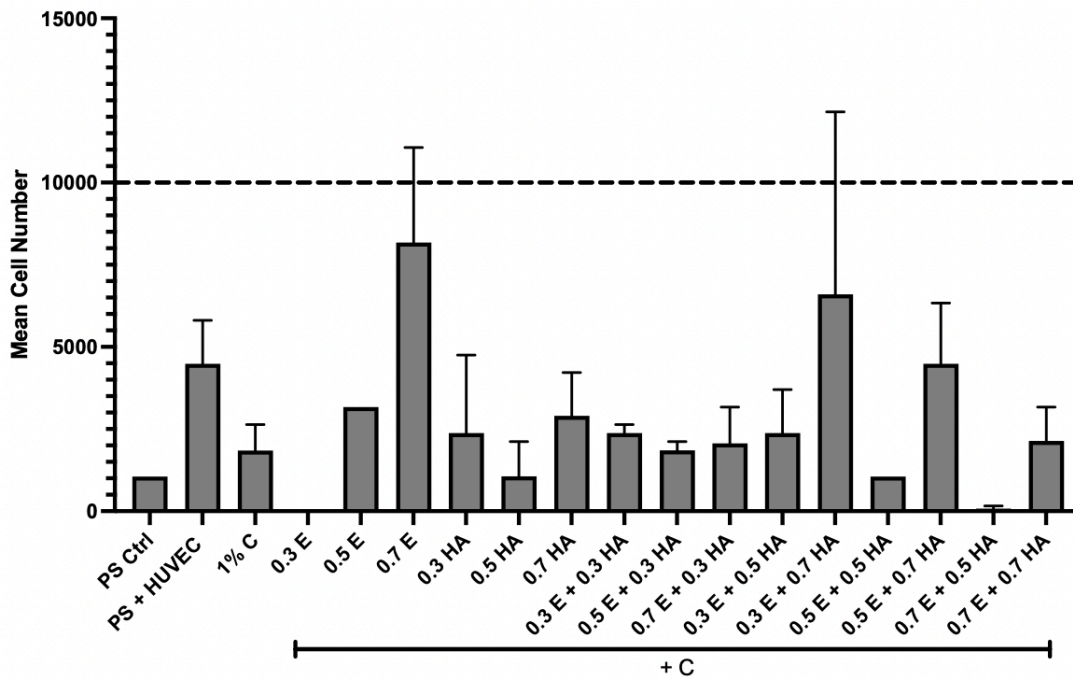


Figure 11. HSPC number per scaffold condition after 48 hours of coculture with HUVECs. Scaffolds consisted of collagen (C), elastin (E), and hydroxyapatite (HA) with polystyrene (PS) controls. Dotted line representative of original plating density of 10,000 cells (dotted line). Data reported as Mean $\pm$ SD, n=2, t-test two-tailed, (*)p<0.05, (**)p<0.01, (***)p<0.001.

2 conditions, one containing E and one containing a combination of E and HA, were notable due to a replicate surpassing the plated density after 24 hours and indicating a population expansion. This is of particular importance to the objective of expanding HSPCs for extended periods. A majority of scaffold conditions demonstrated an increase in recovered cells relative to polystyrene conditions. The increase in recovered cells seen between solo HSPC and co-culture conditions on polystyrene indicates a role for HUVEC-TERT2 cells in HSPC viability in the scaffold culture system.

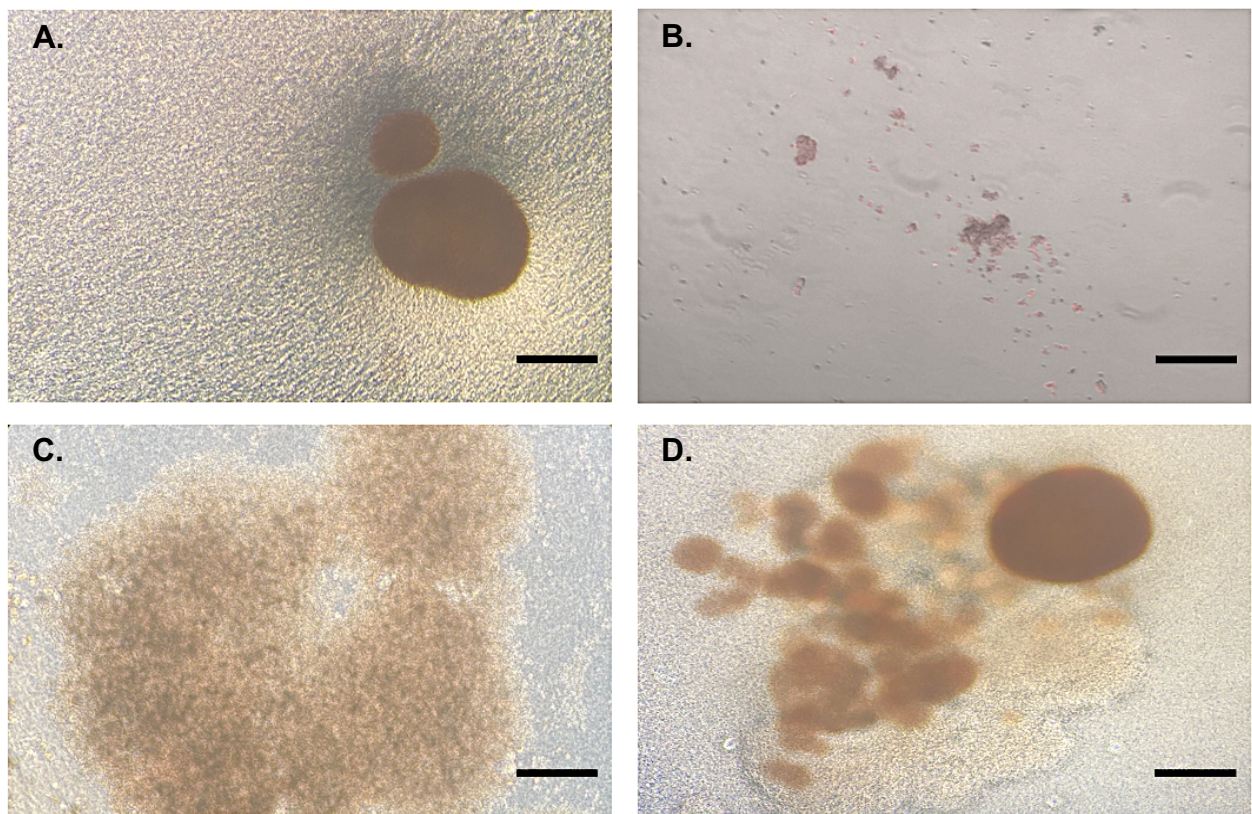


Figure 12. Representative colony images after 14 days of HSPC incubation in methylcellulose, including BFU-E (A), CFU-E (B), CFU-GM (C), and CFU-GEMM (D). Scale: 100 μ m.

Along with viability, functional tests were performed to confirm that the culture system did not have any negative impacts on colony formation potential. HSPCs were scored on both the number and type of colonies present, including BFU-E (Figure 12A), CFU-E (Figure 12B), CFU-GM (Figure 12C), and CFU-GEMM (Figure 12D).

The main criteria for determining if the system benefits HSPCs include the capability of each scaffold condition to both increase colony formation potential and generate all four major HSPC colony types. While cell counts are useful tools, conditions with larger cell populations that are also limited in colony formation potential or restricted to specific fates would not be ideal for translation into transplantation assays due to the limited capability of repopulating all major blood cell types. Polystyrene performed poorly, with both solo and co-culture conditions failing to meet the colony formation potential of day 0 controls immediately after sample thawing and lineage depletion (Figure 13). In contrast, co-culture on scaffolds resulted in a general increase in colony formation potential relative to day 0.

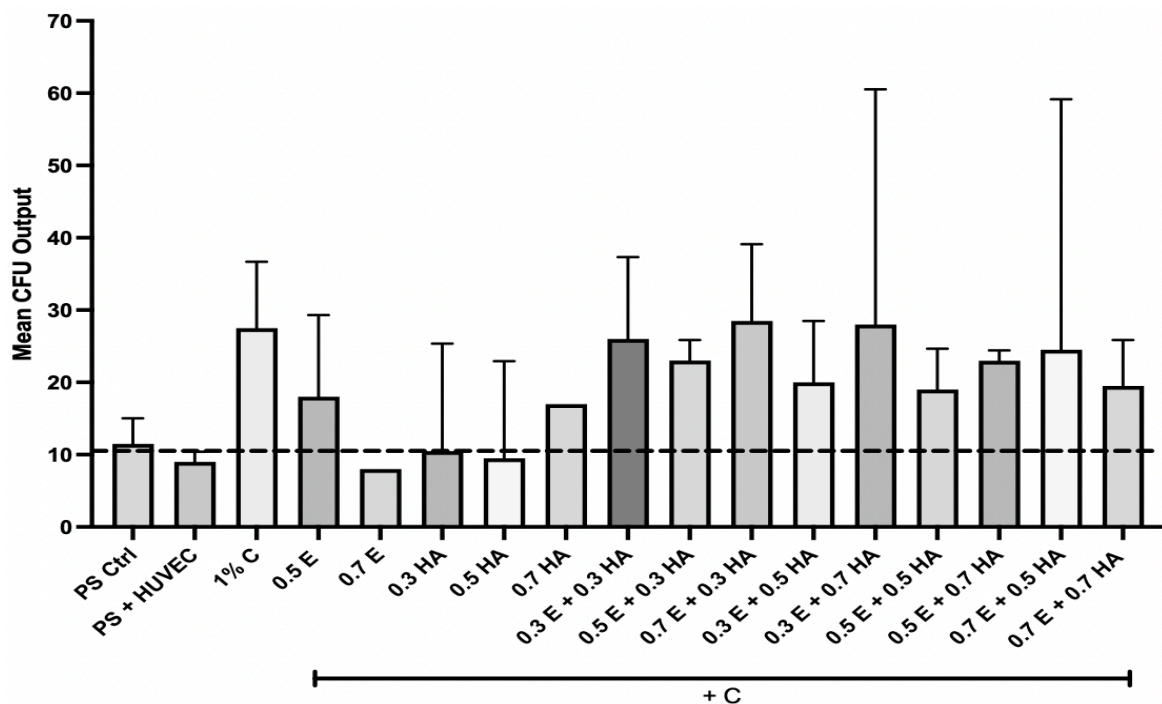


Figure 13. CFU output for each colony type (CFU-GEMM, CFU-GM, BFU-E, CFU-E) after 14 days of incubation in methylcellulose-based medium immediately following 48 hours of HSPC and HUVEC co-culture. Data reported as Mean $\pm$ SD, n=2, t-test two-tailed. Dotted line representative of day 0 colony formation potential.

When examining the types of colonies generated, solo polystyrene controls demonstrated limitations in colony formation potential due to the lack of CFU-E colonies (Figure 14). In contrast, CFU-GEMM, CFU-GM, BFU-E, and CFU-E colonies were identified in every scaffold co-culture condition other than the scaffold consisting of C and 0.7% of either E or HA. This is in line with the colony formation potential of day 0 samples, indicating a functional benefit for HSPCs in the presence of scaffolds. Though quantitative investigations demonstrated the propensity of co-cultured HSPCs on polystyrene to produce more viable cells in comparison to solo HSPCs on polystyrene, this effect did not carry over to functional analyses of CFU output, highlighting the importance of CFU analysis in determining the most ideal conditions for HSPC expansion without limiting function. This also acts as an indicator that the presence of scaffolds in the culture system may play a role in maintaining HSPC function.

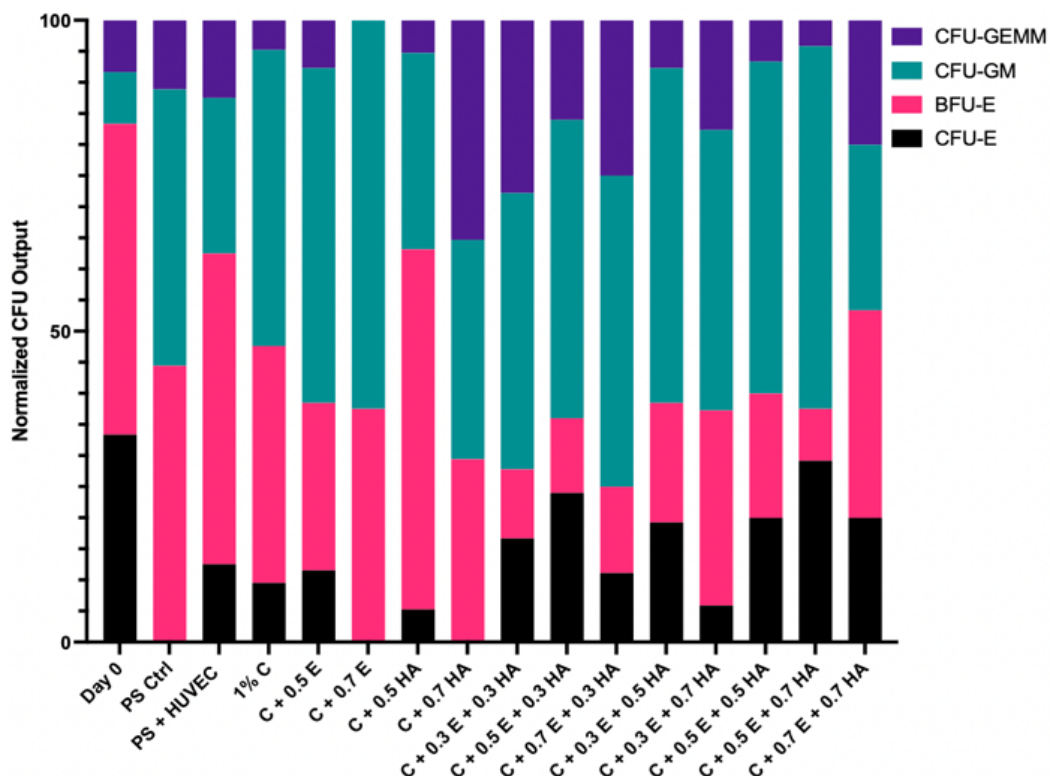


Figure 14. Normalized CFU output after 14 days of incubation represented by proportion of HSPC colony types. Data reported as Mean±SD, n=2, t-test two-tailed, (*)p<0.05, (**)p<0.01, (***)p<0.001.

While there was no statistical significance between mean CFU outputs across all conditions, significance was found when examining specific subsets of colony types. For example, C + 0.5 E + 0.5 HA demonstrated a statistically significant ($p < 0.05$) increase in CFU-GM colonies and C demonstrated a similarly significant ($p < 0.05$) increase in BFU-E colonies relative to solo polystyrene HSPCs. 1% C-based scaffolds as well as combinatorial scaffolds containing both E and HA resulted in both an increase in colony formation and functional diversity. These conditions also contained the largest proportion of CFU-GEMM colonies, indicating that the samples contained multipotent progenitors and had not committed to specific cell lineages. 73% of scaffold culture conditions had a CFU-GEMM proportion equal or greater than that of the day 0 control. Of these, 6 scaffolds were capable of maintaining a more primitive multipotent phenotype of HSPC than those cultured polystyrene. The wide variety of colony types represented in the proof-of-concept experiments is a positive indicator of lineage-negative HSPC expansion being possible within the scaffold co-culture system.

With confirmation that the system could expand HSPCs and maintain multipotency, the pre-fabricated scaffolds with differing collagen concentrations were utilized in order to identify components of importance and scaffold chemistries that should be scaled up for further analysis. For scaffold chemistry screening, 2 patient samples were used to run the experiment in duplicate. The first lineage depletion resulted in 9,680,000 live cells, and the second lineage depletion resulted in 5,060,000 live cells. Since cell counts were not possible at this stage, CFU analysis was used in order to determine if the lineage-negative fraction of HSPCs could survive in an extended co-culture system with incubation up to 7 days. Thus, a sample of 10 μ L of cell suspension per well was used to gauge the colony formation potential of the samples.

3.7 CFU Processing and Imaging

To properly document the CFU output from each scaffold condition, a modified MTT assay was performed with proof-of-concept CFUs to visualize the colorless granulocyte and macrophage progenitor populations. The MTT assay was successful at making larger CFU colonies visible without magnification and repeated imaging being necessary. After 1 hour of incubation, all colony types within the co-culture replicates were dyed purple and appeared on plate scans. This strategy was particularly effective for proof-of-concept samples, which had numerous clusters of colonies around the periphery of the well which moved towards the centre (Figure 15).

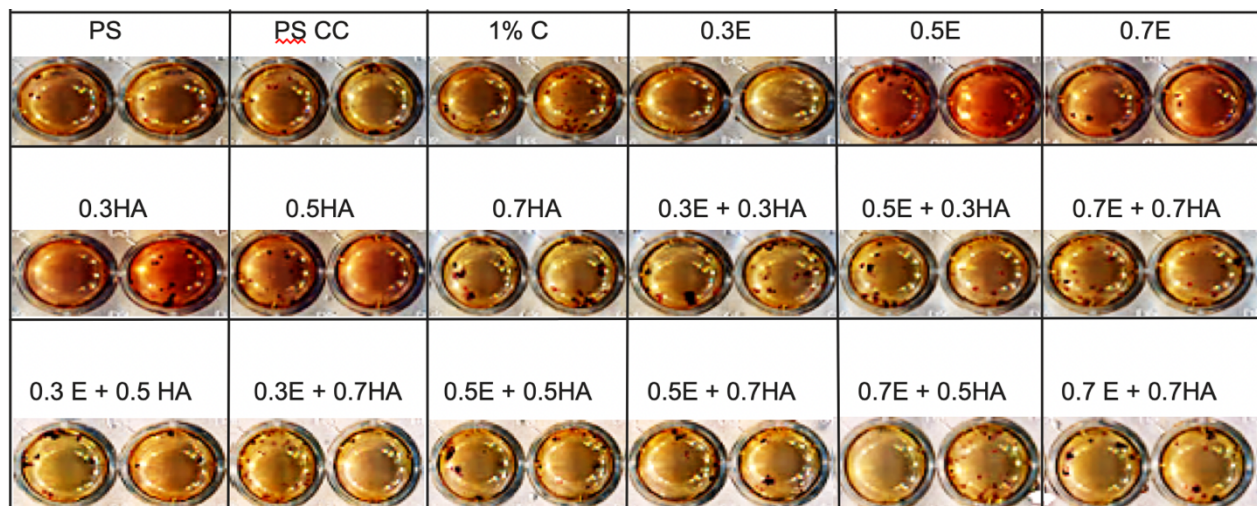


Figure 15. MTT-stained colonies after 14 days of incubation in MethoCult H4434 captured using a conventional scanner (n=2).

Unfortunately, when moving towards scaffold screening colony visibility after 1 hour of MTT exposure was reduced due to the reduced size of the representative colonies. They were excluded in favor of more quantitative CFU analysis values. The use of CFU assays is vital in distinguishing the differentiation potential of cells within the sample, indicating a more primitive or lineage-committed progenitor subtype.

After 3 days of culture, the only condition to reach statistical significance in colony formation was 1% C with the addition of nHA. However, all scaffold co-cultured conditions were all able to produce more colonies in comparison to their HSPC-only culture counterparts (Figure 16A-C). When examining colony composition, HSPCs that were cultured alone tended to commit to a granulocyte and macrophage fate, which was evident particularly on day 3. In contrast, co-cultured HSPCs maintained a greater level of multipotency and formed colonies representative of more diverse fates (Figure 16E-F). Colony formation was also superior on day 5 for HSPCs in co-culture conditions, though colony composition was diverse for both solo and co-cultured HSPCs (Figure 17). After 7 days of culture, polystyrene conditions had entirely exhausted their HSPC population (Figure 18A). This colony formation exhaustion was also noted for solo HSPC conditions. However, co-culture conditions on the same scaffold chemistries still had colony formation after the same incubation period (Figure 18A-C). Relative to day 0 colony formation potential, which only produced one multipotent CFU-GEMM colony, the colonies present for all co-culture samples were representative of erythroid, granulocyte, and macrophage progenitor populations (Figure 18E-F). Low concentrations of collagen within the scaffold appeared to skew cell fate towards a more committed erythroid differentiation program across all 3 time points, while increasing collagen maintained a more multipotent progenitor population.

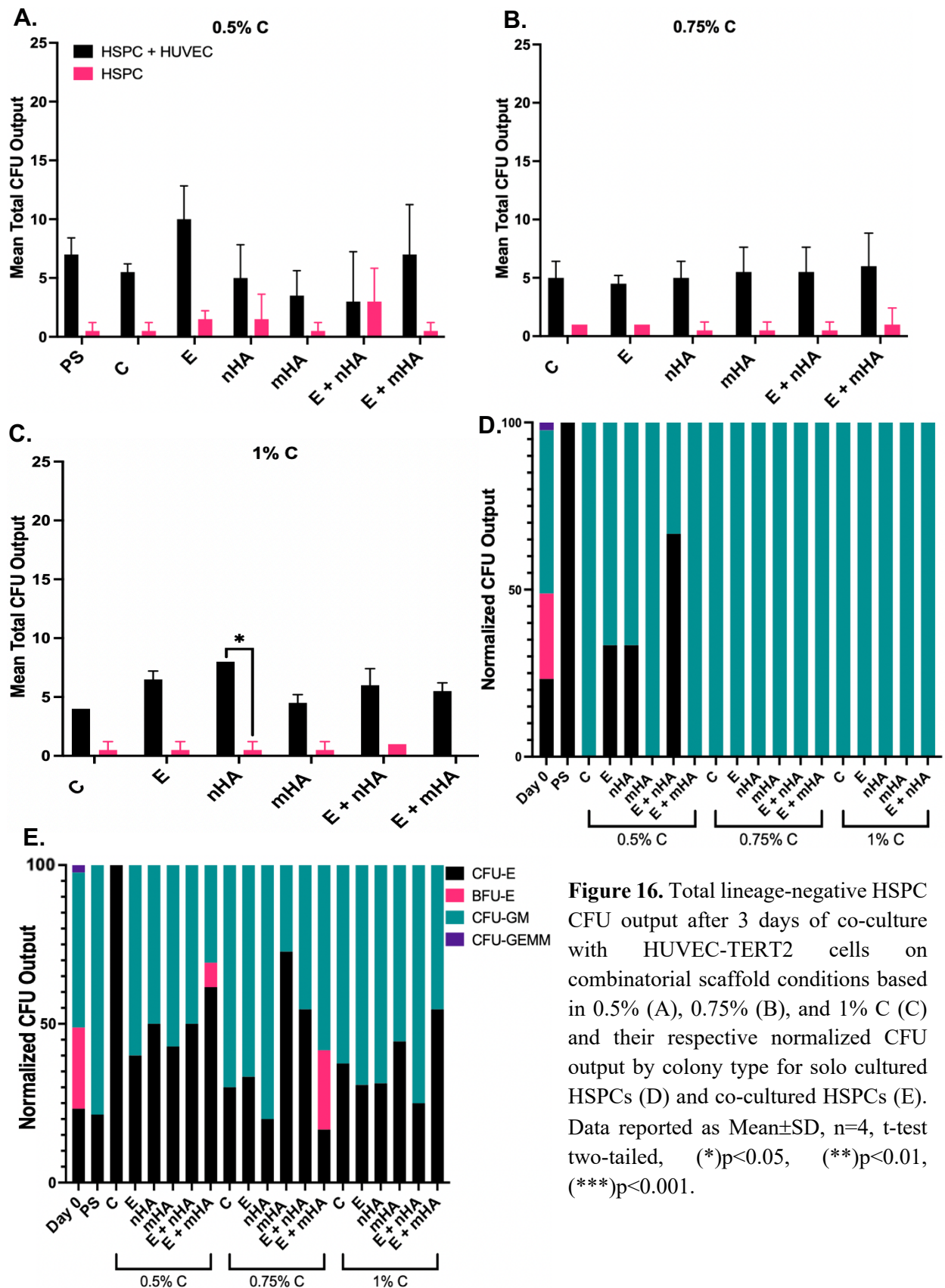


Figure 16. Total lineage-negative HSPC CFU output after 3 days of co-culture with HUVEC-TERT2 cells on combinatorial scaffold conditions based in 0.5% (A), 0.75% (B), and 1% C (C) and their respective normalized CFU output by colony type for solo cultured HSPCs (D) and co-cultured HSPCs (E). Data reported as Mean $\pm$ SD, n=4, t-test two-tailed, (*)p<0.05, (**)p<0.01, (***)p<0.001.

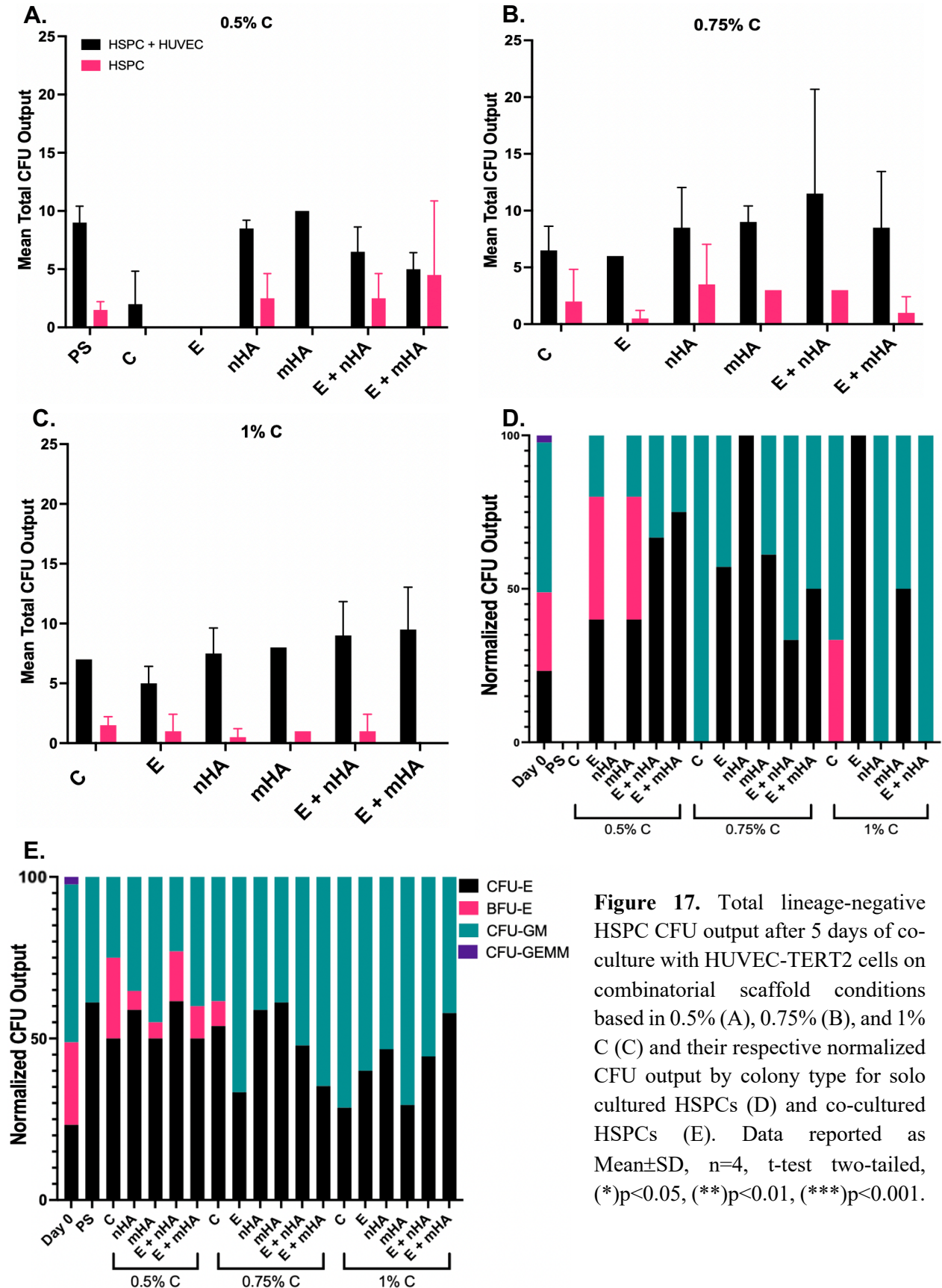


Figure 17. Total lineage-negative HSPC CFU output after 5 days of co-culture with HUVEC-TERT2 cells on combinatorial scaffold conditions based in 0.5% (A), 0.75% (B), and 1% C (C) and their respective normalized CFU output by colony type for solo cultured HSPCs (D) and co-cultured HSPCs (E). Data reported as Mean $\pm$ SD, n=4, t-test two-tailed, (*)p<0.05, (**)p<0.01, (***)p<0.001.

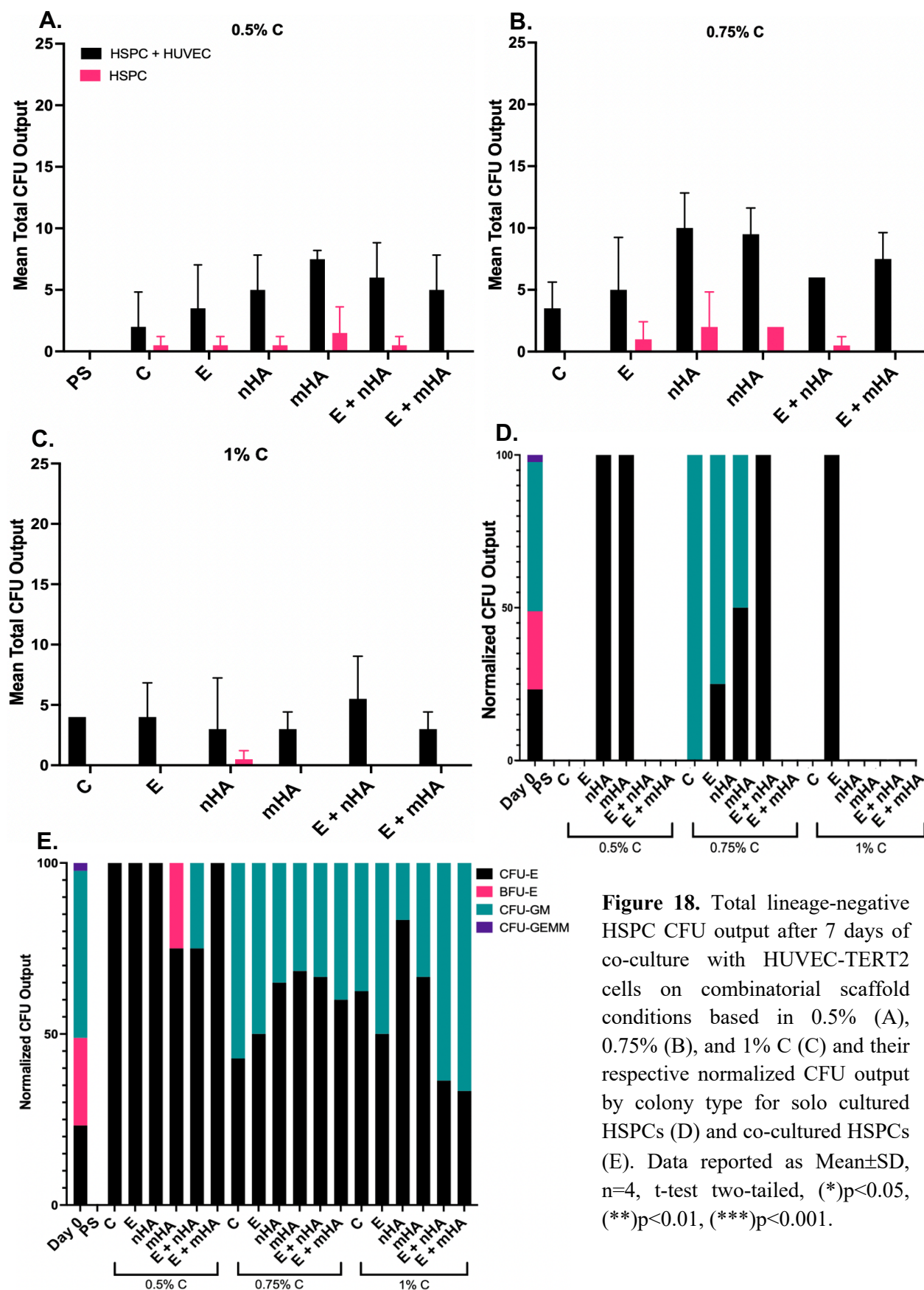


Figure 18. Total lineage-negative HSPC CFU output after 7 days of co-culture with HUVEC-TERT2 cells on combinatorial scaffold conditions based in 0.5% (A), 0.75% (B), and 1% C (C) and their respective normalized CFU output by colony type for solo cultured HSPCs (D) and co-cultured HSPCs (E). Data reported as Mean $\pm$ SD, n=4, t-test two-tailed, (*)p<0.05, (**)p<0.01, (***)p<0.001.

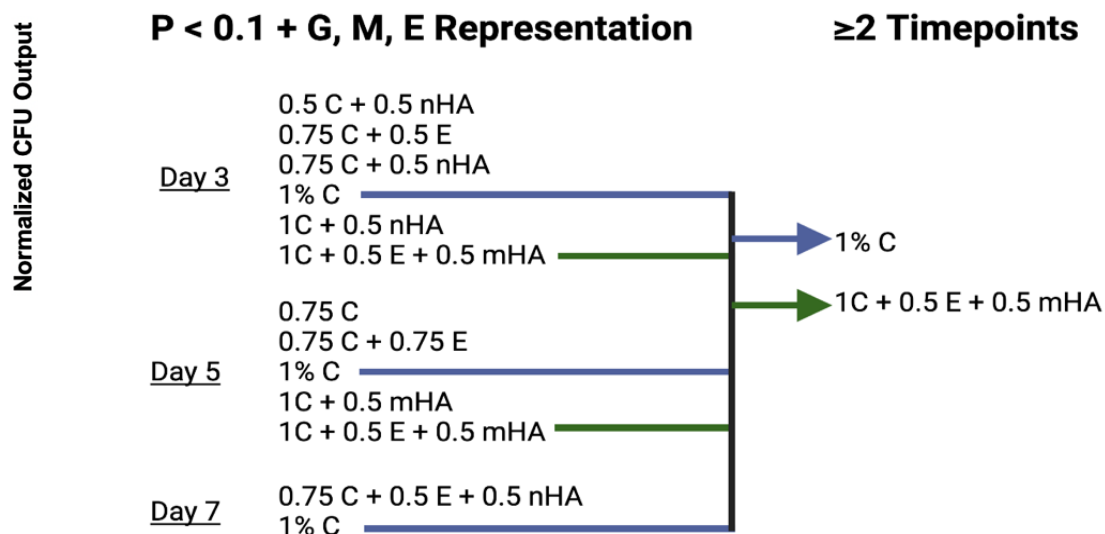


Figure 19. Schematic breakdown of screening analysis. Conditions that approached statistical significance ($p < 0.1$) across 3 timepoints were collected and assessed for multilineage representation. Those that appeared in 2 or more timepoints were selected for further investigation.

In order to determine scaffold conditions for scale-up, a matrix system was used in order to score scaffold chemistries based on both colony number and colony quantity via multiple paired t-tests of solo lineage-negative and co-culture conditions. The chemistries that approached statistical significance ($p < 0.1$) were collected and medians of their performance across the days of incubation determined which scaffolds showed the most promise in scale-up. This was also cross-referenced with the CFU breakdown by colony type to ensure that differentiation potential lined up with growth overall. The two colony types most represented across each time period that approached statistical significance over lineage-negative controls and represented all progenitors were 1% collagen and a combination of 1% C, 0.5% E, and 0.5% HA (Figure 19).

3.8 Scale Up Investigations

To further investigate the role of the system on HSPC behaviour, scaled-up models of the scaffolds were fabricated in a 6-well plate in order to be able to accommodate a larger volume of cells. At day 3, the viability of co-cultured cells significantly surpassed that of cells cultured on scaffold only as well as in HUVEC-conditioned media. This trend continued to day 7, though there was no statistical significance between co-culture and conditioned media (Figure 20A, B). Day 3 colony output was significantly greater for co-cultured conditions relative to the solo and conditioned media conditions, and day 7 colony output between lone HSPCs and co-cultured HSPCs continued this trend (Figure 20C, D). The day 0 CFU output from thaw with no scaffold or cell intervention was limited to only more committed progenitors and showed a bias towards an erythroid fate. In contrast, the most primitive multipotent CFU-GEMM colonies were observed in co-culture conditions seeded on scaffolds at both days 3 and 7. The HSPCs seeded onto PS without HUVEC support did not produce colonies on day 7, indicating an exhaustion of the repopulating fraction of the population. However, HUVEC support was able to mitigate this exhaustion, with CFU-GEMM colonies being observed on PS co-cultured HSPCs on day 7. Though no significant difference was found other than on day 7 on the multi-combinatorial scaffold, conditioned media performed slightly better in maintaining HSPC viability and functional output at both days 3 and 7.

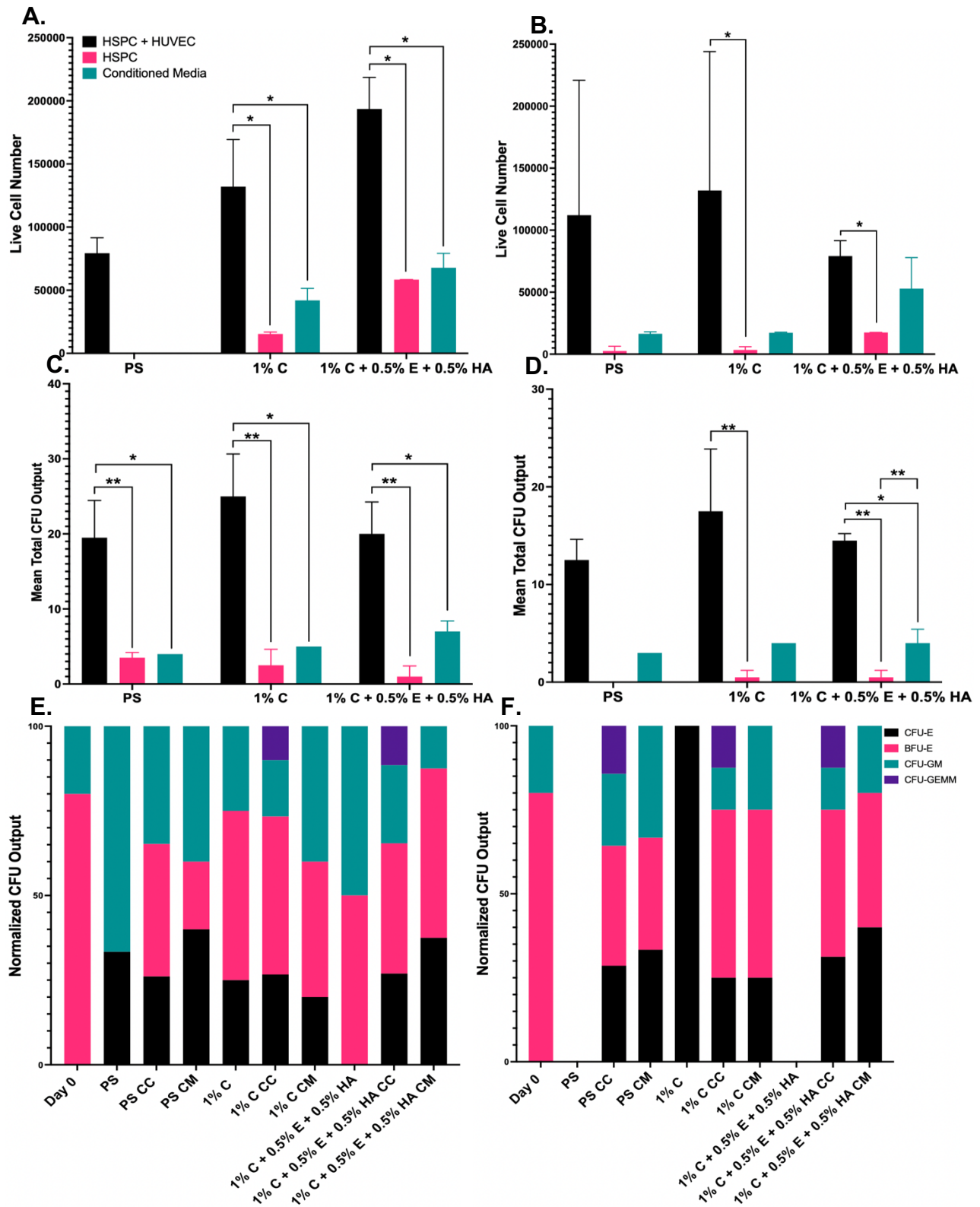


Figure 20. Viability and function of HSPCs cultured on scaffolds with HUVECs or conditioned media. Live cell number was assessed on day 3 (A) and 7 (B). Colony formation potential was assessed at day 3 (C) and 7 (D) for total output as well as respective colony proportions (E, F). Data reported as Mean $\pm$ SD, n=4, t-test two-tailed, (*)p<0.05, (**)p<0.01, (***)p<0.001.

3.9 HUVEC Genetic Analysis

Analysis of mRNA levels of target genes involved in HSPC maintenance is a valuable tool to determine the mechanisms behind the changes in cell viability and function after scaffold co-culture. After being cultured on polystyrene, 1% C, and a multi-combinatorial scaffold, mRNA levels of HUVEC-TERT2 cells were measured. Several changes in the mRNA levels of VEGF1, PECAM1, and SFRP1 were detected over the different scaffold chemistries. Interestingly, no significant changes in expression were observed for each gene between polystyrene and collagen scaffolds; however, there were significant changes when considering the multi-combinatorial C + 0.5 E + 0.5 HA scaffold. Both VEGF1 and SFRP1 expression also increased over 72 hours on both scaffolds in comparison to polystyrene (Figure 21A, C). Significant differences in expression were noted between the two scaffold compositions, with the multifactorial scaffolds demonstrating enhanced expression over collagen scaffolds. While SFRP1 demonstrated the largest fold change of mRNA levels in comparison to VEGF1 and PECAM1, all 3 genes demonstrated at least a 2-fold increase in expression when cells were cultured on multi-combinatorial scaffolds.

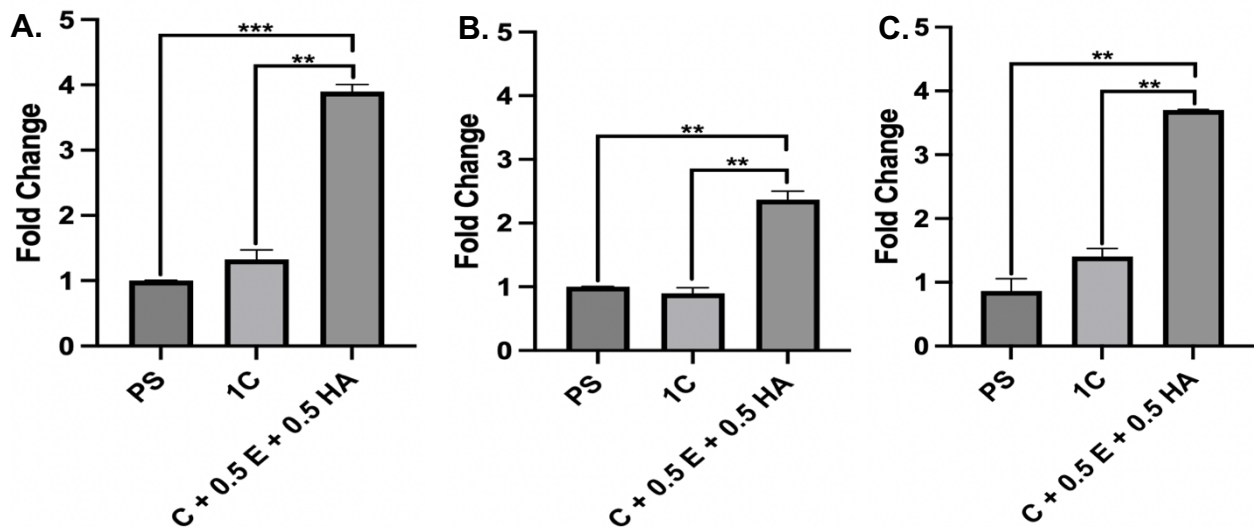


Figure 21. Quantification of mRNA expression after using RT-qPCR. Fold change in VEGF1 (A), PECAM1 (B), and SFRP1 (C). Data reported as Mean $\pm$ SD, n=2, t-test two-tailed, (*)p<0.05, (**)p<0.01, (***)p<0.001.

CHAPTER 4

Discussion

CHAPTER 4. DISCUSSION

4.1 Scaffold Fabrication

The use of biologically compatible scaffolds for *in vitro* cell culture applications has been extensively studied. However, due to the diversity of applications and fabrication processes, it is challenging to identify a singular optimal protocol. Thus, the fabrication of the scaffold system for use in a niche-mimicking culture system required optimization and a system of screening components. Collagen type I, the most abundant collagen in the human body, can be harvested from tissues for use in matrix fabrication due to its ability to self-assemble *in vitro*⁹³. Its mechanical and physical properties have been extensively described, facilitating alterations during the fabrication process. The use of collagen type I has successfully expanded human HSPCs in several different fabrication processes, such as suspension in collagen beads⁹⁴. In addition, HUVECs readily bind with collagen films and scaffolds, which have been shown to facilitate cell-cell interactions within *in vitro* culture systems⁹⁵. These qualities make collagen-based scaffolds an attractive option for sufficiently supporting both cell types. However, the native niche in which HSPCs reside is a heterogeneous combination of a great deal of other components. In order to maintain physiological relevance and recreate the niche with maximal fidelity, a multi-combinatorial approach to scaffold fabrication is necessary.

There are hundreds of structural proteins and factors that can be used to design a multi-combinatorial scaffold. Here, two distinct components, elastin and hydroxyapatite, were selected for screening. Elastin, though not classically associated with the bone marrow, is an important protein for maintaining elasticity within the engineered scaffold. Where collagen-based scaffolds can become incredibly stiff as collagen concentration increases, compliance can be increased with

the addition of elastin⁹⁶. Collagen and elastin scaffolds have been utilized in a wide range of applications, such as skin tissue engineering⁹⁷. However, elastin adhesion has been found to be critical for the long-term survival of HSPCs and the maintenance of a quiescent state, indicating that incorporation into scaffolds may provide a benefit to *ex vivo* viability⁹⁸. The incorporation of elastin into the scaffold can elucidate the importance of mechanical compliance in preventing differentiation in the system.

HA has previously been utilized in the fabrication of combinatorial scaffolds to. As a major component of bone, it has several applications in bone tissue engineering. However, success has been found when implementing it into haematopoietic investigations. For example, previous studies have found evidence that the use of hydroxyapatite scaffolds as a medium for cell growth can reconstitute hematopoiesis in a mouse model⁹⁹. As it is established that osteoblast interactions near the border with hard bone can also benefit HSPC population maintenance *in vivo*, the role of HA in this process should be further investigated to determine if these benefits can be enhanced and the mechanisms behind HA-HSPC interactions can be determined. The use of HA as a scaffold component poses specific challenges, as it is naturally basic and can alter the pH within the system. In addition to altering the environment for cell culture, it can also hinder collagen solubility in acetic acid and cause individual collagenous fibers to precipitate out of the solution, affecting the quality of the media throughout longer-term cultures¹⁰⁰. This alteration in pH is something to consider when attempting to optimize culture conditions, as an ideal range should be between 7.2 and 7.4¹⁰¹. However, repeated washes with PBS can temper these pH affects before cell seeding and allow for their use in long term cultures.

The deposition of solubilized collagen can produce a scaffold framework suitable for cell culture applications. Collagen molecules have demonstrated the capacity to self-assemble into fibrils *in*

vitro, forming a structural basis for cell seeding¹⁰². These fibrils have been observed to grow to several centimeters in length despite beginning from molecules of 1.5 nm in size¹⁰³. However, the stability of the fibrils is mostly imparted by post-translational modifications *in vivo*, which are notably absent if manufactured for cell culture applications¹⁰⁴. In order to maintain a physiologically relevant stiffness mimicking that of the *in vitro* environment, scaffold cross-linking was employed. Cross-linking is extremely useful in providing stability and mechanical strength to engineered scaffolds manufactured with biological proteins¹⁰⁵. In human tissues, cross-linking through post-translational modification is typically catalyzed by enzymes¹⁰⁶. For example, lysyl oxidases are responsible for cross-linking collagen and elastin in the native ECM¹⁰⁷. While this is an effective process *in vivo*, similar efficacy during *in vivo* manufacturing is difficult to achieve due to ATP dependence and the multifaceted interactions of a wide range of enzymes¹⁰⁸. Physical processes have also been employed as cross-linking strategies for engineered scaffolds. For example, UV-A exposure for 30 minutes has been used to cross-link collagen in the human cornea to reverse the effects of keratoconus, where genetically controlled degradation of collagen in the eye lens degrades vision¹⁰⁹. However, physical cross-linking does not provide flexibility towards other scaffold aspects such as pore size, which can result in extremely inconsistent results when used for cell culture¹¹⁰. As an effective alternative, the use of chemical cross-linking agents has been found to produce more potent stabilization of collagen fibers in contrast with physical or biological processes¹¹¹. There are a wide variety of chemical agents that can be used which target and link functional groups of structural proteins, such as genipin or glutaraldehyde¹¹². However, the use of EDC/NHS has been successful in imparting physical strength to engineered scaffolds via increasing interconnectivity of collagen fibrils and enhancing scaffold stiffness¹¹³. EDC/NHS functions by allowing activated carboxyl groups to form zero-length amide bonds without the incorporation of other chemical components to the matrix¹¹⁴. Biological scaffolds cross-linked with

EDC/NHS have been shown to maintain their biocompatibility and do not demonstrate cytotoxicity, permitting their use in cell culture¹¹⁵. Thus, the use of EDC/NHS for scaffold cross-linking was a safe and effective measure to maintain the physical properties of engineered scaffolds.

The fabrication process is one that has room for improvement. With the developments of 3D printing, sacrificial molds can be developed to form perfusable vessels¹¹⁶. This would allow for several alternative investigations, such as using a perfusion system to deliver cytokines to seeded HSPCs or examining the tubular formation potential of HUVECs within the system for tissue engineering applications. For the purpose of high-throughput component screening, the formation of scaffolds in a 96-well format is a viable option. However, the three-component design of the plates fabricated at this stage of the study must be expanded on. Collagen type I, the basis for each scaffold, has been found to be an effective vessel for HSPC expansion as it can both capture and release growth factors to alter cell behavior¹¹⁷. Moving forward, the introduction of new components will be necessary in order to fully encompass the complexity of the vascular niche. In addition, once specific scaffold chemistries are identified in this format that lead to HSPC expansion, multi-component combinations consisting of four- or five-component wells can be fabricated in order to determine if more complex ECM-mimicking conditions can also lead to HSPC expansion and multipotency.

4.2 Co-culture protocol optimization

In addition to scaffolds providing a platform for cytokine binding, the use of endothelial cells in the culture system is critical for continuous cytokine production during incubation. HUVECs are a widely used source of ECs for study due to their faithful representation of typical cell behaviour in contrast with other established cell lines. Their uses range from the study of inflammation and

cardiovascular disease to regenerative medicine¹¹⁸. However, one of the major disadvantages of HUVECs in culture is rapid exhaustion, with morphological characteristics being lost by passage 6 and complete exhaustion by passage 10¹¹⁹. In order to make HUVECs permissible in long-term culture, a series of genetic changes has been used to mitigate the time limitation and fortify the cells against less-than-optimal culture environments. For example, one approach generated a vascular niche using HUVECs altered with the adenoviral E4ORf1 gene, allowing for long term survival without a change in function¹²⁰. Similarly, HUVEC-TERT2 cells immortalized by human telomerase catalytic enzyme (hTERT) show long-term viability without a loss of function or morphology¹²¹. Due to this maintained viability, HUVEC-TERT2 was selected for use in the co-culture system.

HSCs are an extremely rare and elusive cell population, making their study *in vitro* extremely challenging. They comprise anywhere from 0.001% to 0.00001% of a given sample of PBMNCs¹²². However, the use of human cells is vital due to the differences between human and murine HSPCs when it comes like characteristics such as surface markers¹²³. Though murine models are effective for *in vivo* transplantation assays, more in-depth analysis of the mechanisms behind cell behaviour is needed in human cell samples in order to be applicable to human therapeutics. Cord blood is an alternative source of HSPCs and is commonly used in bone marrow transplantation. However, studies have found that cord blood is often less successful when isolating a sufficient stem cell fraction and can have variable output in terms of colony formation potential¹²⁴. To this end, primary samples of PBMNCs derived from human patients were used for co-culture study.

The formation of three-dimensional endothelial spheroids has previously been used in research for applications such as tissue revascularization¹²⁵. Typically, most functional studies define a spheroid to be at least 500 cells in size. When considering behaviour within a CFU assay, detection

of large spheroids could have a negative impact on colony growth or identification. Spheroid generation also poses the possibility of limiting physical space for sufficient colony expansion and accelerating the exhaustion of growth factors in the media. The methylcellulose-based suspension used for producing CFUs contains growth factors tailored towards HSPCs such as recombinant stem cell factor, erythropoietin, and human granulocyte-macrophage colony-stimulating factor. The presence of these factors are optimal for HSPC colony growth, but are not optimized for endothelial expansion or spheroid formation. By discovering that the cell aggregates or spheroids remained relatively small and diffuse throughout the methylcellulose-based medium even at higher cell densities, it could be determined that further protocols such as cell sorting after HUVEC and HSPC co-culture on scaffolds will not be necessary before initiating a CFU assay. This aided in decreasing the length of the protocol and improve efficiency moving forward.

Maintenance of haematopoietic stem cells *in vitro* requires specific protocols, including the requirement of a serum-free environment in order to prevent differentiation of progenitors¹²⁶. In order to ensure the co-culture system could function in a serum-free environment, a media assay for the HUVEC-TERT2 cell line was required. Under normal conditions, HUVEC media will contain serum such as FBS as well as a phenol pH indicator¹²⁷. However, the successful use of 1:1 ratios of supplemented EBM and HSPC media has been demonstrated in previous co-culture studies, warranting its investigation for the proposed scaffold model¹²⁸. The surprising durability of the cells in HSPC complete media over 72 hours of culture indicated that the immortalized cell line was tolerant of serum-free and phenol-free conditions. However, in contrast with previous protocols, the 1:1 ratio of both medias did not generate as large of a population expansion relative to HSPC media only. This may be due to the type of culture model employed, as consistent perfusion in contrast with 2D culture may impact cytokine circulation and availability. The lack of

notable morphological changes and excellent adherence of HUVECs cultured in HSPC complete media reflected a healthy cell population. This informed the use of HSPC complete media during co-culture scaffold screening.

The optimization of cell recovery from scaffolds is vital as no relevant data can be produced if the cells cannot detach from the scaffolds. A wide variety of dissociation strategies have been employed in culture systems such as the use of enzyme cocktails. However, strong enzymatic dissociation can lead to loss of cell surface markers, rendering cells unusable for downstream identification applications such as flow cytometry or single cell RNA-Seq¹²⁹. Mechanical dissociation tools such as the use of physical force to detach cells from scaffolds risks the generation of debris and physically damaging the delicate cells¹³⁰. Two dissociation reagents, Accutase and Trypsin, were examined for their ability to recover adherent cells from the scaffolds. Trypsin-EDTA is a proteolytic enzyme which acts by cleaving lysine and arginine to detach cells from their culture substrate, primarily targeting integrins $\alpha 5\beta 1$ and $\alpha V\beta 3$. Despite common use, there are several drawbacks to its application. Trypsin-EDTA dissociation has been found to damage cell surface markers such as N-cadherin, influencing aggregate formation in kidney cells¹³¹. In addition, Trypsin-EDTA has been found to modify antigenic epitopes, altering the results of cell surface marker identification such as through flow cytometry¹³². While this may translate positively to the presented co-culture system in avoiding the formation of spheroids by HUVECs, it also may detrimentally impact downstream assays. In contrast to the commonly used Trypsin-EDTA which requires close monitoring to prevent apoptosis, Accutase is considered to be a much gentler alternative and is commonly used in the dissociation of stem cells to prevent altering viability and functional output¹³³. Accutase-dissociated embryonic stem cells have been found to have no alteration in viability and proliferation during downstream assays, making Accutase an

attractive option for dissociation with HSPCs present¹³⁴. The preliminary recovery experiment found that Accutase was significantly more effective than Trypsin when dissociating cells from the scaffold. Its demonstrated superior function as well as its mild nature in contact with HSPCs informed the selection of Accutase for all cell dissociation from scaffolds during co-culture experiments. The results of preliminary recovery also helped determine the cell densities of further experiments moving forward. Plating densities of over 5000 cells per well in a 96-well format led to a decrease in recovered cells relative to the original amount plated, indicating that lower densities in this format allow for healthy cell growth and population expansion. The density of 2500 HUVECs per well selected for scaffold screening is consistent with the existing literature using HUVECs as a cell model, as typically densities below 2500 cells per well are used in 96-well formats¹³⁵. This is due to ECs experiencing contact inhibition *in vitro*, where further population growth is hindered under conditions where too many cells are present¹³⁶. Overall, this work was critical for optimizing protocols that will be used going forward to assess how the behaviour of both HUVECs and HSPCs may change in response to experimental parameters.

4.3 Endothelial morphology imaging

Endothelial cell imaging is a useful tool in order to determine if specific conditions cause an alteration in HUVEC morphology. Changes in morphology can indicate concerns regarding cell health or an endothelial-to-mesenchymal transition, which suggests that the cells are under stress¹³⁷. By confirming that cultured ECs maintain a typical cobblestone monolayer morphology, fluorescence imaging can be a predictor of a scaffold condition's effectiveness in cell maintenance¹³⁸. The use of phalloidin to selectively target F-actin of the cell cytoskeleton is an effective means for the detection of changes in morphology and cellular organization under different chemical conditions provided by scaffolds¹³⁹.

There are several challenges to consider when attempting to capture cellular morphology in the current scaffold system, most prominently the presence of the scaffold components themselves and polystyrene. Though polystyrene is commonly used for cell culture, it also displays autofluorescence between 300-400 nm¹⁴⁰. Unfortunately, this wavelength range also overlaps with excitation and emission spectra of numerous commonly used fluorophores, such as DAPI¹⁴¹. Thus, the use of glass coverslips is necessary to prevent polystyrene autofluorescence from distorting resulting images. When considering the chemical composition of the scaffolds, both collagen and elastin have been observed to be sources of structural autofluorescence within the natural human ECM¹⁴². The two components share several similarly structured fluorophores, where collagen autofluorescence has been observed between 400-450 nm¹⁴³ and elastin autofluorescence has been observed between 320-405 nm¹⁴⁴. The cumulative range of these values covers a wide range of wavelengths, which may negatively impact the visualization of selected fluorescent dyes. Due to these considerations, the use of fluorescent markers to examine morphology of cells seeded on autofluorescent scaffold components may not be feasible in a high-throughput context such as scaffold component screening. The assessment of varying collagen concentrations as well as a gelatin control was therefore necessary to determine if scaffold composition could be optimized to allow for efficient fluorescent imaging of cells within the co-culture system.

The strong autofluorescence emitted by the collagen type 1-coated coverslips as well as decreased quality of images through polystyrene in preliminary experiments indicates that imaging of cells grown directly on scaffolds in a 96-well polystyrene plate is not feasible. While the gelatin coverslip demonstrated high resolution and clarity with major structures clearly identifiable, increasing concentrations of collagen led to both a decrease in the number of cells visible as well as an associated increase in autofluorescence. The enhanced autofluorescence occluded cells and

did not allow for distinct cellular compartments to be identified within the image. The overall results from this experiment indicate that cell imaging may not be a suitable investigation moving forward in this study, as both polystyrene and collagen type I pose significant problems related to the optics of the microscope. As the scaffolds themselves are opaque, imaging through both a polystyrene and scaffold layer to focus on the cells seeded on top of the scaffold is extremely challenging. 96-well polystyrene plates are typically not recommended for cell imaging due to the shape and thickness of the wells decreasing image quality, as well as incompatibility with objective lenses of increasing magnification¹⁴⁵. The added complexity of enhanced autofluorescence by scaffold components unfortunately limits the use of this technique during scaffold screening. In addition, recovering the cells from a scaffold and subsequently seeding them onto gelatin-coated coverslips would alter cell morphology and not allow for any meaningful interpretation of results. An alternative use for fluorescence microscopy in this system could be to investigate the deposition of collagen and elastin in polystyrene co-culture conditions. Though the autofluorescence of these components prevents direct scaffold imaging, this characteristic has been used in previous studies as a surrogate marker of ECM deposition by support cells¹⁴⁶. Adapting imaging protocols for this purpose may allow for its use in continued study of the niche mimicking system.

4.4 Co-culture scaffold screening

The most critical component of this study was the analysis of the ability of the niche-mimicking culture system to enhance HSPC viability and function. The preliminary proof-of-concept screen was completed to synthesize the findings of optimization experiments and determine if the co-culture system was feasible.

The elusive HSCs which this system aims to enhance represent only a marginal fraction of the total cell population of a given sample, meaning that further processing is required to enrich for a

multipotent population. However, this is an extremely challenging process given the highly dynamic state of the field. Human HSC markers are still being identified in order to completely purify the stem cell population. For example, the most recent single cell analyses identifying endothelial protein C receptor (EPCR) as a marker for the self-renewing and repopulating stem cell population has still only achieved a one-in-three to one-in-five HSC frequency, leaving room for further characterization¹⁴⁷. In addition, the use of single cell RNA-Seq to analyze the transcriptome of HSPCs has elucidated the extensive heterogeneity exhibited by cells that share the same cell surface markers¹⁴⁸. Differences between murine and human cell surface markers also complicate this isolation process. In order to increase the throughput of scaffold screening, lineage depletion was selected as a population enrichment method. By using negative selection to bind committed progenitors, precursors, and mature haematopoietic cells, the lineage-negative stem and progenitor population is left unmarked. This allows for the purity of the stem and progenitor fraction to be maintained, permitting surface marker analysis downstream of initial experiments. Similar considerations were taken in using automated cell counting rather than manual trypan blue exclusion, as best practices for downstream assays encourage automated counting to maintain reproducibility and accuracy¹⁴⁹.

The lineage-negative HSPC fraction used for co-culture with HUVEC-TERT2 cells responded positively to the combined effects of endothelial and scaffold support. The ability for two scaffold conditions to produce a net population expansion is extremely promising when taken along with the enhanced functional output of the population relative to day 0 controls, indicating that a more primitive HSC phenotype was present. To contextualize the functional output of the cells, perspective within the haematopoietic hierarchy is necessary. Given the increasing frequency of cells proportional to maturity, it is expected that the most primitive CFU-GEMM colonies would

be infrequent, while committed progenitors such as CFU-GM, BFU-E, and CFU-E would appear more frequently¹⁵⁰. In addition, enhancing functional output beyond day 0 controls is often an extremely difficult process. Relative to untreated cells immediately after thawing and lineage depletion, several scaffold chemistries demonstrated an increase in the multipotent fraction of the cell population through enhanced CFU-GEMM production. By targeting cellular function through the use of a CFU assay, more relevant insight can be deduced as opposed to dependence on cell count alone.

4.5 CFU processing and imaging

In order to make the most impactful observations, a focus on cell function was established. While cell counting can be used as a general indicator of the viable population present, it is also not sufficient to determine if an HSPC population is truly present or if stem and progenitor exhaustion has occurred. In addition, assays dependent on metabolic activity such as the use of resazurin-based reagents are not viable options due to the typically dormant and metabolically inactivate state of HSPCs¹⁵¹. The use of a CFU assay is critical to generate a functional output indicating the multipotency of the resulting population, acting as an indicator of cell identity within the sample.

The use of MTT as an imaging stain was successfully adopted into this protocol. While more primitive HSPCs have low metabolic activity, more committed precursor cells that make up the colonies visible in a CFU assay generate enough metabolic activity to convert MTT to insoluble blue formazan¹⁵². This colour change was exploited to visualize colorless granulocyte and macrophage progenitors along with the bright red erythroid progenitors by eye as opposed to being dependent on microscopic visualization. This also allowed for the use of a conventional scanner to capture images of each replicate condition, facilitating data analysis. Despite success in visualizing conditions with numerous large colonies, there were several constraints to this imaging modality.

As CFU-E and CFU-GM have lower cell number thresholds for what is considered to be a colony, at 8 and 20 cells respectively, these smaller colonies were only made visible under higher magnification and thus were not clearly visible with the naked eye in the resulting scanned images. These colonies are also reflective of a slightly more mature progenitor population rather than a lineage negative stem cell population, which tend to form much larger CFU-GEMM colonies representative of multilineage potential. MTT is an extremely effective imaging solution available for documenting CFU results along with manual counts and analysis. In this case, the MTT assay was secondary to using the CFU counts and identities in order to support colony analysis data and make an informed decision on which scaffold conditions should be scaled up.

The resulting colony formation potential of both co-cultured and solo HSPCs continued the previous trend showing a marked functional increase when supported by HUVECs. The exhaustion of the colony-forming population in many HSPCs cultured without HUVEC support by day 7 reflects expected behaviour. HSPC exhaustion is still a top issue in the field, continuing from *in vitro* cell cultures to *in vivo* transplantation assays, which has yet to be resolved. However, this system allows for high-throughput functional screening in order to identify conditions that not only maintain and expand HSPC populations, but also maintain their critical function for therapeutic translation. The presence of primitive CFU-GEMM colonies in co-culture conditions is also of great importance. *In vivo*, the increased production of primitive progenitors is associated with the presence of SCF as mutations affecting the cytokine or its receptors lead to perinatal mortality¹⁵³. Its role as a master regulator of haematopoiesis makes it a logical supplement for HSPC culture *in vitro*. However, while HSPC complete media contains supplemental SCF to encourage population expansion, this supplementation was not sufficient in maintaining HSPCs in culture without significant differentiation and exhaustion. The maintenance and expansion of the most primitive

progenitors may then be a result of continuous cytokine production and supplementation provided by HUVECs.

In order to better understand the interactions within the culture system, scaling up of top scaffolds was necessary. In addition to approaching statistical significance in colony production, scaffolds for larger scale culture should provide a functional benefit over extended periods of time. Another critical component of the selection process is the ability of the scaffold to support all progenitor types and avoid a lineage bias towards a myeloid or lymphoid fate. The top hit scaffolds selected, 1% collagen and a combination of 1% C and 0.5% E and HA, were recreated in a 6-well format to continue examining the specific effects of the system. The use of a 1% collagen type I scaffold alone allowed for the role of collagen in facilitating HSPC-HUVEC interactions to be further interpreted. The inclusion of 2 other components is also logical, as elastin has been shown to play a role in cell signaling and cell fate¹⁵⁴ while hydroxyapatite scaffolds have shown promise in reconstituting the functional niche after irradiation¹⁵⁵. Scale-up of the selected scaffold chemistries allowed for in-depth analysis into the impacts of the scaffold co-culture system moving forward.

4.6 Scale up investigations

Conditioned media has been applied in a variety of HSPC expansion techniques, to varying degrees of success. BMEC-conditioned media has been used to successfully expand murine yolk sac HSPCs, but was less successful in the expansion of adult bone marrow HSPCs¹⁵⁶. A recent study focused on the optimization of long-term murine HSPC expansion found that conditioned media conditions induce an overall decrease in the stem cell population, and suggests that factors involved in inducing cycling and differentiation may be soluble¹⁵⁷. Similar studies have found that despite the presence of HSC-supportive cytokines, HSPC populations do not demonstrate an overall expansion without direct cell-cell contact between ECs and HSPCs¹⁵⁸. The results of the CFU assay

are consistent with previous findings, as the most primitive CFU-GEMM colonies were not generated unless HSPCs were in direct contact with HUVECs. While the cytokine content of conditioned media is an attractive option for HSPC expansion, cell-cell contact has been established as an essential mediator of this process¹⁵⁹.

While polystyrene is a common culture plate constituent, it is not an optimal medium for HSPC expansion. For example, one study found that despite the total nucleated cell population seemingly expanding over 10 days of HSPC culture on polystyrene, less than six percent of those cells represented a multipotent fraction¹⁶⁰. The use of typical culture materials is insufficient in the expansion of HSPCs due to their lack of consideration of cell-cell and cell-matrix interactions, which are known to regulate expansion and differentiation *in vivo*¹⁶¹. This was demonstrated through the rapid exhaustion of the HSPC culture population in polystyrene conditions as well as the lack of functional output reflecting that of a multipotent cell population.

The mechanisms behind HSPC expansion and differentiation are elusive as their expansion and isolation remains challenging. Their behaviour and fate are highly dependent on a complex network of interactions with local cells and ECM of the native niche. While *in vivo* human experiments remain limited due to HSPC localization within hard bone, a viable strategy for elucidating the critical pathways controlling cell fate is the recreation of the human bone marrow vascular niche *in vitro*. The incorporation of natural ECM components into the culture system maintains fidelity to the native niche, as does the addition of native vascular niche cells.

4.7 HUVEC genetic expression

The use of RT-qPCR allows for insight into the molecular basis of previous findings. While the probes selected have been previously linked to HSPC function and viability, their expression has

not been explicitly linked to ECM composition or to a particular component. The hypoxanthine phosphoribosyl transferase 1 (HPRT1) gene encodes for a transferase enzyme primarily responsible for purine generation¹⁶². The use of HPRT1 as a housekeeping gene has been validated in several previous studies¹⁶³. It has specifically been demonstrated to maintain stability in HUVECs over a wide range of experimental conditions, making it suitable for use¹⁶⁴.

Secreted frizzled related protein 1 (SFRP1) is a Wnt antagonist associated with the mediation of cell senescence and tumor suppression¹⁶⁵. In ECs, SFRP1 has been shown to be a regulator of neovascularization by enhancing cell spreading on the ECM¹⁶⁶. A recent study found that SFRP1 in the vascular niche regulates HSPC repopulation through controlling cell cycling, assisting in the maintenance of the stem cell pool¹⁶⁷. This is achieved through β -catenin regulation, by which SFRP1 binds to Wnt family members to activate its transcriptional pathway¹⁶⁸. SFRP1 knockout in mice models has led to a premature exhaustion of LT-HSPCs, further underscoring its critical role in HSPC maintenance¹⁶⁹. In particular, the hormonal regulation of lymphopoiesis has been linked to the presence of SFRP1¹⁷⁰. It has been suggested that SFRP1 regulation is highly dependent on the environment in the local niche *in vivo*, meaning that enhanced SFRP1 expression in the scaffold co-culture system may then be a result of EC-ECM interactions¹⁷¹. While collagen-based scaffolds have been used to explore the role of SFRP1, the direct link between ECM composition and SFRP1 expression has not yet been explored¹⁷².

Vascular endothelial growth factor-1 (VEGF1) is considered to be a master regulator of vascular permeability and angiogenesis¹⁷³. It acts directly on ECs to stimulate the production of vasodilators such as nitric oxide, which in turn also antagonize vasoconstrictors to extend and enhance the cellular response¹⁷⁴. Specifically in the vascular niche of the bone marrow, VEGF1 has been demonstrated to stimulate Notch expression in sinusoidal ECs of the native bone marrow, which

aids in the prevention of stem cell exhaustion¹⁷⁵. These functions make VEGF1 an important component of vascular niche homeostasis, especially after myeloablation preceding bone marrow transplant to sufficiently revascularize the tissue and allow for the HSC pool to be maintained¹⁷⁶. The enhanced colony formation potential seen on scaffold co-cultured HSPCs may be related to VEGF1 expression. An alternative method for enhancing VEGF1 expression includes EC culture under hypoxic conditions, which also coincides with conditions that enhance HSPC population maintenance. While this has been done on collagen-based scaffolds, the role of the scaffold composition on cytokine production has not been explored¹⁷⁷.

Platelet endothelial cell adhesion molecule (PECAM1) is commonly expressed in both vascular and haematopoietic cells, and in ECs specifically is evenly distributed over the entire cellular surface¹⁷⁸. Its primary function is the mobilization of mature haematopoietic cell types such as neutrophils and monocytes, which it achieves by translocating integrins responsible for ECM adhesion to the cellular surface. It is expressed diffusely across the entire bone marrow vascular niche, and enhances HSPC adhesion to local vasculature¹⁷⁹. Studies have found that it is able to influence HSC progenitor population maintenance, and necessary for proper mobilization between the marrow and bloodstream¹⁸⁰. Its notable increase in expression on multi-combinatorial scaffolds in contrast with polystyrene may play a role in population maintenance in this system. Previous studies have found that ECs cultured on ECM scaffolds including heparin and elastin had similar expression of adhesion molecules including PECAM1 to controls¹⁸¹. The role of PECAM1 in endothelial biology has been studied in collagen-based suspensions, but the influence of PECAM1 on HSPCs has yet to be explored in the context of the ECM¹⁸².

CHAPTER 5

Concluding Remarks

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5.1 Conclusion

In summary, we have developed a co-culture system capable of maintaining live HSPCs and inclusive of HUVECs. By using the HUVEC-TERT2 cell line, the use of serum-free media is possible to prolong HSPC culture without inducing differentiation. We demonstrated that populations of HSPCs can be maintained throughout 7 days of co-culture with support from HUVECs and collagen-based scaffolds. The enhanced expression of several genes related to HSPC maintenance and viability in support HUVECs may explain the efficacy of the scaffold co-culture system.

This work has built the foundation of a co-culture model system capable of supporting *ex vivo* HSPCs for up to seven days in culture. However, in order to translate successfully into a cell therapy application, it is vital to strive to understand how endothelial behaviour impacts HSPCs. The ultimate goal of this work would be the removal of the immortalized HUVEC-TERT2 cell line in order to move towards a cell-free and reproducible system that can be adapted for use with patient samples. With translation in mind, the use of patient PBMNCs is critical to establish the effectiveness of the system in humans. The use of these individual human samples provides a broader representation of primary cell behaviour during experimentation. The model can also be adapted for malignant cells, such as acute myeloid leukaemia (AML) patient samples. The use of both healthy and malignant HSPCs can be employed in drug screens to scan for cytotoxicity, accelerating the process of drug discovery in primitive stem cell subpopulations that are typically challenging to maintain *ex vivo*. This can extend into personalized medicine applications, where a patient's own cells can be utilized to determine an optimal treatment protocol that limits side effects

and increases efficacy. Overall, the results suggest that the development of a niche-mimicking co-culture model can be utilized for extended primary human HSPC viability and function maintenance *ex vivo*.

5.3 Limitations & Future Directions

Sustained experimentation will continue to reveal the intricate molecular framework at play that leads to the improved colony formation potential and viability seen in scaffold co-culture models. Transcriptome analysis will have the potential to reveal the genetic basis of cell survival within the culture system, while the use of rt-QPCR using alternative probes can be employed to understand what soluble factors are present and how their abundance may correlate with scaffold composition.

Experimentation is ongoing to develop optimized scaffold chemistries in alternative culture models, such as co-culture models with mesenchymal stem cells. Collaborative work with these models has the potential to reveal a unified scaffold composition that can be utilized in a multi-cell culture model that better mimics the native HSPC niche. In addition, differences in optimal composition can be used to reveal the distinct molecular framework of cytokine binding and presentation for support cell types and their ECM. With composition in mind, it is vital to screen additional chemical components that can provide benefits to cytokine retention and HSPC viability. Components such as hyaluronic acid are currently being used to fabricate additional scaffolds in an effort to optimize the *in vitro* environment.

The development of two-dimensional films to reconstruct the niche is in itself limited, as niche models have shown greater success using three-dimensional culture suspensions¹⁸³. By adjusting the fabrication process, the films can be developed into three-dimensional porous scaffold structures that would allow for HSPCs to reside in a more structured environment similar to that of

the native niche. While three-dimensional scaffolds pose other considerations such as controlling pore size or topography, their use will bring the co-culture system closer to maintaining fidelity to the native niche.

While scaffold composition is vital, there are several other attributes of the system that can be altered to achieve maximal HSPC growth and proliferation. The use of hypoxic chambers can be applied to alter the oxygenation of the culture environment, with potential to improve HSPC viability. In addition, 3D printing can be applied to create perfusable channels within the scaffold to provide continuous nutrient and soluble factor supply for long-term extended cultures. Further optimization of the culture model will be focused on the amalgamation of these considerations in order to best recapitulate the bone marrow vascular niche and support native HSPCs *ex vivo*.

REFERENCES

1. Mirantes, C., Passegué, E. & Pietras, E. M. Pro-inflammatory cytokines: Emerging players regulating HSC function in normal and diseased hematopoiesis. *Exp Cell Res* **329**, 248–254 (2014).
2. Hoggatt, J., Kfoury, Y. & Scadden, D. T. Hematopoietic Stem Cell Niche in Health and Disease. <https://doi-org.ezproxy.library.yorku.ca/10.1146/annurev-pathol-012615-044414> **11**, 555–581 (2016).
3. Herbert, K. E., Lévesque, J. P., Haylock, D. N. & Prince, M. The use of experimental murine models to assess novel agents of hematopoietic stem and progenitor cell mobilization. *Biol Blood Marrow Transplant* **14**, 603–621 (2008).
4. Rocha, V. & Gluckman, E. Improving outcomes of cord blood transplantation: HLA matching, cell dose and other graft- and transplantation-related factors. *Br J Haematol* **147**, 262–274 (2009).
5. Marquez-Curtis, L. A., Turner, A. R., Sridharan, S., Ratajczak, M. Z. & Janowska-Wieczorek, A. The Ins and Outs of Hematopoietic Stem Cells: Studies to Improve Transplantation Outcomes. *Stem Cell Rev* **7**, 590 (2011).
6. TILL, J. E. & McCULLOCH, E. A. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* **14**, 213–222 (1961).
7. Christensen, J. L. & Weissman, I. L. Flk-2 is a marker in hematopoietic stem cell differentiation: A simple method to isolate long-term stem cells. *Proceedings of the National Academy of Sciences* **98**, 14541–14546 (2001).

8. Morrison, S. J., Wandycz, A. M., Hemmati, H. D., Wright, D. E. & Weissman, I. L. Identification of a lineage of multipotent hematopoietic progenitors. *Development* **124**, 1929–1939 (1997).
9. Laurenti, E. & Göttgens, B. From haematopoietic stem cells to complex differentiation landscapes. *Nature* 2018 553:7689 **553**, 418–426 (2018).
10. Buenrostro, J. D. *et al.* Integrated Single-Cell Analysis Maps the Continuous Regulatory Landscape of Human Hematopoietic Differentiation. *Cell* **173**, 1535–1548.e16 (2018).
11. Ema, H. Successful ex vivo expansion of mouse hematopoietic stem cells. *Blood Science* **1**, 116–118 (2019).
12. Briddell, R. A., Kern, B. P., Zilm, K. L., Stoney, G. B. & Mcniece, I. K. Purification of CD34⁺ cells is essential for optimal ex vivo expansion of umbilical cord blood cells. *J Hematother* **6**, 145–150 (1997).
13. Sotnezova, E. v., Andreeva, E. R., Grigoriev, A. I. & Buravkova, L. B. Ex Vivo Expansion of Hematopoietic Stem and Progenitor Cells from Umbilical Cord Blood. *Acta Naturae* **8**, 6 (2016).
14. Grskovic, B., Ruzicka, K., Karimi, A., Qujeq, D. & Müller, M. M. Cell cycle analysis of the CD133⁺ and CD133[–] cells isolated from umbilical cord blood. *Clinica Chimica Acta* **343**, 173–178 (2004).
15. Qian, H. *et al.* Critical Role of Thrombopoietin in Maintaining Adult Quiescent Hematopoietic Stem Cells. *Cell Stem Cell* **1**, 671–684 (2007).

16. Broxmeyer, H. E. *et al.* Flt3 ligand stimulates/costimulates the growth of myeloid stem/progenitor cells. *Exp Hematol* **23**, 1121–1129 (1995).
17. Fukuda, S., Broxmeyer, H. E. & Pelus, L. M. Flt3 ligand and the Flt3 receptor regulate hematopoietic cell migration by modulating the SDF-1 α (CXCL12)/CXCR4 axis. *Blood* **105**, 3117–3126 (2005).
18. Delaney, C., Varnum-Finney, B., Aoyama, K., Brashem-Stein, C. & Bernstein, I. D. Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. *Blood* **106**, 2693 (2005).
19. Peled, T. *et al.* Pre-clinical development of cord blood-derived progenitor cell graft expanded ex vivo with cytokines and the polyamine copper chelator tetraethylenepentamine. *Cytotherapy* **6**, 344–355 (2004).
20. Boitano, A. E. *et al.* Aryl Hydrocarbon Receptor Antagonists Promote the Expansion of Human Hematopoietic Stem Cells. *Science* **329**, 1345 (2010).
21. Peled, T. *et al.* Nicotinamide, a SIRT1 inhibitor, inhibits differentiation and facilitates expansion of hematopoietic progenitor cells with enhanced bone marrow homing and engraftment. *Exp Hematol* **40**, (2012).
22. Choi, J. S. & Harley, B. A. C. The combined influence of substrate elasticity and ligand density on the viability and biophysical properties of hematopoietic stem and progenitor cells. *Biomaterials* **33**, 4460–4468 (2012).
23. Bresnick, E. H. *et al.* Mechanisms of erythrocyte development and regeneration: implications for regenerative medicine and beyond. *Development* **145**, (2018).

24. Chivu-Economescu, M. & Rubach, M. Hematopoietic Stem Cells Therapies. *Curr Stem Cell Res Ther* **12**, 124–133 (2017).
25. Kumar, S. & Geiger, H. HSC Niche Biology and HSC Expansion Ex Vivo. *Trends Mol Med* **23**, 799–819 (2017).
26. Himburg, H. A. *et al.* Distinct Bone Marrow Sources of Pleiotrophin Control Hematopoietic Stem Cell Maintenance and Regeneration. *Cell Stem Cell* **23**, 370–381.e5 (2018).
27. Rafii, S., Mohle, R., Shapiro, F., Frey, B. M. & Moore, M. A. S. Regulation of hematopoiesis by microvascular endothelium. *Leuk Lymphoma* **27**, 375–386 (1997).
28. Davis, T. A., Robinson, D. H., Lee, K. P. & Kessler, S. W. Porcine Brain Microvascular Endothelial Cells Support the In Vitro Expansion of Human Primitive Hematopoietic Bone Marrow Progenitor Cells With a High Replating Potential: Requirement for Cell-to-Cell Interactions and Colony-Stimulating Factors. *Blood* **85**, 1751–1761 (1995).
29. de Bruijn, M. F. T. R., Speck, N. A., Peeters, M. C. E. & Dzierzak, E. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J* **19**, 2465–2474 (2000).
30. Yun, H. J. & Jo, D. Y. Production of stromal cell-derived factor-1 (SDF-1) and expression of CXCR4 in human bone marrow endothelial cells. *J Korean Med Sci* **18**, 679–685 (2003).
31. Fliedner, T. M., Graessle, D., Paulsen, C. & Reimers, K. Structure and Function of Bone Marrow Hemopoiesis: Mechanisms of Response to Ionizing Radiation Exposure. <https://home-liebertpub-com.ezproxy.library.yorku.ca/cbr> **17**, 405–426 (2004).

32. Mazo, I. B. *et al.* Hematopoietic progenitor cell rolling in bone marrow microvessels: parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. *J Exp Med* **188**, 465–474 (1998).
33. van Meeteren, L. A. & ten Dijke, P. Regulation of endothelial cell plasticity by TGF- β . *Cell Tissue Res* **347**, 177 (2012).
34. Pan, L., Liu, Z., Chen, Y., Yang, B. & Cheng, B. Angiopoietin-1: Can be produced by endothelial cells and act in an autocrine agonistic manner? *Clin Hemorheol Microcirc* **74**, 341–345 (2020).
35. Kobayashi, H. *et al.* Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol* **12**, 1046 (2010).
36. Gori, J. L. *et al.* Endothelial Cells Promote Expansion of Long-Term Engrafting Marrow Hematopoietic Stem and Progenitor Cells in Primates. *Stem Cells Transl Med* **6**, 864 (2017).
37. Srivastava, S. K. *et al.* Comparative engraftment and clonality of macaque HSPCs expanded on human umbilical vein endothelial cells versus non-expanded cells. *Mol Ther Methods Clin Dev* **20**, 703–715 (2021).
38. Frantz, C., Stewart, K. M. & Weaver, V. M. The extracellular matrix at a glance. *J Cell Sci* **123**, 4195–4200 (2010).
39. Bosman, F. T. & Stamenkovic, I. Functional structure and composition of the extracellular matrix. *J Pathol* **200**, 423–428 (2003).
40. Rozario, T. & DeSimone, D. W. The extracellular matrix in development and morphogenesis: A dynamic view. *Dev Biol* **341**, 126–140 (2010).

41. Gattazzo, F., Urciuolo, A. & Bonaldo, P. Extracellular matrix: A dynamic microenvironment for stem cell niche. *Biochim Biophys Acta* **1840**, 2506 (2014).
42. Song, Y. Function of membrane-associated proteoglycans in the regulation of satellite cell growth. *Adv Exp Med Biol* **900**, 61–95 (2016).
43. Gordon, M. K. & Hahn, R. A. Collagens. *Cell Tissue Res* **339**, 247–257 (2010).
44. Mak, K. M. & Mei, R. Basement Membrane Type IV Collagen and Laminin: An Overview of Their Biology and Value as Fibrosis Biomarkers of Liver Disease. *Anat Rec* **300**, 1371–1390 (2017).
45. Franzke, C. W., Tasanen, K., Schumann, H. & Bruckner-Tuderman, L. Collagenous transmembrane proteins: Collagen XVII as a prototype. *Matrix Biology* **22**, 299–309 (2003).
46. Francomano, C. A. Key role for a minor collagen. *Nat Genet* **9**, 6–8 (1995).
47. Wise, S. G. & Weiss, A. S. Tropoelastin. *Int J Biochem Cell Biol* **41**, 494–497 (2009).
48. Callaghan, T. M. & Wilhelm, K. P. A review of ageing and an examination of clinical methods in the assessment of ageing skin. Part 2: Clinical perspectives and clinical methods in the evaluation of ageing skin. *Int J Cosmet Sci* **30**, 323–332 (2008).
49. Chow, M. J., Turcotte, R., Lin, C. P. & Zhang, Y. Arterial Extracellular Matrix: A Mechanobiological Study of the Contributions and Interactions of Elastin and Collagen. *Biophys J* **106**, 2684–2692 (2014).

50. Halper, J. & Kjaer, M. Basic components of connective tissues and extracellular matrix: elastin, fibrillin, fibulins, fibrinogen, fibronectin, laminin, tenascins and thrombospondins. *Adv Exp Med Biol* **802**, 31–47 (2014).
51. Moore, J. & Thibeault, S. Insights into the role of elastin in vocal fold health and disease. *Journal of Voice* **26**, 269 (2012).
52. Schaefer, L. & Schaefer, R. M. Proteoglycans: From structural compounds to signaling molecules. *Cell Tissue Res* **339**, 237–246 (2010).
53. Jiang, D., Liang, J. & Noble, P. W. Hyaluronan in Tissue Injury and Repair. <https://doi-org.ezproxy.library.yorku.ca/10.1146/annurev.cellbio.23.090506.123337> **23**, 435–461 (2007).
54. Cavalcante, F. S. A. *et al.* Mechanical interactions between collagen and proteoglycans: Implications for the stability of lung tissue. *J Appl Physiol* **98**, 672–679 (2005).
55. Muramatsu, T., Muramatsu, H. & Kojima, T. Identification of Proteoglycan-Binding Proteins. *Methods Enzymol* **416**, 263–278 (2006).
56. Lin, X., Patil, S., Gao, Y. G. & Qian, A. The Bone Extracellular Matrix in Bone Formation and Regeneration. *Front Pharmacol* **11**, 757 (2020).
57. Tavafoghi, M. & Cerruti, M. The role of amino acids in hydroxyapatite mineralization. *J R Soc Interface* **13**, (2016).
58. Ohgushi, H., Dohi, Y., Tamai, S. & Tabata, S. Osteogenic differentiation of marrow stromal stem cells in porous hydroxyapatite ceramics. *J Biomed Mater Res* **27**, 1401–1407 (1993).

59. Fujita, A. *et al.* Hematopoiesis in regenerated bone marrow within hydroxyapatite scaffold. *Pediatr Res* **68**, 35–40 (2010).
60. Zhang, P. *et al.* The physical microenvironment of hematopoietic stem cells and its emerging roles in engineering applications. *Stem Cell Research & Therapy* 2019 10:1 **10**, 1–13 (2019).
61. Zhang, Q., Gerlach, J. C., Schmelzer, E. & Nettleship, I. Effect of Calcium-Infiltrated Hydroxyapatite Scaffolds on the Hematopoietic Fate of Human Umbilical Vein Endothelial Cells. *J Vasc Res* **54**, 376–385 (2017).
62. Choi, J. S. & Harley, B. A. C. Marrow-inspired matrix cues rapidly affect early fate decisions of hematopoietic stem and progenitor cells. *Sci Adv* **3**, (2017).
63. Choi, J. S. & Harley, B. A. C. The combined influence of substrate elasticity and ligand density on the viability and biophysical properties of hematopoietic stem and progenitor cells. *Biomaterials* **33**, 4460–4468 (2012).
64. Li, H. *et al.* Biomechanical cues as master regulators of hematopoietic stem cell fate. *Cellular and Molecular Life Sciences* 2021 78:16 **78**, 5881–5902 (2021).
65. Kang, Y. G., Jeong, J. Y., Lee, T. H., Lee, H. S. & Shin, J. W. Synergistic Integration of Mesenchymal Stem Cells and Hydrostatic Pressure in the Expansion and Maintenance of Human Hematopoietic/Progenitor Cells. *Stem Cells Int* **2018**, (2018).
66. Notta, F. *et al.* Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* **333**, 218–221 (2011).
67. Papayannopoulou, T. & Nakamoto, B. Peripheralization of hemopoietic progenitors in primates treated with anti-VLA4 integrin. *Proc Natl Acad Sci U S A* **90**, 9374–9378 (1993).

68. Winkler, I. *et al.* Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nat Med* **18**, 1651–1657 (2012).
69. Witjas, F. M. R., Berg, B. M. van den, Berg, C. W. van den, Engelse, M. A. & Rabelink, T. J. Concise Review: The Endothelial Cell Extracellular Matrix Regulates Tissue Homeostasis and Repair. *Stem Cells Transl Med* **8**, 375–382 (2019).
70. Travlos, G. S. Normal Structure, Function, and Histology of the Bone Marrow. <http://dx.doi.org/10.1080/01926230600939856> **34**, 548–565 (2016).
71. Mangialardi, G., Cordaro, A. & Madeddu, P. The bone marrow pericyte: an orchestrator of vascular niche. *Regenerative Med* **11**, 883–895 (2016).
72. Ding, L., Saunders, T., Enikolopov, G. & Morrison, S. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* **481**, 457–462 (2012).
73. Kunisaki, Y. *et al.* Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* **502**, 637–643 (2013).
74. Xu, C. *et al.* Stem cell factor is selectively secreted by arterial endothelial cells in bone marrow. *Nat Commun* **9**, (2018).
75. Luo, W. *et al.* Laminar shear stress delivers cell cycle arrest and anti-apoptosis to mesenchymal stem cells. *Acta Biochim Biophys Sin (Shanghai)* **43**, 210–216 (2011).
76. Kusumbe, A. *et al.* Age-dependent modulation of vascular niches for haematopoietic stem cells. *Nature* **532**, 380–384 (2016).

77. Butler, J. M. *et al.* Endothelial Cells Are Essential for the Self-Renewal and Repopulation of Notch-Dependent Hematopoietic Stem Cells. *Cell Stem Cell* **6**, 251–264 (2010).
78. Ottersbach, K. Endothelial-to-haematopoietic transition: an update on the process of making blood. *Biochem Soc Trans* **47**, 591–601 (2019).
79. Taqvi, S. & Roy, K. Influence of scaffold physical properties and stromal cell coculture on hematopoietic differentiation of mouse embryonic stem cells. *Biomaterials* **27**, 6024–6031 (2006).
80. Ventura Ferreira, M. S. *et al.* Cord blood-hematopoietic stem cell expansion in 3D fibrin scaffolds with stromal support. *Biomaterials* **33**, 6987–6997 (2012).
81. O'Brien, F. J. Biomaterials & scaffolds for tissue engineering. *Materials Today* **14**, 88–95 (2011).
82. Hussey, G. S., Dziki, J. L. & Badylak, S. F. Extracellular matrix-based materials for regenerative medicine. *Nature Reviews Materials* 2018 3:7 **3**, 159–173 (2018).
83. Carvalho, M. S., Cabral, J. M. S., da Silva, C. L. & Vashishth, D. Bone Matrix Non-Collagenous Proteins in Tissue Engineering: Creating New Bone by Mimicking the Extracellular Matrix. *Polymers* 2021, Vol. 13, Page 1095 **13**, 1095 (2021).
84. Porzionato, A. *et al.* Tissue-Engineered Grafts from Human Decellularized Extracellular Matrices: A Systematic Review and Future Perspectives. *International Journal of Molecular Sciences* 2018, Vol. 19, Page 4117 **19**, 4117 (2018).

85. Rico-Llanos, G. A., Borrego-González, S., Moncayo-Donoso, M., Becerra, J. & Visser, R. Collagen Type I Biomaterials as Scaffolds for Bone Tissue Engineering. *Polymers (Basel)* **13**, 1–20 (2021).
86. Koide, T. Designed triple-helical peptides as tools for collagen biochemistry and matrix engineering. *Philosophical Transactions of the Royal Society B: Biological Sciences* **362**, 1281–1291 (2007).
87. Łopianiak, I. & Butruk-Raszeja, B. A. Evaluation of Sterilization/Disinfection Methods of Fibrous Polyurethane Scaffolds Designed for Tissue Engineering Applications. *International Journal of Molecular Sciences* 2020, Vol. 21, Page 8092 **21**, 8092 (2020).
88. Sun, Y., Lovric, V., Wang, T., Oliver, R. A. & Walsh, W. R. Effects of SCCO₂, Gamma Irradiation, and Sodium Dodecyl Sulfate Treatments on the Initial Properties of Tendon Allografts. *International Journal of Molecular Sciences* 2020, Vol. 21, Page 1565 **21**, 1565 (2020).
89. Dong, C. & Lv, Y. Application of Collagen Scaffold in Tissue Engineering: Recent Advances and New Perspectives. *Polymers (Basel)* **8**, (2016).
90. Kao, G. S. & Sloan, S. R. Peripheral Blood Stem Cells. *Handbook of Pediatric Transfusion Medicine* 283–293 (2004) doi:10.1016/B978-012348776-6/50027-3.
91. Heng, B. C. *et al.* Effect of cell-seeding density on the proliferation and gene expression profile of human umbilical vein endothelial cells within ex vivo culture. *Cytotherapy* **13**, 606–617 (2011).

92. Stamov, D. R. & Pompe, T. Structure and function of ECM-inspired composite collagen type I scaffolds. *Soft Matter* **8**, 10200–10212 (2012).
93. Kim, H. S. *et al.* Ex Vivo Expansion of Human Umbilical Cord Blood CD34+ Cells in a Collagen Bead—Containing 3-Dimensional Culture System. *International Journal of Hematology* 2003 78:2 **78**, 126–132 (2003).
94. Malcor, J. D. *et al.* Collagen scaffolds functionalized with triple-helical peptides support 3D HUVEC culture. *Regen Biomater* **7**, 471–482 (2020).
95. Daamen, W. F., Veerkamp, J. H., van Hest, J. C. M. & van Kuppevelt, T. H. Elastin as a biomaterial for tissue engineering. *Biomaterials* **28**, 4378–4398 (2007).
96. Jimenez Vazquez, J. & San Martín Martínez, E. Collagen and elastin scaffold by electrospinning for skin tissue engineering applications. *J Mater Res* **34**, 2819–2827 (2019).
97. Krause, D. S. Regulation of hematopoietic stem cell fate. doi:10.1038/sj/onc/1205316.
98. Fujita, A. *et al.* Hematopoiesis in Regenerated Bone Marrow Within Hydroxyapatite Scaffold. *Pediatric Research* 2010 68:1 **68**, 35–40 (2010).
99. Lewandowska, K., Szulc, M. & Sionkowska, A. Effect of Solvent on the Hydrodynamic Properties of Collagen. *Polymers (Basel)* **13**, (2021).
100. Michl, J., Park, K. C. & Swietach, P. Evidence-based guidelines for controlling pH in mammalian live-cell culture systems. *Communications Biology* 2019 2:1 **2**, 1–12 (2019).

101. Helseth, D. L. & Veis, A. Collagen self-assembly in vitro. Differentiating specific telopeptide-dependent interactions using selective enzyme modification and the addition of free amino telopeptide. *Journal of Biological Chemistry* **256**, 7118–7128 (1981).
102. Revell, C. K. *et al.* Collagen fibril assembly: New approaches to unanswered questions. *Matrix Biol Plus* **12**, 100079 (2021).
103. Depalle, B., Qin, Z., Shefelbine, S. J. & Buehler, M. J. Influence of cross-link structure, density and mechanical properties in the mesoscale deformation mechanisms of collagen fibrils. *J Mech Behav Biomed Mater* **52**, 1–13 (2015).
104. Haugh, M. G., Murphy, C. M., McKiernan, R. C., Altenbuchner, C. & O'Brien, F. J. Crosslinking and Mechanical Properties Significantly Influence Cell Attachment, Proliferation, and Migration Within Collagen Glycosaminoglycan Scaffolds. <https://home-liebertpub-com.ezproxy.library.yorku.ca/tea> **17**, 1201–1208 (2011).
105. Heck, T., Faccio, G., Richter, M. & Thöny-Meyer, L. Enzyme-catalyzed protein crosslinking. *Appl Microbiol Biotechnol* **97**, 461 (2013).
106. Lucero, H. A. & Kagan, H. M. Lysyl oxidase: an oxidative enzyme and effector of cell function. *Cellular and Molecular Life Sciences CMLS* **63**, 2304–2316 (2006).
107. Heck, T., Faccio, G., Richter, M. & Thöny-Meyer, L. Enzyme-catalyzed protein crosslinking. *Appl Microbiol Biotechnol* **97**, 461 (2013).
108. Arbelaez, M. C., Sekito, M. B., Vidal, C. & Choudhury, S. R. Collagen cross-linking with riboflavin and ultraviolet-A light in keratoconus: One-year results. *Oman J Ophthalmol* **2**, 33 (2009).

109. Parhi, R. Cross-Linked Hydrogel for Pharmaceutical Applications: A Review. *Adv Pharm Bull* **7**, 515 (2017).
110. Zeugolis, D. I., Paul, G. R. & Attenburrow, G. Cross-linking of extruded collagen fibers—A biomimetic three-dimensional scaffold for tissue engineering applications. *J Biomed Mater Res A* **89A**, 895–908 (2009).
111. Adamiak, K. & Sionkowska, A. Current methods of collagen cross-linking: Review. *Int J Biol Macromol* **161**, 550–560 (2020).
112. Lin, Y. L., Chen, C. P., Lo, C. M. & Wang, H. S. Stiffness-controlled three-dimensional collagen scaffolds for differentiation of human Wharton’s jelly mesenchymal stem cells into cardiac progenitor cells. *J Biomed Mater Res A* **104**, 2234–2242 (2016).
113. Everaerts, F., Torrianni, M., Hendriks, M. & Feijen, J. Biomechanical properties of carbodiimide crosslinked collagen: Influence of the formation of ester crosslinks. *J Biomed Mater Res A* **85A**, 547–555 (2008).
114. Pieper, J. S., Hafmans, T., Veerkamp, J. H. & van Kuppevelt, T. H. Development of tailor-made collagen–glycosaminoglycan matrices: EDC/NHS crosslinking, and ultrastructural aspects. *Biomaterials* **21**, 581–593 (2000).
115. Sachlos, E. & Czernuszka, J. T. MAKING TISSUE ENGINEERING SCAFFOLDS WORK. REVIEW ON THE APPLICATION OF SOLID FREEFORM FABRICATION TECHNOLOGY TO THE PRODUCTION OF TISSUE ENGINEERING SCAFFOLDS. *Eur Cell Mater* **5**, 29–40 (2003).

116. L, S. *et al.* Prolonged transendothelial migration of human haematopoietic stem and progenitor cells (HSPCs) towards hydrogel-released SDF1. *Ann Hematol* **90**, 865–871 (2011).
117. Bachetti, T. & Morbidelli, L. ENDOTHELIAL CELLS IN CULTURE: A MODEL FOR STUDYING VASCULAR FUNCTIONS. *Pharmacol Res* **42**, 9–19 (2000).
118. Medina-Leyte, D. J., Domínguez-Pérez, M., Mercado, I., Villarreal-Molina, M. T. & Jacobo-Albavera, L. Use of Human Umbilical Vein Endothelial Cells (HUVEC) as a Model to Study Cardiovascular Disease: A Review. *Applied Sciences* 2020, Vol. 10, Page 938 **10**, 938 (2020).
119. Seandel, M. *et al.* Generation of a functional and durable vascular niche by the adenoviral E4ORF1 gene. *Proc Natl Acad Sci U S A* **105**, 19288–19293 (2008).
120. Bian, C., Zhao, K., Tong, G. X., Zhu, Y. L. & Chen, P. Immortalization of human umbilical vein endothelial cells with telomerase reverse transcriptase and simian virus 40 large T antigen. *J Zhejiang Univ Sci B* **6**, 631 (2005).
121. Harrison, D. E. & Zhong, R. K. The same exhaustible multilineage precursor produces both myeloid and lymphoid cells as early as 3-4 weeks after marrow transplantation. *Proc Natl Acad Sci U S A* **89**, 10134–10138 (1992).
122. Lai, S. *et al.* Comparative transcriptomic analysis of hematopoietic system between human and mouse by Microwell-seq. *Cell Discovery* 2018 4:1 **4**, 1–4 (2018).
123. Beksac, M. Is There Any Reason to Prefer Cord Blood Instead of Adult Donors for Hematopoietic Stem Cell Transplants? *Front Med (Lausanne)* **2**, 11 (2015).

124. Chen, D. Y. *et al.* Three-dimensional cell aggregates composed of HUVECs and cbMSCs for therapeutic neovascularization in a mouse model of hindlimb ischemia. *Biomaterials* **34**, 1995–2004 (2013).
125. Frisch, B. J. & Calvi, L. M. Hematopoietic Stem Cell Cultures and Assays. *Methods Mol Biol* **1130**, 315 (2014).
126. Baudin, B., Bruneel, A., Bosselut, N. & Vaubourdolle, M. A protocol for isolation and culture of human umbilical vein endothelial cells. *Nat Protoc* **2**, 481–485 (2007).
127. Glaser, D. E. *et al.* Organ-on-a-chip model of vascularized human bone marrow niches. *Biomaterials* **280**, 121245 (2022).
128. Johnson, G., Dastagir, N., Beal, Z., Hart, A. & Godwin, J. Cell Dissociation Techniques in Salamanders. *Methods Mol Biol* **2562**, 389–403 (2023).
129. Volovitz, I. *et al.* A non-aggressive, highly efficient, enzymatic method for dissociation of human brain-tumors and brain-tissues to viable single-cells. *BMC Neurosci* **17**, 1–10 (2016).
130. Tachibana, K. N-cadherin-mediated aggregate formation; cell detachment by Trypsin-EDTA loses N-cadherin and delays aggregate formation. *Biochem Biophys Res Commun* **516**, 414–418 (2019).
131. Mutin, M., George, F., Lesaule, G. & Sampol, J. Reevaluation of Trypsin-EDTA for Endothelial Cell Detachment before Flow Cytometry Analysis. <http://dx.doi.org.ezproxy.library.yorku.ca/10.3109/10623329609024704> **4**, 289–295 (2009).

132. Bajpai, R., Lesperance, J., Kim, M. & Terskikh, A. v. Efficient propagation of single cells accutase-dissociated human embryonic stem cells. *Mol Reprod Dev* **75**, 818–827 (2008).
133. Bajpai, R., Lesperance, J., Kim, M. & Terskikh, A. v. Efficient propagation of single cells accutase-dissociated human embryonic stem cells. *Mol Reprod Dev* **75**, 818–827 (2008).
134. Fu, J., Wang, W., Liu, Y.-H., Lu, H. & Luo, Y. In vitro anti-angiogenic properties of LGD1069, a selective retinoid X-receptor agonist through down-regulating Runx2 expression on Human endothelial cells. *BMC Cancer* **11**, 227 (2011).
135. Suzuki, E. *et al.* Reentry into the Cell Cycle of Contact-inhibited Vascular Endothelial Cells by a Phosphatase Inhibitor. *Journal of Biological Chemistry* **275**, 3637–3644 (2000).
136. Piera-Velazquez, S. & Jimenez, S. A. Endothelial to Mesenchymal Transition: Role in Physiology and in the Pathogenesis of Human Diseases. <https://doi.org/10.1152/physrev.00021.2018> **99**, 1281–1324 (2019).
137. Kadish, J. L., Butterfield, C. E. & Folkman, J. The effect of fibrin on cultured vascular endothelial cells. *Tissue Cell* **11**, 99–108 (1979).
138. Stricker, J., Falzone, T. & Gardel, M. L. Mechanics of the F-actin Cytoskeleton. *J Biomech* **43**, 9 (2010).
139. Young, E. W. K., Berthier, E. & Beebe, D. J. Assessment of enhanced autofluorescence and impact on cell microscopy for microfabricated thermoplastic devices. *Anal Chem* **85**, 44 (2013).
140. Karg, T. J. & Golic, K. G. Photoconversion of DAPI and Hoechst dyes to green and red emitting forms after exposure to UV excitation. *Chromosoma* **127**, 235 (2018).

141. Huang, A. S., Gonzalez, J. M., Le, P. v., Heur, M. & Tan, J. C. H. Sources of Structural Autofluorescence in the Human Trabecular Meshwork. *Invest Ophthalmol Vis Sci* **54**, 4813–4820 (2013).
142. Chai, D. *et al.* Quantitative Assessment of UVA-Riboflavin Corneal Cross-Linking Using Nonlinear Optical Microscopy. *Invest Ophthalmol Vis Sci* **52**, 4231–4238 (2011).
143. Deyl, Z., Macek, K., Adam, M. & Vancikova. Studies on the chemical nature of elastin fluorescence. *Biochimica et Biophysica Acta (BBA) - Protein Structure* **625**, 248–254 (1980).
144. Douglas S. Auld, Ph. D. *et al.* Microplate Selection and Recommended Practices in High-throughput Screening and Quantitative Biology. *Assay Guidance Manual* (2020).
145. Paganelli, A. *et al.* Use of confocal microscopy imaging for in vitro assessment of adipose-derived mesenchymal stromal cells seeding on acellular dermal matrices: 3D reconstruction based on collagen autofluorescence. *Skin Research and Technology* **28**, 133–141 (2022).
146. Anjos-Afonso, F. *et al.* Single cell analyses identify a highly regenerative and homogenous human CD34⁺ hematopoietic stem cell population. *Nature Communications* 2022 13:1 **13**, 1–13 (2022).
147. Velten, L. *et al.* Human haematopoietic stem cell lineage commitment is a continuous process. *Nat Cell Biol* **19**, 271 (2017).
148. Pamphilon, D. *et al.* Current practices and prospects for standardization of the hematopoietic colony-forming unit assay: a report by the cellular therapy team of the Biomedical Excellence for Safer Transfusion (BEST) Collaborative. *Cytotherapy* **15**, 255–262 (2013).

149. Ema, H., Morita, Y. & Suda, T. Heterogeneity and hierarchy of hematopoietic stem cells. *Exp Hematol* **42**, 74-82.e2 (2014).
150. Wilson, A., Laurenti, E. & Trumpp, A. Balancing dormant and self-renewing hematopoietic stem cells. *Curr Opin Genet Dev* **19**, 461–468 (2009).
151. Chiba, K., Kawakami, K. & Tohyama, K. Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells. *Toxicology in Vitro* **12**, 251–258 (1998).
152. Broudy, V. C. Stem Cell Factor and Hematopoiesis. *Blood* **90**, 1345–1364 (1997).
153. Ozsvar, J., Mithieux, S. M., Wang, R. & Weiss, A. S. Elastin-based biomaterials and mesenchymal stem cells. *Biomater Sci* **3**, 800 (2015).
154. Fujita, A. *et al.* Hematopoiesis in regenerated bone marrow within hydroxyapatite scaffold. *Pediatr Res* **68**, 35–40 (2010).
155. Na, X. D., Wang, Q. R. & Zhao, Z. P. Analysis of the different effects of murine bone marrow endothelial cell conditioned medium on the growth of embryonic and adult hematopoietic stem/progenitor cells in vitro. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* **12**, 255–260 (2004).
156. Wilkinson, A. C. *et al.* Long-term ex vivo hematopoietic stem cell expansion affords nonconditioned transplantation. *Nature* **571**, 117 (2019).
157. Kobayashi, H. *et al.* Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol* **12**, 1046 (2010).

158. Chen, Q. The niche for hematopoietic stem cell expansion: a collaboration network. *Cellular & Molecular Immunology* 2017 14:10 **14**, 865–867 (2017).
159. Chua, K. N. *et al.* Functional nanofiber scaffolds with different spacers modulate adhesion and expansion of cryopreserved umbilical cord blood hematopoietic stem/progenitor cells. *Exp Hematol* **35**, 771–781 (2007).
160. Bello, A. B., Park, H. & Lee, S. H. Current approaches in biomaterial-based hematopoietic stem cell niches. *Acta Biomater* **72**, 1–15 (2018).
161. Sedano, M. J. *et al.* Hypoxanthine Phosphoribosyl Transferase 1 Is Upregulated, Predicts Clinical Outcome and Controls Gene Expression in Breast Cancer. *Cancers* 2020, Vol. 12, Page 1522 **12**, 1522 (2020).
162. Wei, R., Stewart, E. A. & Amoaku, W. M. Suitability of endogenous reference genes for gene expression studies with human intraocular endothelial cells. *BMC Res Notes* **6**, 46 (2013).
163. Zyzyńska-Granica, B. & Koziak, K. Identification of Suitable Reference Genes for Real-Time PCR Analysis of Statin-Treated Human Umbilical Vein Endothelial Cells. *PLoS One* **7**, e51547 (2012).
164. Elzi, D. J., Song, M., Hakala, K., Weintraub, S. T. & Shiio, Y. Wnt Antagonist SFRP1 Functions as a Secreted Mediator of Senescence. *Mol Cell Biol* **32**, 4388–4399 (2012).
165. Dufourcq, P. *et al.* Regulation of Endothelial Cell Cytoskeletal Reorganization by a Secreted Frizzled-Related Protein-1 and Frizzled 4- and Frizzled 7-Dependent Pathway: Role in Neovessel Formation. *Am J Pathol* **172**, 37–49 (2008).

166. Hettler, F. *et al.* MICROENVIRONMENTAL SFRP1 REGULATES REPOPULATING ACTIVITY OF HEMATOPOIETIC STEM CELLS VIA PP2A-MEDIATED REGULATION OF CTNNB1/EP300. *Exp Hematol* **88**, S31 (2020).
167. Renström, J. *et al.* Secreted Frizzled-Related Protein 1 Extrinsically Regulates Cycling Activity and Maintenance of Hematopoietic Stem Cells. *Cell Stem Cell* **5**, 157–167 (2009).
168. Celso, C. lo & Scadden, D. T. The haematopoietic stem cell niche at a glance. *J Cell Sci* **124**, 3529–3535 (2011).
169. Yokota, T. *et al.* Soluble Frizzled-Related Protein 1 Is Estrogen Inducible in Bone Marrow Stromal Cells and Suppresses the Earliest Events in Lymphopoiesis. *The Journal of Immunology* **181**, 6061–6072 (2008).
170. Bric, A. *et al.* Functional Identification of Tumor-Suppressor Genes through an In Vivo RNA Interference Screen in a Mouse Lymphoma Model. *Cancer Cell* **16**, 324–335 (2009).
171. Martin-Manso, G. *et al.* sFRP-1 binds via its netrin-related motif to the N-module of thrombospondin-1 and blocks thrombospondin-1 stimulation of MDA-MB-231 breast carcinoma cell adhesion and migration. *Arch Biochem Biophys* **509**, 147–156 (2011).
172. Eming, S. A. & Krieg, T. Molecular Mechanisms of VEGF-A Action during Tissue Repair. *Journal of Investigative Dermatology Symposium Proceedings* **11**, 79–86 (2006).
173. Pandey, A. K. *et al.* Mechanisms of VEGF (Vascular Endothelial Growth Factor) Inhibitor–Associated Hypertension and Vascular Disease. *Hypertension* **71**, E1–E8 (2018).

174. Kobayashi, H. *et al.* Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nature Cell Biology* 2010 12:11 **12**, 1046–1056 (2010).
175. Stucker, S., Chen, J., Watt, F. E. & Kusumbe, A. P. Bone Angiogenesis and Vascular Niche Remodeling in Stress, Aging, and Diseases. *Front Cell Dev Biol* **8**, 1433 (2020).
176. Szot, C. S., Buchanan, C. F., Freeman, J. W. & Rylander, M. N. 3D in vitro bioengineered tumors based on collagen I hydrogels. *Biomaterials* **32**, 7905–7912 (2011).
177. Feng, D., Nagy, J. A., Pyne, K., Dvorak, H. F. & Dvorak, A. M. Ultrastructural Localization of Platelet Endothelial Cell Adhesion Molecule (PECAM-1, CD31) in Vascular Endothelium. *Journal of Histochemistry and Cytochemistry* **52**, 87–101 (2004).
178. Sipkins, D. A. *et al.* Specialized Bone Marrow Endothelium Defines Microdomains for Tumor and Stem Cell Engraftment. *Blood* **104**, 663 (2004).
179. Ross, E. A. *et al.* A Novel Role for PECAM-1 (CD31) in Regulating Haematopoietic Progenitor Cell Compartmentalization between the Peripheral Blood and Bone Marrow. *PLoS One* **3**, e2338 (2008).
180. Kurane, A. & Vyavahare, N. Endothelial cells seeded on elastin–heparin matrices express normal EC markers and resist detachment on exposure to shear stress: a histological study. <http://dx.doi.org/10.1179/014788811X12949268296004> **34**, 11–19 (2013).
181. Yang, S., Graham, J., Kahn, J. W., Schwartz, E. A. & Gerritsen, M. E. Functional Roles for PECAM-1 (CD31) and VE-Cadherin (CD144) in Tube Assembly and Lumen Formation in Three-Dimensional Collagen Gels. *Am J Pathol* **155**, 887–895 (1999).

182. Bessy, T., Itkin, T. & Passaro, D. Bioengineering the Bone Marrow Vascular Niche. *Front Cell Dev Biol* **9**, 897 (2021).
183. Vanuytsel, K., Yeung, A. K., Dowrey, T. W., Murphy, G. J. & Belkina, A. C. CPHEN-013: Comprehensive phenotyping of hematopoietic stem and progenitor cells in the human fetal liver. *Cytometry Part A* **101**, 903–908 (2022).