Utilising mass-spectrometry based proteomics to improve current in vitro haematopoietic stem cell expansion methods

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Abstract

Haematopoietic stem cells (HSCs), the multipotent cells found at the top of the haematopoietic hierarchy, are capable of reconstituting the entire blood system. Their potential for therapeutic gene-editing and transplantation in clinical settings are hindered by their scarcity and lack of robust methods for their *ex vivo* expansion. The discovery of polyvinyl alcohol (PVA)-based serum-free conditions to expand murine HSCs *in vitro* by up to 899-fold, opened new avenues of HSC research; however, a majority of the cells within the culture were still differentiated progeny. To improve this strategy, this project used mass-spectrometry based secretome analysis, and functional transplantation studies, to identify proteins that were enriched in *in vitro* expanded HSC clones with high functional stem cell content. This revealed several targets, such as *Tut4* and *Sema7a*, that were strongly associated with functional HSC activity and future research could interrogate such molecules in the context of PVA-based media to remove the variability between batches and decrease the loss of homogeneity that is seen. For example, of the potential targets investigated in this thesis, angiotensin-II enhanced HSC expansion most successfully. This is in marked contrast to other molecular modulation approaches (e.g. transforming growth factor beta (TGF-β) inhibition by SB-431542) which did not have a significant impact on HSC expansion. This project utilised the complementary nature of proteomics and functional transplant studies to highlight potential improvements to the current gold-standard of HSC expansion and provides strong evidence for further exploration of angiotensin-II in this context.

Author's declaration:

I declare that this is an independent piece of work, written independently with data analysed independently. Laboratory work was performed with bench supervision from Dr Maria Jassinskaja: animal tissue was provided by trained staff, but all, tissue processing, flow cytometry and tissue culture was performed by myself with the supervision of Dr Jassinskaja. Work presented in this thesis has not previously been published or submitted for a qualification at this, or any other, University.

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Introduction

Adult haematopoiesis

The complex formation of blood components, a process known as haematopoiesis, is a vitally regulated part of homeostasis required to constantly replenish the active blood cells necessary for oxygen transport and immunity (1) . Approximately, 2.5 x 10^{11} haematopoietic cells are produced each day in the average human adult, thus there is a high rate of cellular turnover meaning blood cell homeostasis is tightly controlled and if dysregulated, can result in disease (2). The cells found at the bottom of the haematopoietic system have been classically divided into two: the myeloid lineage and the lymphoid lineages (3) . Cells of the innate immune system are of the myeloid lineage which includes, monocytes, macrophages, eosinophils, neutrophils and dendritic cells (4). Both neutrophils and macrophages are phagocytes which engulf foreign pathogens such as bacteria (5); monocytes are capable of recognising 'danger signals' and once recruited they can differentiate into macrophages and dendritic cells (6); dendritic cells can also phagocytose pathogens but can also function as antigen presenting cells in order to initiate the adaptive immune system (7, 8) and eosinophils are involved in parasitic defence and promoting allergic reactions (against pollen, for example) (9). A further two cell types often grouped within the myeloid lineage are erythrocytes, the imperative cells within blood responsible for oxygen delivery to tissues (10), and megakaryocytes which produce platelets (thrombocytes) that form clots and stop bleeding (11) . On the other side, the lymphoid lineage is responsible for adaptive immunity that targets specific pathogens. Lymphoid cells are comprised of B lymphocytes, T lymphocytes and innate lymphoid cells (12). Dendritic cells, acting as antigen presenting cells, can interact with naïve $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells in order to activate them from a quiescent state into a state whereby they undergo clonal expansion in order for accumulation of these antigen-specific T cells, enhancing the immune response (13). $CD4^+$ T helper cells support the expansion of the cytotoxic $CD8^+$ T cells allowing them to express ligands for death receptors (e.g. Fas ligand) in order to induce apoptosis in infected cells (14, 15). B cells are responsible for generating plasma cells that produce antibodies able to bind antigens specifically to allow targeting of foreign pathogens in a variety of ways: they neutralise by blocking viruses in order to render them unable of attack, they can stick pathogens together via agglutination to attract phagocytes, and they can activate the Complement system resulting in cell lysis of pathogens

and inflammation (16-19). Innate lymphoid cells (ILCs) are a group of cells that do not express antigen receptors but secrete inflammatory cytokines similar to the ones secreted by T cells (20). The vast number of cells that can be generated, with their wide range of functions, demonstrates the importance of tight regulation and balance in their production (Figure 1).

The process of haematopoiesis is hierarchical and at the top of the hierarchy is a rare set of multipotent cells known as haematopoietic stem cells (HSCs). HSCs are the most potent cells within the blood system, able to form every cell in the system from erythrocytes through to B and T cells. Haematopoiesis is best defined in mice, where the process has been studied for many decades and well-defined cell isolation and characterisation protocols exist, including for HSCs which reside in the bone marrow (BM). A typical experiment using mouse BM can isolate around 1,500-5,000 HSCs from a single mouse (21). Years of transplantation studies, the gold-standard for assessing HSC function, have demonstrated the single HSCs are able to replenish the whole haematopoietic system, while differentiated short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs) can only maintain multipotency for a short period (~ 1-2 months) and thus have limited self-renewal capacity (22). HSCs can divide in either a symmetrical or asymmetrical manor; symmetrically, the two daughter cells produced will either both commit to differentiating or be maintained as stem cells; asymmetrically, the one daughter will be committed to differentiating and one will be an HSC.

Following this, the ST-HSCs and MPPs give rise to other progenitors which become more and more specialised as they split into common myeloid progenitors (CMPs, which can differentiate into megakaryocyte-erythroid progenitors (MEPs) and esosinophil- basophil progenitors/granulocyte-monocyte progenitors (GMPs)) and lymphoid-primed multipotent progenitors (LMPPs, which can differentiate into common lymphoid progenitors and esosinophil- basophil progenitors/GMPs) (23-25). A wide range of terminally differentiated cells is the result, including erythrocytes, megakaryocytes, B cells, T cells, ILCs, monocytes, macrophages, eosinophils, neutrophils, basophils and dendritic cells (Figure 1). Historically, haematopoiesis was thought of as a step-by-step process; however, it has more recently been described as a much more fluid procedure with different cytokines and growth factors at different stages allowing cells to become more specialised in a specific lineage (26) (Figure 1). For example, recent research has arisen demonstrating the capability of megakaryocytes to be developed straight from HSCs (11, 27-29). Moreover, single cell molecular profiling has

allowed the distinction of highly diverse cell states within traditional cell phenotypes defined by cell surface marker strategies.

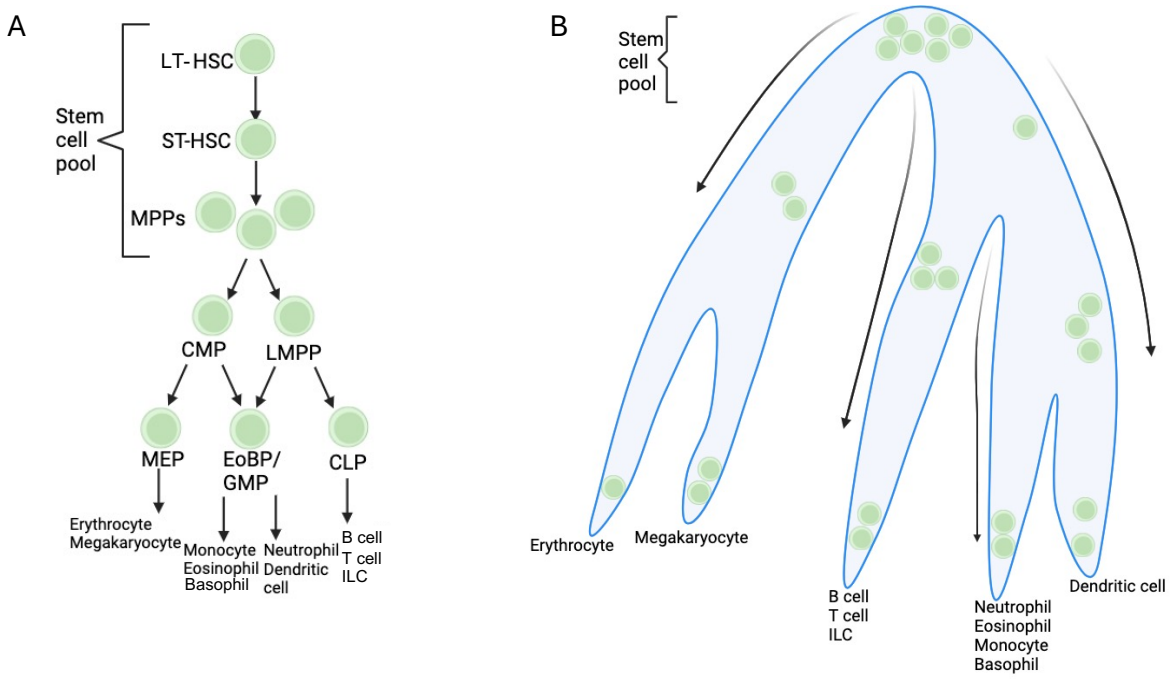


Figure 1: The haematopoietic mechanism as a continuum model vs traditional step-by-step model. A) The step-by-step heterogenous process that was used until ~2016, B) The more continuous model used since 2016. Abbreviations: LT-HSC, long term- HSC; ST-HSC, short-term HSC; MPP, multipotent progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-monocyte progenitor; CLP, common lymphoid progenitor; LMPP, lymphoid-primed multipotent progenitor; EoBP, esosinophil- basophil progenitor; ILC, innate lymphoid cell.

Developmental haematopoiesis

As mentioned above, haematopoiesis is required throughout life in order to replenish cells frequently, but how do the HSCs created during embryogenesis relate to those produced in the BM in adulthood? Classically, developmental haematopoiesis was thought to occur in two waves, then new evidence suggested a multi-wave process, and most recently more HSC-independent waves have been suggested (30-32). The first wave observed in developmental haematopoiesis is the primitive wave whereby the cells produced, supply the embryo with

immunity and oxygen. The definitive wave is the second traditional wave of development that generates the self-renewing HSCs that produce mature progeny throughout life (33). Of the three germ layers, the mesoderm is the one responsible for the formation of blood (as well as bone and muscle etc.) and is formed ~day 6.5 after fertilisation in mice (34). Primitive erythroblasts, the first haematopoietic cells, are found at ~day 8 in blood islands (in the yolk sac) (31). At the same time, the vascular system has been established and a process called endothelial to haematopoietic transition (EHT) begins where endothelial cells from blood vessels transition into blood cells (35, 36). These blood cells that are generated form haematopoietic clusters that are primarily found in the dorsal aorta but also in the yolk sac (36, 37). The cells formed from EHT are initially erythromyeloid progenitors (EMPs), then lymphomyeloid progenitors (LMPs) and finally haematopoietic stem and progenitor cells (HSPCs) (32).

The first HSCs are found in the aorta-gonad-mesonephros (AGM) at day 10 as embryogenesis occurs in small clusters in the dorsal aorta (38). These clusters contain both HSCs and pre-HSCs (cells that only garner effective transplant capacity after co-culture with stromal cells) (39, 40) . These HSCs then migrate and colonise the foetal liver (FL) and replace the EMPs and the cells they produce (e.g. macrophages and monocytes) (41). Until recently, it was believed that these HSCs in the FL were responsible for generating most of the progenitors at the late stages of pregnancy; however, it was unclear how these cells differentiate and self-renew so quickly. Recently, studies have shown that progenitors can be produced in an HSC-independent manner, directly from endothelial cells, thus saving the HSC supply from consumption, allowing the HSCs to expand and not become exhausted (42, 43). HSCs are found within the BM at around day 18.5 of gestation and the FL stops supplying HSCs soon after birth, which occurs at ~day 20 depending on the mouse breed/strain (44-46).

Much like adult haematopoiesis, developmental haematopoiesis is highly influenced from protein signalling and transcription factors. *Runx1* is a transcription factor necessary for EHT to take place. In *Runx1* knock out mice, yolk sac and embryo proper endothelial cells could not support haematopoietic cell production *ex vivo* in conditions optimal for producing them (47). Hepatic leukaemia factor (*Hlf*) is a transcription factor that has been demonstrated to play a role in developmental haematology. Yokomizo et al. demonstrated that *Hlf* is expressed

during EHT, not in EMPs but it is enriched in the FLs with high levels of HSCs, suggesting that *Hlf* expression can differentiate between the production of EMPs and HSCs during EHT (48). $Hlf^{+}cKit^{+}$ cells are defined as pre-HSCs and Hlf expression can be used to separate HSCs from pre-HSCs (42). Interestingly, *Hlf* is also an adult human HSC marker with all HSCs expressing it (49). Also, *Ecotropic viral integration site 1* (*Evi1*) expression in these $Hlf^{+}cKit^{+}$ pre-HSCs determines whether they become more defined progenitors or become HSCs (31, 42). *Procr* is expressed homogenously in both HSCs and pre-HSCs and encodes endothelial protein C receptor (EPCR), suggesting roles in HSC formation process (50, 51). Interestingly EPCR is a marker for adult HSCs which is discussed later in the '*Adult murine HSCs and markers'* section of the thesis (52).

Dynamics of quiescent HSCs

After birth, HSCs are actively cycling and proliferating until the majority become quiescent by week 4 of life (53). An important distinction to discuss is quiescent vs cycling HSCs. Most HSCs are quiescent and divide infrequently (reportedly every 145 days), implicating rapidly dividing ST-HSCs and MPPs as the primary cells responsible for maintaining daily blood production (54-57). Stresses, such as infection, bleeding, or inflammation, trigger HSCs to enter the cell cycle, enabling the timely production of blood progeny and once homeostasis is reinstated, HSCs can exit the cell cycle and return to quiescence in G_0 (58). This key dynamic between quiescence and cell cycling means there is tight regulation in these processes to ensure there is no potential of HSC exhaustion, or at the other end of the spectrum, aberrant haematopoiesis (59). Therefore, it is unsurprising that the regulators of the cell cycle play a huge role: cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs). The CKIs p21 and p57 have important roles inhibiting CDK2 and 4, and their loss leads to exit from G_0 resulting in HSC exhaustion (60). Cyclin A is essential for cell cycle entry in HSCs as its knock-out in mice reduced cell numbers of all lineages of blood as well as leading to mouse death in most cases (61). Furthermore, CDK6 plays a role in quiescence of HSCs. LT-HSCs and ST-HSCs are equally quiescent, but LT-HSCs do not express CDK6 while ST-HSCs, that require quick entry into the cell cycle, express higher levels of CDK6, thus demonstrating that this regulation is controlled by CDK6 (62, 63). Interestingly, proteins known to be involved in foetal haematological development also play roles in regulation of HSC quiescence. Goyama et al. found that *Evi1* is essential for maintaining proliferation of HSCs *in vivo* as knock-out murine

experiments resulted in a reduced number of Lineage^{-}Sca1^{+}cKit^{+} (LSK) cells (a way to mark for murine HSCs) after 4 weeks (64). Furthermore, Kataoka et al. found that *Evi1* expression represents a HSPC population that remain in a quiescent and undifferentiated state (65). They found that overexpression of *Evi1* increased the self-renewal of HSCs and impaired differentiation, and even heterozygous expression of *Evi1* resulted in an almost total loss of HSC self-renewal (65). Quiescent HSCs are believed to utilise glycolytic energy production over oxidative phosphorylation to prevent the use of mitochondria and reactive oxygen species (ROS) generation (66). This is marked by an upregulation of Hif1α in LT-HSCs (66). Additionally, *ID2* ablation was shown to reduce the expression of Hif1α and resulted in increased cell cycle entry and ROS production. It has been reported that Hif1α promotes the expression of *ID2* which promotes a positive feedback loop to maintain quiescence in HSCs (67).

Aging HSCs

As mice age, it is known that their HSCs have a reduced capacity for self-renewal although they increase in number; they have less regenerative potency, and a depletion of functional power. This has been demonstrated through competitive transplant experiments where aged HSCs are outcompeted by HSCs from a young mouse (68-70). Another hallmark of aging HSCs is a skew towards the myeloid lineage, as LT-HSCs from older mice have upregulated expression of myeloid related genes and a downregulation of lymphoid related genes (69). Age-associated clonal haematopoiesis (CH) refers to the expansion of HSCs that have acquired somatic mutations- resulting in all progeny carrying that mutation. This can result in the mutated cells having a selective advantage, allowing them to outcompete unmutated cells. A common mutation that is seen is within the gene *Tet methylcytosine dioxygenase 2* (*Tet2*) which is commonly mutated in myeloid malignancies such as acute myeloid leukaemia (71). Furthermore, when comparing aged HSCs to young HSCs, 5% of the genes upregulated in aged HSCs have relation to leukemic transformation (69). A driver of the aging phenotype seen is believed to be chronic inflammation, known as inflammaging, where HSC self-renewal is impaired (72).

Isolation and characterisation of HSCs

Flow cytometry

Another important aspect of haematological studies, as well as immunological investigations, is identifying cells through their cell surface markers, known as immunophenotyping. Throughout haematopoiesis, different cells have different markers that can affect their function and development through what they bind since they can act as receptors or ligands in order for a signalling cascade to initiate. These markers are often referred to as clusters of differentiation, or CD markers. Each type of leukocyte has a defined set of markers that allow them to be identified in the laboratory. For example, CD45 is a pan blood cell marker with the exception of erythrocytes, CD19 and CD20 are pan B-cell markers and CD11b is expressed on phagocytes such as macrophages (73-75).

To identify cells in a population, multi-parameter flow cytometry can be used. This laboratory technique involves interrogating single cells with multiple lasers as they flow through a tube. Both visible light and multi-colour parameters can be analysed through the size (forward scatter) and granularity (side scatter) and, the colour and intensity of the colour fluorescence in order to identify populations with ease. Specific dyes can be used to bind directly to DNA to identify if cells are dead and antibodies with specific fluorophores attached can be used to bind to specific proteins on cells that will emit light when excited by a certain wavelength of light. This can allow isolation of rare cells such as HSCs. Flow cytometry has long been used in haematological studies, even the first iterations of flow cytometers in the late 1950s were used to count blood cells (76-78). Since then, flow cytometry has become a mainstay of any haematology lab and as a tool in cellular medicine. It can be utilised to aid in the diagnoses of haematological malignancies, monitor HIV infection and detect foetal erythrocytes in mother's blood (to test for foetal-maternal haemorrhage) (79).

Adult murine HSCs and markers

Most stages of differentiation have a unique expression profile that is able to be identified via flow cytometry (Table 1). In humans, more primitive immature cells are lineage marker negative, $CD34^+$, $CD45RA^-$, $CD38^-$, $CD90^+$ and $CD49f^+$, although a range of markers may be used

(80, 81). In mice, the markers differ slightly compared to the human population; surprisingly, there is a lack of CD34 expression in the most primitive HSCs (82, 83). A commonly used marker set to define murine HSCs is lineage$^-$, Sca1$^+$, cKit$^+$ (LSK), however within this LSK population, only ~2.5% are true LT-HSCs (21). In the early 2000s, the SLAM phenotype was discovered, which includes the markers CD150$^+$CD244$^-$CD48$^-$, and highlighted a novel method for selecting for HSCs (84). Finally, the introduction of EPCR to the SLAM marker-set enriched the purity of HSC selection to ~50%, which is the most optimal method, currently (EPCR+, CD45+,CD48-, CD150+, Sca1+ (ESLAM)), and is the method currently used in the lab and these experiments (21, 52).

Table 1: An outline of different markers for murine haematopoietic progenitors

Cell type	**Markers**	**Reference/s**
LT-HSC	Lin$^-$, Sca1$^+$, cKit$^+$, CD150$^+$, CD48$^-$, CD34$^-$, Flk2$^-$	(85, 86)
ST-HSC	Lin$^-$, Sca1$^+$, cKit$^+$, CD34$^+$, Flk2$^-$	(21, 87)
MPP	Lin$^-$, Sca1$^+$, cKit$^+$, CD150$^{+/-}$, CD48$^{+/-}$, CD135$^{+/-}$ (different MPPs have different CD150, CD48 and CD135 expression)	(22, 55, 88-92)
CMP	Lin$^-$, Sca1$^-$, c-Kit$^+$, CD34$^+$, CD16/32$^-$	(93)
GMP	Lin$^-$, Sca1$^-$, cKit$^+$, CD34$^+$, CD16/32$^+$	(93)
MEP	Lin$^-$, Sca1$^-$, cKit$^+$, CD34$^-$, CD16/32$^-$, CD127$^-$	(93, 94)

Functional methods for studying HSCs

Since the 1960s, colony forming unit (CFU) assays have been utilised in HSC research to identify the differentiation capacity and proliferation ability of early haematopoietic progenitors (95). One of the earliest assays, colony forming spleen unit assays (CFU-S) is performed where the blood cell suspension of interest is isolated and transplanted into an irradiated host. Between one and two weeks later, the spleen is harvested from the recipient and the colonies are counted and analysed under a microscope. The colonies are then dissected and put into a single cell suspension where they can be analysed using flow cytometry to determine their lineage (95, 96). In the 1970s and 1980s, developments utilising semi-solid media permitted the growth of colonies *in vitro* and these assays continue to function as robust tools to study cell differentiation, whilst removing the need for additional mice to be used in experiments (97). HSCs are known as long term culture- initiating cells

(LTC-ICs) through their capability of being able to maintain *in vitro* cultures for over 5 weeks, when supplied with a stromal feeder layer, in experiments called LTC-IC assays (98).

These *in vitro* assays are complemented well by long term repopulation assays. For quantification of stem cells, limiting dilution assays are the gold standard and were used to demonstrate that the transplant of a single HSC ($CD34^-KSL$) could reconstitute blood for over 3 months in over 1/5 of lethally irradiated recipient mice when transplanted on its own in the 1990s by the Nakauchi group, and are still used to this day (83, 99, 100). Transplantation of donor HSPCs into recipient irradiated hosts allows long-term reconstitution to be measured through repeated bleeds. They can confirm that LT-HSCs are found within the transplanted population, if they can self-renew and maintain contribution to blood-lineages for over 16 weeks in the transplanted host, and furthermore throughout secondary transplantation (as less potent progenitors will not support long-term reconstitution) (99, 101, 102). Another type of transplant experiment that is frequently utilised in HSC research is competitive transplants, whereby cells from two different mice are transplanted into an irradiated mouse at a 1:1 ratio, in order to compare the two cell populations' HSC capacity (103). These *in vivo* studies are possible due to the alleles of CD45, the pan leukocyte marker, CD45.1 and CD45.2. These allow the transplanted cells to express one allele (e.g. CD45.2) and the recipient cells to express the other (e.g. CD45.1) which can be both quantified through flow cytometry analysis.

Molecular methods for studying HSCs

As HSCs are such a rare and heterogeneous population of cells, coupled with the difficulties with the *in vitro* expansion of the cells, their study has often been hindered by the large number of cells required for studies such as -omics. Single cell-based sequencing has tackled this issue, but it does require a large cost, again making research on HSCs more difficult. A number of studies have utilised these single cell techniques for the HSC transcriptomics such as RNA-sequencing (RNAseq) and quantitative polymerase chain reaction (qPCR). For example, single cell (sc) RNAseq of HSPCs and their progeny in zebrafish gave increased evidence for the continuous model of haematopoiesis (104). Furthermore, these techniques have also highlighted the potential for megakaryocytes to develop straight from HSCs. Sanjuan-Pla et al. utilised sc-qPCR to do this by characterising that HSCs expressing von

Willebrand factor (*vWF*), a megakaryocyte associated gene, are primed for megakaryocyte differentiation (105). *vWF* knock out transcriptome comparison with normal HSCs as well as functional transplant studies were the bases of those experiments performed. Furthermore, scRNAseq comparing the transcriptome of young and old mice demonstrated that old mice express higher levels of platelet specific genes (106). ATACseq is a method for studying epigenomics that maps chromatin availability. Martin et al. compared the accessibility of the genome in HSCs and differentiated progeny. They found out of all terminally differentiated cells, lymphoid (B and T) cells were the least similar to HSCs and megakaryocytes were the most similar (107). This could be inferred as further evidence to megakaryocytes being developed straight from HSCs as their chromatin availability is the most similar, indicating there are less steps epigenetically for megakaryocytes to be developed from HSCs. More recently, studies have emerged linking molecular studies to cell state together. TrackSeq is a method developed by Wehling et al. which utilises scRNASeq with imaging of daughter cells of HSCs in order to track them and identify molecular changes in asymmetric HSC division (108).

Mass spectrometry-based proteomics

Interrogating the transcriptome of HSCs has been simpler than the proteome as RNA and DNA can be amplified i*n vitro* which is not the case for proteins. However, proteomics is one of the most powerful forms of -omics as proteins are the most functional units of cells, as proteins are related highly to phenotype compared to RNA and DNA, thus it is imperative it is interrogated. Mass spectrometry (MS) based proteomics is the gold-standard for doing this. The most commonly used MS-based proteomics method involves lysing cells to isolate the proteins, using trypsin to digest the proteins into peptides and then measurement on the spectrometer in order to infer the identity of proteins of the proteome (109).

When tandem mass spectrometry (MS/MS) is used, analysis of the resulting data can either occur in a data-dependent acquisition (DDA) manner or a data-independent acquisition (DIA) manner. In DDA, the most abundant ions are selected for fragmentation. Conversely, in DIA, all the ions within a certain range are fragmented (110). DIA has become more popular in recent years as it has been shown to have higher reproducibility and accuracy, whilst also

being better at quantifying small amounts of proteins, something that is key when investigating a small cell set such as HSCs (111).

Mass spectrometry-based studies have been utilised in haematological studies for understanding the proteomic profile of cells, whether normal or malignant (112-115). Much like sc approaches with studying the transcriptome, proteome analysis has highlighted interesting discoveries with megakaryocyte-associated cells. When comparing the proteome to the transcriptome, it was found that megakaryocyte-committed progenitors are kept in a quiescent state but are primed for differentiation expressing mRNA but not translating them into proteins so that when under stress (inflammation) they can be quickly translated so platelet number is maintained and they are not exhausted (116). This study highlights the importance of both proteomic studies as well as transcriptomic studies and neither should be omitted from studies. There have been a handful of studies interrogating the differences of the proteome of foetal and adult HSCs. These studies found that foetal HSCs are enriched for aerobic respiration whilst adult HSCs utilise glycolysis more and foetal HSCs are enriched for cell cycle related proteins compared to adult HSCs indicating that foetal HSCs are not as quiescent as adult HSCs, which is of no surprise considering there is an increased demand for both HSCs and terminally-differentiated cells before birth when HSCs become quiescent (116, 117). Outside of findings from these studies, there have been concerted efforts to reduce the number of cells needed for mass spectrometry-based proteomics experiments, especially in the context of HSCs. A breakthrough study by Amon et al. in 2019 highlighted a novel DIA-MS method to analyse human HSCs by only using 25,000 cells (118). Most recently, although not published, Porse et al. presented work at the International Society for Experimental Haematology conference of 2024, that demonstrated the first work using sc mass spectrometry (sc-MS) on human $CD34^+$ cells (119). Sc-MS has been made possible in recent years through the tagging of proteins and using carrier cells to provide enough peptides to allow for identification and relative quantification (120). The work of Porse et al. is the first time sc-MS has been performed on HSCs, on over 2,500 cells with an average of 1,000 proteins identified in each cell. These advancements in proteomics using small numbers of cells opens up rare cell populations to be interrogated in further detail. This may highlight differences between normal haematopoiesis and malignant haematopoiesis, and thereby signify

targetable proteins as therapeutics, or even to improve the reporter system for HSCs to make FACS-sorted HSCs purer.

Expanding HSCs ex vivo

As previously mentioned, the haematopoietic system is best described in mice, and that is reflected in the greater successes in *ex vivo* expansion of HSCs in murine systems compared to humans. The expansion of these cells in both species has long been an active and dynamic part of haematological research. As HSCs are such a rare cell population, the expansion of these cells in the laboratory is imperative to allow for large scale drug screening, as well as gene editing studies. In terms of research, mice are often used to model humans, as there are similarities between the species, and they are more readily available compared to human samples. Therefore, often, techniques that are successful are then applied to human cells, often with slight modifications, in order to ensure human samples are not wasted and there are more successful attempts.

There are many different methods to expand HSCs *in vitro* with varying rates of success. Until recently, there were rare successes in expanding human HSCs with studies focussing on murine techniques. Commonly, the cytokines stem cell factor (SCF) and thrombopoietin (TPO) are added to HSC culture medias in order to aid expansion. SCF is the ligand for cKit (CD117) which is expressed on the surface membrane of HSCs. It has been shown that depleting levels of cKit in HSCs can reduce numbers of HSCs, and blocking the interaction of SCF and cKit can increase HSC clearance (121, 122). TPO and its receptor Mpl are primarily known to have roles in megakaryocyte function and platelet development but is also expressed on all HSCs (123). As TPO binds, it induces the dimerisation of Mpl causing JAK2 molecules to phosphorylate each other (124, 125). The active forms of JAK2 are then able to phosphorylate Mpl resulting in the recruitment of further signalling proteins to the SH2 domain. For example, Stat (3 and 5) transcription factors are able to be phosphorylated by JAK2 allowing entry of the Stats into the nucleus to bind to specific promoters of genes, leading to expression of proteins involved in proliferation and the avoidance of apoptosis, such as cyclin D1 and BCLxl (126-128). Another pathway that is activated through TPO is the PI3K/Akt pathway resulting in the progression of the cell cycle (129). The interleukin (IL)-6 family of cytokines use β-receptor glycoprotein 130

(gp130) as a signal transducer (130). Of the cytokines within the family, IL-6 is a cytokine whose expression is primarily activated by tumour necrosis factor-α (TNFα) as well as interleukin 1 β (IL-1 β signalling). It is produced by a range of mature blood cells including, B and T cells, macrophages and DCs (131). IL-6 has been found to play a role in developmental haematology and the specialisation of HSCs. Upon activation of PDGFRβ by Hif1α, there is increased expression of IL-6 as it is a downstream target. In experiments where PDGFRβ was overexpressed, there was an increased production of HSPCs. When IL-6 was ablated in the same setting, that production of HSPCs was decreased, demonstrating its key role in HSC development (132). Through this discovery in developmental haematology, IL-6 was interrogated as a cytokine to enhance the *ex vivo* expansion of HSCs. It was found to be a greater expander of $CD34^+$ human progenitor cells than IL-3 (in combination with other cytokines TPO, SCF and Flt3L) (133). In mice, the combination of IL-6, IL-3 and SCF induced a large expansion of LSKs over a two-week period, however, a vast majority were found to be mast cells ($FcεRIα^+$ LSKs) (134). Another member of the IL-6 family is IL-11 which has also been investigated as a molecule for the *in vitro* expansion of HSCs. Audet et al. found that IL-11 and SCF improved HSC expansion the most and IL-11 has also been shown to play a role in maintaining hibernation cultures of HSCs, keeping HSCs in a quiescent state for up to 7-days *ex vivo* (135-137).

The bone marrow, as the home of HSCs, has long inspired researchers searching for the most optimal way to expand them *ex vivo*. It has been demonstrated that the co-culture of HSCs with bone marrow derived stromal cells, can support expansion modestly (138, 139), and even enhance transplant outcomes of CRISPR-edited HPSCs (140). Another method for modelling the BM microenvironment is utilising hydrogels to mimic the extra-cellular matrix (ECM), achieving this modelling in a cell-free technique. These hydrogels are composed to mimic the BM niche through the inclusion of ECM proteins and well as having the same 'hardness' as standard BM (141). Hydrogels made from polyethylene glycol (PEG) have been used to expand HSC *ex vivo* as proteins can be attached to the network (142). For example, Cuchiara et al. demonstrated that the addition of SCF, SDF1α and RGDS into a PEG-based hydrogel better modelled the HSC microenvironment (143) . Then, zwitterionic hydrogels were utilised (144). This method found an over 70-fold expansion of LT-HSCs over a 24-day period. It was believed this effect was due to inhibited reactive oxygen species (ROS) production via a suppression of

oxygen-based metabolism, a known impairment of many *ex vivo* HSC cultures due to increased DNA damage (144). Recently, a novel aliginate porous hydrogel was compared against a X-Vivo 20 liquid culture system and a non-porous hydrogel. The porous hydrogel was the most effective at expanding $CD34^+$ cells, as even though the liquid culture had the largest cell number after culture, it experienced the most differentiation (145). Although there have been some exciting improvements in using hydrogels to expand HSCs, more work is necessary for them to become standard practice in the field.

As recombinant cytokines can induce variability within these expansion systems and promote differentiation, small molecules have been investigated on their effect in HSC expansion. StemRegenin-1 (SR-1) is an aryl hydrocarbon receptor antagonist that has been proved to enhance $CD34^+$ human progenitor cell expansion *in vitro* (146, 147). In a clinical trial in which 20 leukaemia/myelodysplastic syndrome patients received $CD34^+$ umbilical cord blood cells expanded *in vitro* with SCF, TPO, Flt3L, IL-6 and SR-1. The $CD34^+$ cells had an over 200-fold increase in cell number after 15-days, and after transplantation, time for engraftment was shorter in the expanded cell fraction compared to those treated with unmanipulated cells (148). Valproic acid (VPA) is a histone deacetylase inhibitor that has proved effective at improving HSC expansion in both mice and humans (149). VPA treatment has shown to increase phenotypic HSCs rapidly (up to 63- fold only 24 hours after treatment) (150). UM171 is another molecule that has been used in clinical trials to expand HSCs. Although only a small trial, it found that patients transplanted with UM171 expanded HSCs had an increased relapse -free survival (in terms of graft vs host disease) and decreased mortality rate compared to transplants of non-treated cord blood (151, 152).

Difficulties with *ex vivo* expansion of HSCs

There are many reasons for the struggles seen when it comes to expanding HSCs *ex vivo*. The largest issue has been avoiding cell differentiation, which is frequently observed in these methods. Many studies have highlighted vast expansion of cells but when that expansion is interrogated for the cell types produced, the majority of cells are differentiated progeny and upon transplantation fail to give substantial contribution or are skewed towards long lived lymphoid cells (153). Even in the most advanced PVA-based conditions, substantial clone-to-

clone variability is seen as some HSC clones will expand well and some will not. It is possible that not all of the FACS-sorted cells are true HSCs, or that the early stage of the culture has an element of random chance with respect to differentiation, or that the media change mechanically remove the key cells or do not remove factors that induce differentiation. As previously mentioned, sorted ESLAMs have a purity of ~50%, thus it is not surprising some ESLAMs differentiate in culture, and some do not, but clone success in these cultures is typically <20%, so there is some merit in figuring out what drives the previously 'good' HSCs to differentiation (154).

With this in mind, improving the reporter strategy for murine LT-HSCs may aid in the *in vitro* expansion of the cells themselves. Furthermore, there has only recently been a method discovered for marking *in vitro* expanded functional HSCs. It had long been reported that HSCs change their cell surface markers during *in vitro* culture (155). For example, the increase of LSK expressing cells in culture does not correlate with a higher number of transplantable HSCs (155, 156). The different types of cell division that can be performed by HSCs also brings up interesting problems that need to be resolved for the *ex vivo* expansion of these cells. In an ideal world, cultured HSCs would expand in a symmetrical manner whereby both daughter cells produced are HSCs. Currently, however, most methods seem to induce HSCs to differentiate (or at best asymmetrically divide) (157). Therefore, methods of culture which allow HSCs to enter the cell cycle and divide in the desirable manner, is of great need.

Recent advancements in *in vitro* HSC expansion

Replacing serum for HSC expansion medias

There is a large effort to enable the expansion of HSCs without serum as if these cells could be expanded *in vitro* – ideally for clinical use without involving undefined animal products – they would have the potential to be used clinically, expanding the chance of patients with haematological malignancies having the access to effective therapy. Serum albumin, in its many forms, has been frequently added to HSC cultures, but it can induce variability between batches, and has been found to be necessary for inducing quiescence in ex vivo cultures (158). Recently, a novel method for expanding HSCs in a serum-free system was developed by Wilkinson et al. (99). Polyvinyl alcohol (PVA) was found to support HSC growth with up to 899-

fold expansion in functional transplantable HSCs (99). In competitive transplantation studies performed, the PVA based cultures performed better than recombinant human serum cultures. Although the complete mechanism how this polymer functions as a replacement for serum is not known, it is believed it can similarly stabilise recombinant cytokines (159). This serum-free media is the one utilised in the experiments throughout this thesis.

In vitro markers for murine HSCs

In 2022, our lab published a novel method for marking *in vitro* expanded HSCs (154). Through use of the Fgd5^{+}/ZsGreen reporter mouse, along with the expression of EPCR, functional and translatable HSCs can be identified and isolated (154). This method can be utilised with non-reporter mice, by using EPCR^{+}LSKs (ELSK) (141) or even exchanged for the marker ESAM (to mark for ESAM^{+}ELSKs [EELSK]) (154). In their work, after single HSCs were grown for 28-days in the PVA-based media, the cells were split into ELSKs (phenotypic HSCs) and non-ELSKs (non-HSCs) with half of the cells used for RNA-seq and half for transplantation analysis. Within the transplant studies, they found that even though the mice transplanted with non-ELSKs were transplanted with ~26-fold more cells, chimerism was rarely seen, thus non-ELSKs were found to mostly lack reconstitution capacity. Conversely, in the ELSK fraction, 40% of clones engrafted well and %ELSK of the clone correlated highly with transplanted cell contribution. In the RNAseq analysis, groups were further fractionated into clones that repopulated mice (Pos) and those that did not (Neg). Interestingly, Pos-non-ELSKs overlapped with Neg-ELSKs, and Pos-ELSKs did not cluster with Neg-ELSKs. Then, when comparing the gene signature to that of freshly isolated HSCs, ELSKs were more similar than non-ELSKs, and Pos-ELSKs were more similar than Pos-Non-ELSKs, revealing that ELSK is the most optimal way to mark for *in vitro* HSCs as they are most similar to freshly isolated HSCs and have the highest reconstitution capacity. A repopulation molecular signature (termed RepopSig) containing 23 genes was generated of the top genes found to drive reconstitution potential which are capable of identifying *in vitro* expanded HSCs, as well as HSCs in other cellular states: cell cycling HSCs, quiescent HSCs or even FL HSCs. This study revealed the ability to couple functional studies and molecular studies in the HSC context that are generally hindered by low cell number.

Aims

This project had two main aims:

Identify proteins enriched in clones with high HSC

Similar to the approach in Che et al. (154), with transcriptomic studies of 'good' and 'bad' clones, we aimed to identify 'good' and 'bad' clones and profile their secretome to determine whether the expression of specific molecules induce the differentiation of HSCs in 28-day cultures.

Further improve the PVA-based media used to expand murine HSCs in a serum free system

Although the PVA-based method for expanding HSCs can lead up to 899-fold expansion, a large proportion of the cells are not HSCs, thus there is a room for improvement in the expansion system (99). Inspired by the HSC secretomic studies and interrogation of molecules in the context of the literature, different molecules were added to bulk ESLAM cultures in order to determine their effect on HSC retention.

Together we hypothesise that coupling functional assays with molecular assays will lead to more robust candidate identification and will use this information to undertake studies that are described in the results chapter below.

Methods

A simple workflow of basic experiments is outlined in Figure 2.

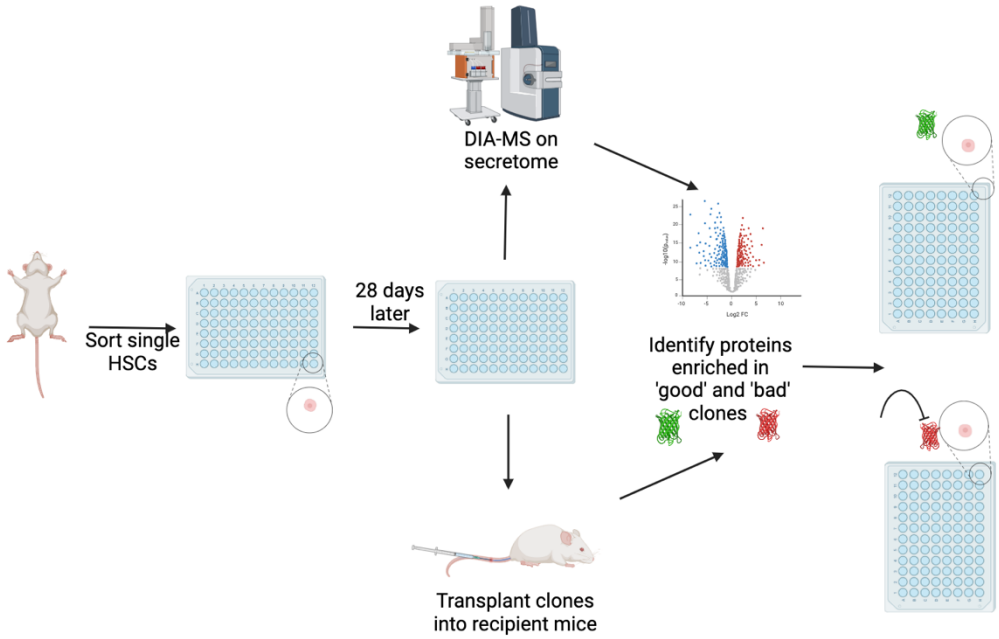


Figure 2: Workflow used to determine the secretome of *in vitro* expanded HSC clones and whether the current F12/PVA based media can be improved. Single ESLAM HSCs were sorted per well of a 96-well plate before growth for 28 days. Throughout media changes, the media the cells were grown in was kept for mass spectrometry analysis. At day 28, the clones were transplanted into recipient mice and maintained for 24 weeks to analysis the long-term repopulation capacity of the HSC clones. This allowed identification of clones that could reconstitute blood in the long-term and those that did not, thus permitting the identification of proteins that were enriched in 'good' HSC clones, and 'bad' HSC clones that did not reconstitute blood well. Then, bulk HSC cultures were treated with molecules that either inhibited proteins enriched in non-repopulating clones, or agonising proteins enriched in repopulating clones.

Mice

Wild-type (wt) mice were CD45.2 C57BL/6 and these were used for both single-cell and bulk ESLAM experiments. All recipients of transplants were C57BL/6 W41/41-Ly5.1. All mice were kept in the animal facility at the University of York, and provided continuously with food,

bedding and water in sterile conditions. All investigations were performed according to the UK Home Office regulations.

Fluorescent Activated Cell Sorting and flow cytometry

ESLAM sorts

Hind legs and sternum were crushed using a pestle and mortar and bone marrow (BM) was transferred into a falcon tube through a cell strainer. Crushing was repeated with further PBS/2 mM EDTA to a total of 30 mL and the crushing solution was clear. Cells were centrifuged for 5 min at 300 xg and supernatant removed. To lyse cells, 3 mL PBS/2 mM EDTA and 5 mL ammonium chloride solution was added without resuspending the cells, for 5 mins on ice. After vortexing to resuspend the cells, they were incubated for a further 5 mins on ice. Lysing was quenched using PBS/2 mM EDTA to total the volume to 25 mL and solution centrifuged for 5 min at 300xg with the supernatant removed. To lineage deplete the cells, samples were resuspended to 500 µL total using PBS/2 mM EDTA, transferred to FACS tube, and 20 µL of StemCell HSPC cocktail was added to each sample and incubated for 15 mins on ice. 50 µL of StemCell magnetic beads were added and incubated for 15 mins on ice. 2 mL PBS/2 mM EDTA was added, lid removed and placed into magnet for 3 mins. Depleted cell solution was poured into clean FACS tube, centrifuged for 5 min at 300xg and supernatant removed. Samples were resuspended in 100 µL of antibody mastermix (Table 2) and incubated for 30 mins on ice in the dark. 3 mL of PBS/2 mM EDTA was added to the staining cells, then centrifuged for 5 mins at 300 xg and cells resuspended in PBS/2 mM EDTA with 7AAD (for live/dead staining) at the correct concentration, immediately before sorting.

Table 2: Antibodies with associated fluorophores used in ESLAM sorts.

Antibody- fluorophore	**Concentration**	**Clone**	**Company**
CD45 – FITC	1:100	30-F11	BioLegend
CD48 – APC	1:100	HM48-1	BioLegend
CD150 – BV605	1:100	TC15-12F12.2	BioLegend
Sca-1 – PE-Cy7	1:100	D7	BioLegend
EPCR – PE	1:100	1560	eBioscience
7AAD	1:1000		Invitrogen

ELSK panel

At the end of the culture period, whether 28 days or 10 days, cells were transferred to a U-bottom plate and centrifuged for 5 min at 350xg with the supernatant flicked off. 50 µL of ELSK antibody cocktail used to resuspend cells and left to stain in the dark, on ice for 30 mins (Table 3). 100 µl PBS was added, plates centrifuged for 5 mins at 300xg and resuspended in 120 µl PBS/1:1000 7AAD and wells were analysed on a Fortessa (gating for ELSK cells represented in Figure 3).

Table 3: ELSK antibody mastermix with associated fluorophores

Antibody - fluorophore	**Concentration**	**Clone**	**Company**
CD45 – BV785	1:500	30-F11	BioLegend
EPCR – PE	1:500	1560	eBioscience
cKit – APC-Cy7	1:500	2B8	BioLegend
CD11b – PE-Cy7	1:250	M1/70	BioLegend
Gr-1 – PE-Cy7	1:250	RB6-8C5	BioLegend
CD42d – APC	1:500	1C2	BioLegend
Sca-1 – BV605	1:250	D7	BioLegend
CD41 - FITC	1:500	MWReg30	BD Pharmingen
7AAD	1:1000		Invitrogen

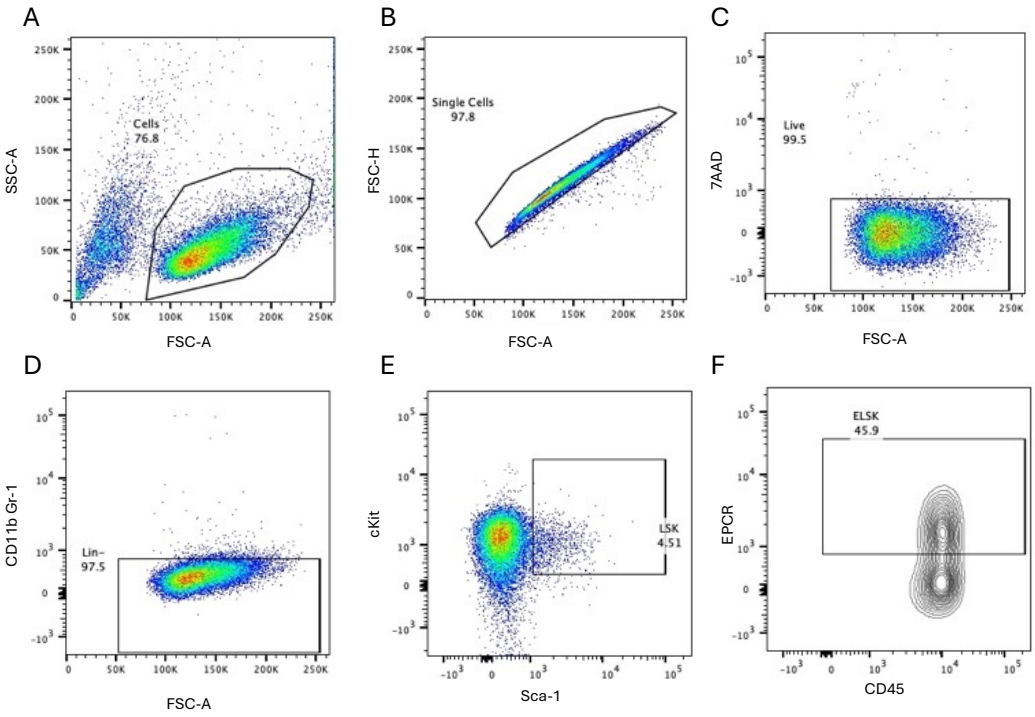


Figure 3: Representative gating to isolate ELSK population from *in vitro* expanded HSCs. After gating on cells (A), single cells are selected (B) and viable cells are gated (C). Then, lineage negative ($CD11b^{-}Gr1^{-}$) cells are selected (D), and $Sca1^{+}cKit^{+}$ are selected (E), and finally $EPCR^{+}$ cells are gated (F).

ELSK HSC Transplants

Mice were transplanted as previous described by Che and Bode et al. into recipient C57BL/6 W41/41-Ly5.1 mice (154). Single doses of Cesium (400 cGy) irradiation were used to sub-lethally irradiate recipients. Intravenous tail vein injections were performed of HSC clones suspended in 200 µL PBS.

Peripheral bleed analysis

Tail vein bleeds of recipient mice were performed at 4-week time points using microvette tubes coated in EDTA. Red blood cell lysis was performed with 1 mL ammonium chloride with samples incubated for 5 min on ice, vortexed, then incubated for a further 5 min on ice. After quenching with PBS/2% FBS samples were centrifuged at 300 xg for 5 min and resuspended in 100 µL of antibody mastermix (Table 4) and left to stain for 30 min, on ice, in the dark. Then, 3 mL of PBS/2% FBS was added to the samples and they were then centrifuged at 300 xg for 5 min. Samples were then resuspended in 400 µL PBS/2% FBS and 7AAD was spiked into samples at the appropriate concentration immediately before analysis.

Table 4: Peripheral bleed antibody mastermix with relevant clones and concentrations

Antibody - fluorophore	**Concentration**	**Clone**	**Company**
CD45.1- PE-Cy7	1:1000	A20	BioLegend
CD45.2- AF700	1:1000	104	BioLegend
Ly6G- BV421	1:1000	1A8	BioLegend
CD11b- BV605	1:1000	M1/70	BioLegend
CD3e- PE	1:1000	145-2C11	BioLegend
B220- APC	1:1000	RA3-6B2	BioLegend

7AAD	1:1000		Invitrogen

DIA-MS: Mass spectrometry on expanded HSC secretome

At timepoints of interest, supernatant was collected from cultures during media changes by transferring the liquid to a fresh U-bottom plate. Plates were spun at 350xg for 5 mins and supernatant transferred to a fresh U-bottom plate, taking care not to aspirate any cells and stored at -80 °C. When needed, plates were placed in a fridge to thaw overnight. 4x the sample volume of acetone was cooled to -20 °C and samples were transferred into Protein Lo-Bind tubes and acetone was added. Then, tubes were vortexed and incubated for one hour at -20 °C. Samples were centrifuged for 10 min at 15,000 xg and the supernatant removed. 6 µL of 0.1 M Dithiothreitol (DTT) was added to samples and incubated for 20 mins at 56 °C and then 6 µL of 0.2 M Iodoacetamide (IAA) was added and incubated in the dark for 30 mins. 0.5 µg trypsin was added and incubated o/n at 37 C. Digestion was stopped by adding 6 µL of 10% trifluoroacetic acid (TFA) and samples were dried in a SpeedVac and stored at -20 °C. When ready to use, samples were thawed and made up to 20 µl TFA. Evotips were prepped by rinsing 20 µL of 0.1% FA in acetonitrile (ACN) and centrifuged for 1 min at 800xg. 100 µL Isopropyl Alcohol (IPA) was added and tips soaked until tips were translucent. Then, 20 µL 0.1% FA in H_2O was added to each tip and was spun for 1 min at 800xg. Samples were then loaded and centrifuged at 800 xg for 1 min. 50 µL of 0.1% FA in H_2O was added to each tip and were centrifuged at 800xg for 1 min, this step was repeated for a total of three times to wash the tips. To preserve samples, 100 µL 0.1% FA in H_2O was added to each tip and centrifuged at 800 xg for 10 seconds. Each sample was introduced onto a 15 cm endurance column with elution using a pre-set 30 samples per day gradient on an EvoSep One UPLC system. DIA-PASEF (data-independent aquisiton parallel accumulation–serial fragmentation) data was acquired using a Bruker timsToF HT MS with a 1.1 second cycle time, between 400-1200 m/z whilst using 25 m/z DIA windows. Resulting DIA data was searched using DIA-NN against an in-silico generated library produced from the murine subset of SwissProt. DIA-NN searching was run with a target 1% FDR and match between runs applied. DIA-NN results were collated and pivoted to a non-normalised protein-group centric output. The resulting data was further filtered to require protein group q-values <0.01, and a peptide length >1.

Cell culture

ESLAM cells

ESLAM cells were grown in 200 µl per well in a 96 well plate coated in 10 mm/cm^2 fibronectin, in media containing: Ham's F12 Nutrient Mix (Gibco), 1x penicillin-streptomycin-glutamine (PSG) (Gibco), 10 mM HEPES buffer (Gibco), 1 mg/mL Polyvinyl acetate (PVA) (Sigma), 1x insulin-transferrin-selenium-ethanolamine (ITSX) (Gibco), 10 ng/mL SCF (Peprotech), 100 ng/mL TPO (Peprotech) Plates are kept at 37°C, 5% CO_2 in sterile conditions.

Bulk plates

50 cells were sorted per well of a 96 well U-bottom plate. Plates were kept at 37°C, 5% CO_2 for up to 11 days, with no media changes. For investigations into the effect of certain molecules on HSC expansion the molecule and concentrations are outlined in Table 5:

Table 5: Molecules tested in bulk ESLAM cultures to determine their effect on

Reagent	**Concentration/s**
Olaparibol	20 nM
Salidroside	20 µM, 250 µM, 500 µM, 1 mM
N-acetyl cysteine	100 µM, 500 µM
Resveratrol	10 µM, 25 µM
Transforming growth factor beta	1 ng/mL
SB-431542	10 µM
Tumour necrosis factor alpha	10 ng/mL, 100 ng/mL
Angiotensin-II	2 µM

Single cell plates

One ESLAM was sorted per well in a 96 well flat-bottomed plate. Plates were kept at 37°C, 5% CO_2 for 28 days, with media changes occurring every 2/3 days with the first after day 7.

Data analysis and statistics

Proteomics data was analysed using the limma package in RStudio. All other statistics tests (unpaired T tests and ANOVA tests) were calculated and plotted using Graphpad Prism 10.

Results

With larger cell numbers available through the *in vitro* expansion of ESLAMs, work in this project utilised mass-spectrometry based proteomics to investigate the secretome of HSC clones with reconstitution capacity. Here, single ESLAM clones were grown for 28 days whilst the media removed at each media change was stored for DIA-MS analysis. At the end of the 4 weeks, half the cells from each well were sent for flow cytometry analysis to quantify the HSC content of the respective clones, and the other half was transplanted into irradiated mice in order to measure the effectiveness of the clones to reconstitute blood. Through linking the functional analyses to the proteomics data, we have identified novel proteins that may play a role in HSC regulation *in vitro*.

Refining the range of clones containing functional HSCs in expansion cultures

Single ESLAM HSCs sorted into a well of a 96 well plate that were grown for 28 days and then the cells from the wells of 11 clones (with varying %ELSK content) were transplanted into sub-lethally irradiated mice, where repopulation capacity was measured for each clone through the proportion of CD45.2 cells in recipient mice (with recipient cells expressing CD45.1). Transplanted mice were longitudinally followed for 24 weeks but serial transplantations have not taken place yet due to the short timescale of this project. Tail vein bleeds were performed every 4 weeks to acquire peripheral blood samples, as CD45.1 vs CD45.2 expression can be analysed in different blood cell populations, namely, T cells ($CD3e^+$), B cells ($B220^+$) and cells of a granulocyte-macrophage (GM) ($Ly6G^+CD11b^+$) lineage. This data can be used to categorise the HSC clones transplanted to either ‘good’ clones that can reconstitute a transplanted host well, and ‘bad’ clones which cannot. The threshold for a mouse to be considered repopulated is that it has >1% overall donor chimerism at week 16 or later, and at least 0.5% CD45.2 expression to each of the lineages at some point during the transplantation period (154). Of this basis, of the 11 initially transplanted clones, we had 5 available ‘good’ clones and 6 ‘bad’

clones (Table 6, Figure 4). It should be noted that mouse H1017/23_3 died between weeks 12 and 16 but showed no chimerism up to this point and was therefore classified as a 'bad' clone.

Table 6: Table of classification to determine repopulation capacity of clones. Green indicates clones with over 1% overall donor chimerism at week 16 (%CD45.2 at week 16), and over 0.5% CD45.2 expression to each lineage at any time point (B cell: $B220^+$, T cell: $CD3e^+$, Granulocyte-macrophage (GM): $Ly6G^+CD11b^+$), indicating a 'good' clone; orange indicates below the threshold.

	%chimerism wk16	B cell						T cell						GM						Overall Rank
		wk 4	wk 8	wk 12	wk 16	wk 20	wk 24	wk 4	wk 8	wk 12	wk 16	wk 20	wk 24	wk 4	wk 8	wk 12	wk 16	wk 20	wk 24	
H988/23_1	3.26	2.75	0.95	0.68	0.67	0.48	0.32	1.01	19.6	23.3	21.2	15.9	12.6	48.5	0.02	0	0	0	0.16	
H988/23_2	75.2	80	87.8	90.5	91.1	93.1	94.4	28.1	66.3	77.5	76.3	78.7	81.7	97.3	98.5	99	98.7	97.8	97.8	
H988/23_3	0.64	0.35	0.14	0.14	0.031	0.02	0.12	4.64	8.74	4.79	3.04	1.81	1.79	3.14	0	0.049	0.041	0.13	0	
H988/23_4	65	12.2	22.3	33.4	33.9	43.5	47.2	1.06	24.5	44.4	54.3	52.3	44.8	90.4	93.6	99	98.7	97.4	88	
H989/23_3	0.82	0.11	0.057	0.029	0.035	0.011	0	3.75	13.3	6.33	3.97	2.24	2.97	0.042	0.058	0.31	0.087	0	0	
H989/23_4	73.7	22	60.4	70.5	72.6	73.1	67.3	4.56	56.8	72.6	76.1	79.9	64.6	91.7	92	98	98	96.2	92.4	
H989/23_5	0.013	0	0	0.00567	0.023	0	0.00844	0	0	0.00463	0.00698	0	0	0	0	0	0	0	0	
H1017/23_1	79.4	47.2	70.8	76.9	81	86.9	90.8	5.13	56.2	73	81.7	81.7	51.7	90.2	98.6	99.8	96.4	97.9	94.1	
H1017/23_2	1.59	1.37	0.28	0.22	0.15	0.12	0.054	11.6	14.3	6.95	5.78	4.11	4.17	0.1	0	0	0.028	0.066	0.069	
H1017/23_3	0	0.00829	0	0	N/A	N/A	N/A	0	0	0.013	N/A	N/A	N/A	0.088	0	0	N/A	N/A	N/A	
H1017/23_4	2.66	20.4	6.83	5.09	3.23	2.14	3.02	14.9	24.6	21.3	11.8	6.01	6.83	0.04	0.081	0	0	0.11	0.17	

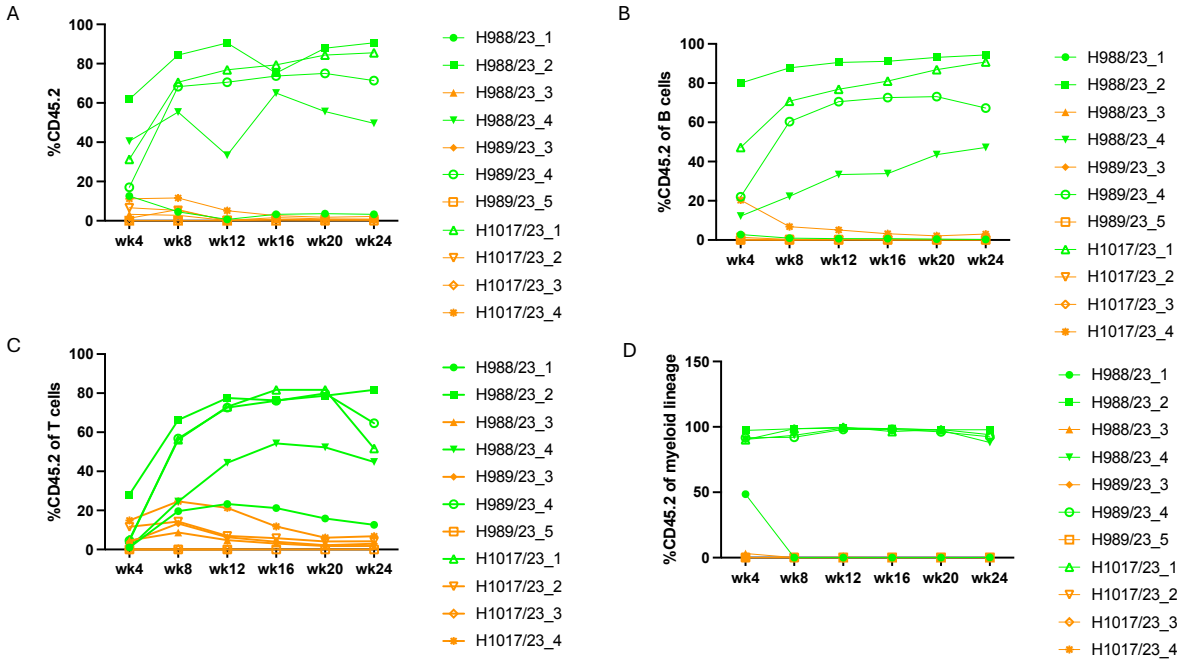


Figure 4: Contribution of transplanted ELSKs to mouse peripheral blood throughout 24 weeks post-transplant. A) overall CD45.2 (transplanted ELSKs) contribution over time with B) outlining CD45.2 contribution to B cells ($B220^+$), C) to T cells ($CD3e^+$) and D) within the myeloid lineage ($CD11b^+Ly6G^+$). Each mouse is indicated through a different shape as indicated in the key, with green indicating 'good' clones and orange indicating 'bad' clones.

The distinction of 'good' versus 'bad' clones was further supported by assessment of the transplant over time. As demonstrated in Figure 4, in almost all lineages there is a split contribution from the host HSCs and the transplanted HSCs, apart from in the myeloid lineage where it is almost completely either donor or recipient derived. This corroborates with other studies where GM contribution has been suggested as an indicator of a repopulating donor HSC clones (due to the high turnover of cells in the myeloid lineage relative to long-lived lymphoid cells) (154, 160). At the end of the 28-day *in vitro* culture period, a stem and progenitor cell panel (ELSK) analysis took place, using flow cytometry to analyse each clone. ESAM was also added to the panel (to mark for $ESAM^+$ELSKs (EELSK)) to allow us to concomitantly assess EELSK content as it has been shown to also mark for clones further enriched for functional HSCs (154, 161). Using this end of culture data along with the peripheral blood data allows comparison of effectiveness as a clone and %ELSK at transplant (Figure 5). Whereas previous transplantations of 28-day clones had suggested that 20% ELSK

content marks for clones containing HSCs, this analysis suggests that this could be lowered to >5% for *in vitro* expanded markers of ELSKs (154).

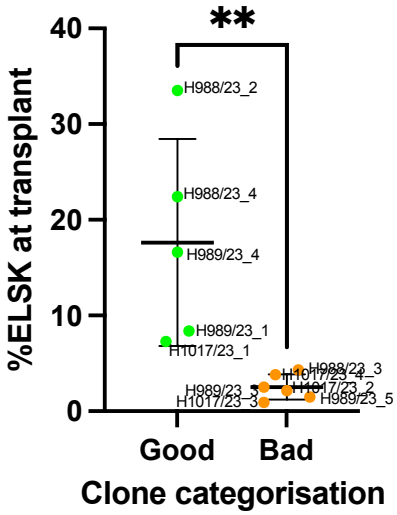


Figure 5: Assessment of the difference between 'good' and 'bad' HSC clones and their %ELSK content on day 28 of culture before transplant into sub-lethally irradiated hosts. Unpaired T-test. **: P ≤ 0.01

Identifying proteins enriched in repopulating HSC clones

Throughout the *in vitro* culture of the HSCs, the media (which contained the molecules secreted by the cells in culture) was removed from the wells at each media change and stored at -20 °C. Using DIA-MS, the secretome of the clones could be analysed and the contents compared against the successful (or not) engraftment of the cells transplanted into the mice. This allows identification of proteins that are enriched in the clones that could repopulate a transplanted host well, and vice versa with clones that did not contain HSCs. The secretome was analysed from media collected on day 14, day 21 and day 26 of culture. However, high data variability was observed, likely due to low overall protein content on day 14, thus analysis was performed on days 21 and 26. After normalisation (both sample loading normalisation, to account for loading errors and to ensure the total abundance of protein between samples is the same, and trimmed mean of M values (TMM) normalisation to equate the medians of the samples) of the day 21 and day 26 data was performed, and outliers were removed, principal component analysis (PCA) was performed of the top 90% variables using Horn and

Elbow tests to show which components are important in the variability between samples (Figure 6). From both tests it was clear that the key PCs involved are from PC1-5 when both days are analysed, and from each day on their own, PC1-3 were sufficient. Through interrogation of PCs 1 and 2 via biplots of both days (21 and 26) data alone and grouped together, the differing classification of clones appear to group together, demonstrating their variation (Figure 7) and verifying the method of classification.

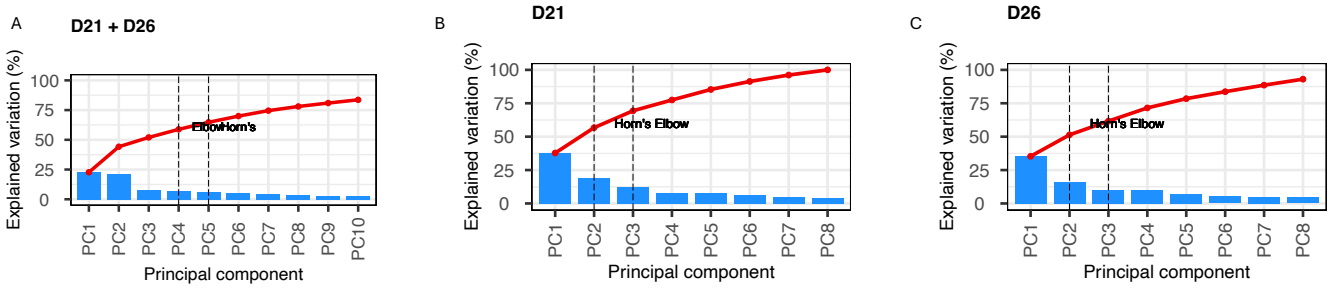


Figure 6: SCREE plot of principal component analyses showing Elbow and Horn's testing determining key principal components. A) PCA plots of day 21 and day 26, B) day 21 alone, C) day 26 alone.

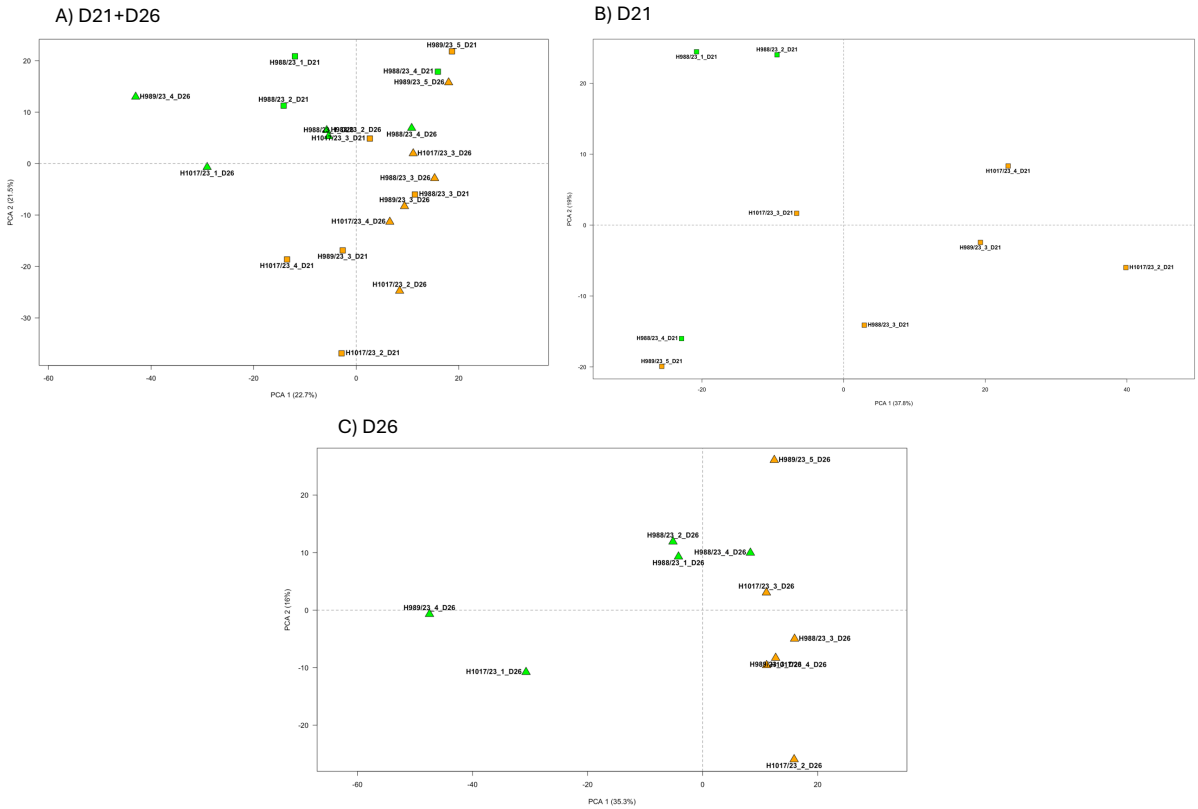
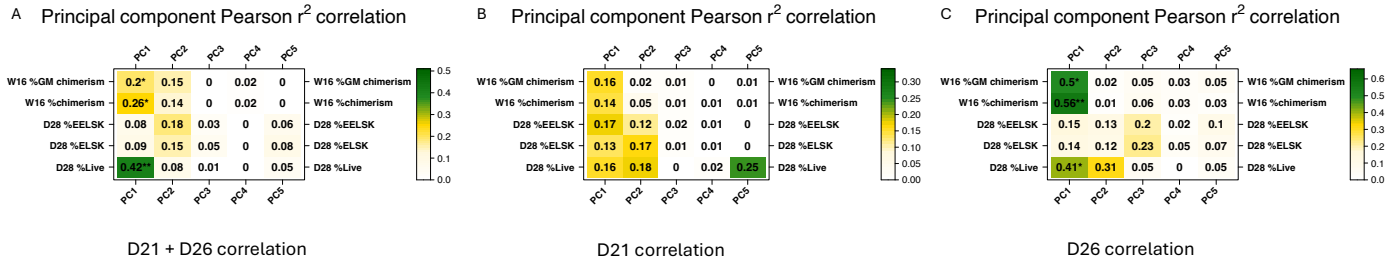


Figure 7: Biplot of principal components 1 and 2, showing grouping by 'good' and 'bad' clones A) Biplots plots of day 21 and day 26, B) day 21 alone, C) day 26 alone. Colour of points demonstrates the classification of clones; green: 'good'; orange: 'bad'. Shapes demonstrate the day of culture the sample was taken; square: day 21; triangle: day 26.

Taking this data forward, the metadata from the transplants (both donor chimerism and %ELSK or %live at transplant) can be used to explain where the variability between the secretome of samples comes from through utilising Pearson correlation tests. These analyses found that most of the variability between samples comes from the overall donor chimerism at week 16, the donor GM chimerism at week 16 and the %live cells at transplant (Figure 7). Furthermore, looking a day 21 and day 26 of culture, it shows that only day 26 has significant variabilities, thus showing that perhaps all 28 days of culture are necessary for the clones to be effective re-populators.

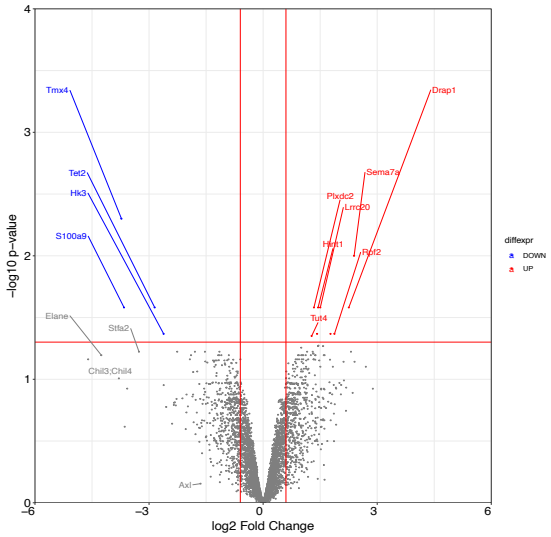


D21 + D26 correlation D21 correlation D26 correlation

Figure 7: Heat map plots indicating which metadata from the transplanted data contributes most to the variability within the secretome DIA-MS data. A) demonstrates the combined secretome data from day 21 and 26 of culture, B) including only the secretome data from day 21 and C) from the secretome data from day 26 only.

Next, with the use of the limma package in RStudio, comparison between the secretome of clones that repopulate blood well and those that do not is possible. In the comparison from these clones, it was found that there were 4 proteins downregulated, and 9 proteins upregulated in the 'good' clones compared to the 'bad' clones at day 26 of culture, and there were no significant differences found at day 21 (Figure 8 and Table 7).

A



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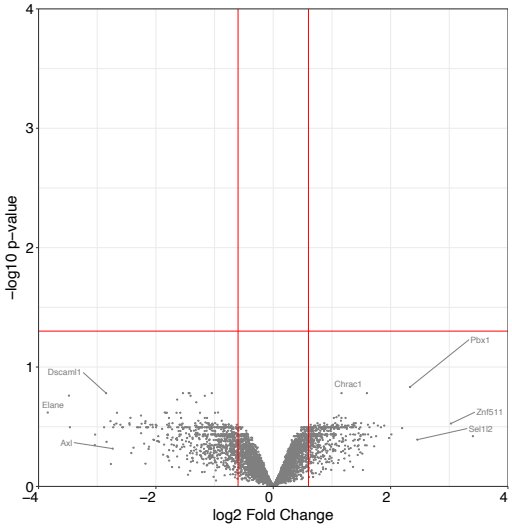

Figure 8: Proteins enriched in repopulating HSC clones: Volcano plot showing proteins upregulated and downregulated in repopulating HSC clones compared to non-repopulating clones. Proteins above threshold include fold change >0.6 and <-0.6 with a p value <0.05 (-log p value >1.30). A) showing differences at Day 26 and B) differences at Day 14.

Table 7: Identified upregulated and downregulated proteins within the secretome of engrafting HSC clones.

Gene ID	Up/Downregulated	Function
Drap1	Up	DR-1 associated protein 1. Encoding a protein that prevents the formation of an active transcription complex, repressing transcription globally (162)
Tut4	Up	Encodes a poly(A) polymerase, Tut4, that functions as a uridyl transferase for pre-microRNA. It is recruited to pre-let 7 by lin28, and its oligouridylation of pre-let 7 prevents its activation (163)
Hspa1	Up	Encoding heat shock 70kDa protein 1, representing two proteins HSPA1a and HSPA1b that are functionally interchangeable but differ by two amino acids, both being necessary for protein refolding generated under stress (164)
Lrrc20	Up	Encoding leucine rich-repeat containing protein 20. A currently poorly characterised protein known to function in most murine tissues (165)
Rpf2	Up	Encoding ribosome production factor 2 homolog. Key protein involved in the assembly of the major ribosomal subunit, 60S (166)
Hint1	Up	Encodes histidine triad nucleotide binding protein 1. Protein involved in many cellular processes, involving, mast cell activation, neuronal signalling regulation and tumour suppression (167)
Cpsf6	Up	Cleavage and polyadenylation specific factor 6. A part of the cleavage factor Im complex that acts as an activator of 3' end cleavage of pre-mRNA and polyadenylation for maturation into mRNAs (168, 169)
Plxdc2	Up	Encoding plexin domain containing protein 2, a type 1 transmembrane protein, vital for controlling proliferation within the brain (170, 171)
Sema 7a	Up	Encodes semaphorin 7A (also known as CD105) and is expressed throughout development and in a range of tissues. Notably, in erythrocytes, T lymphocytes, and within the nervous system (172)
Tet2	Down	Encoding tet (ten-eleven translocation) methylcytosine dioxygenase 2, a protein involved in myeloid differentiation within the haematopoietic system. Believed to act as a tumour suppressor gene through altering DNA methylation by converting 5-methyl cytosine to 5-hydroxymethylcytosine (173, 174)

S100a9	Down	S100 calcium binding protein A9/myeloid-related protein 14. Protein involved in pro-inflammatory signalling and regulating leukocyte migration (175)
Tmx4	Down	Thioredoxin-related transmembrane protein 4. Although not well characterised, the protein is a member of the endoplasmic reticulum family known as the protein disulfide isomerases, responsible for regulating the formation and disassembly of the bonds between cysteine residues (176)
Hk3	Down	Encodes hexokinase 3, in the family of hexokinases responsible for the production of glucose-6-phosphate from glucose in glycolysis. This protein in particular is expressed on haematopoietic cells (primarily myeloid) and is responsible for myeloid cell survival (177)

Using these data, including others from Dr M. Jassinskaja's previous work using this technique to find other genes that have differential expression between 'good' clones and 'bad' clones, as well as other investigations on HSC expansion, the next part of the project focussed on whether the PVA-based media could be improved to enhance HSC expansion.

Investigating the role of oxidative stress on HSC expansion

Previous secretome analyses in the Kent lab found that poly (ADP-ribose) polymerase 1 (Parp1) is enriched in repopulating HSC clones (unpublished data). Therefore, inhibiting Parp1 may impair HSC expansion, and using a Parp1 agonist, may improve expansion. Parp1 is a protein involved in DNA repair and has been shown to have a protective role for murine HSCs *in-vivo* (178, 179). As HSCs are found in the BM, taking them *ex vivo,* to a normoxic environment, makes them more vulnerable to oxidative stress and reactive oxygen species (ROS) compared to the hypoxic environment they are usually found in. Therefore, it was hypothesised that Parp1 aids in healing the damage from these stresses. To test the role of Parp1 in the PVA-based system *ex-vivo*, bulk ESLAMs were treated with Olaparibol (a Parp1 inhibitor) or Salidroside (a Parp1 agonist) for 10 days. After completing a dose analysis with a range of treatments of 0 nM to 100 nM, 20 nM of Olaparibol was selected for further repeats as it was tolerated the best by cells. With this 20 nM treatment of Olaparibol, there was no

significant effect on the viability (Figure 9B, 9D) of the cells or the expansion of HSCs (Figure 9A, 9C).

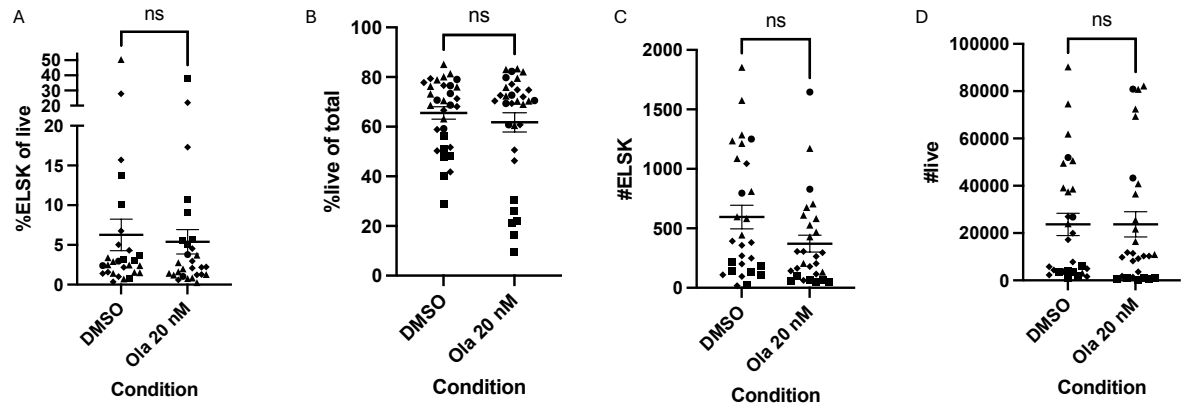

Figure 9: Olaparibol has no effect on cellular proliferation of ESLAMs in F12/PVA media. A) the %ELSK of live in the separate conditions. B) the %live of total per condition. C) # of ELSK per condition. D) the # of live cells per condition. Each shape represents a separate biological repeat. Unpaired T-test, ns: non-significant P>0.05.

When treating the cells with Salidroside, it was a fine line between using a high enough concentration of drug to exhibit an effect and using too much that the cells cannot survive (Figure 10). The treatment of 250 µM significantly increased the raw number of LSKs and live cells in culture, but did not enhance the number of ELSKs, the %ELSK of live, %LSK of live or %live of these populations. Lower concentrations had no significant effect on expansion and higher concentrations impaired the viability of the cells. Furthermore, the treatment of 250 µM was only performed in one biological repeat, thus more repeats would be needed to confirm the results.

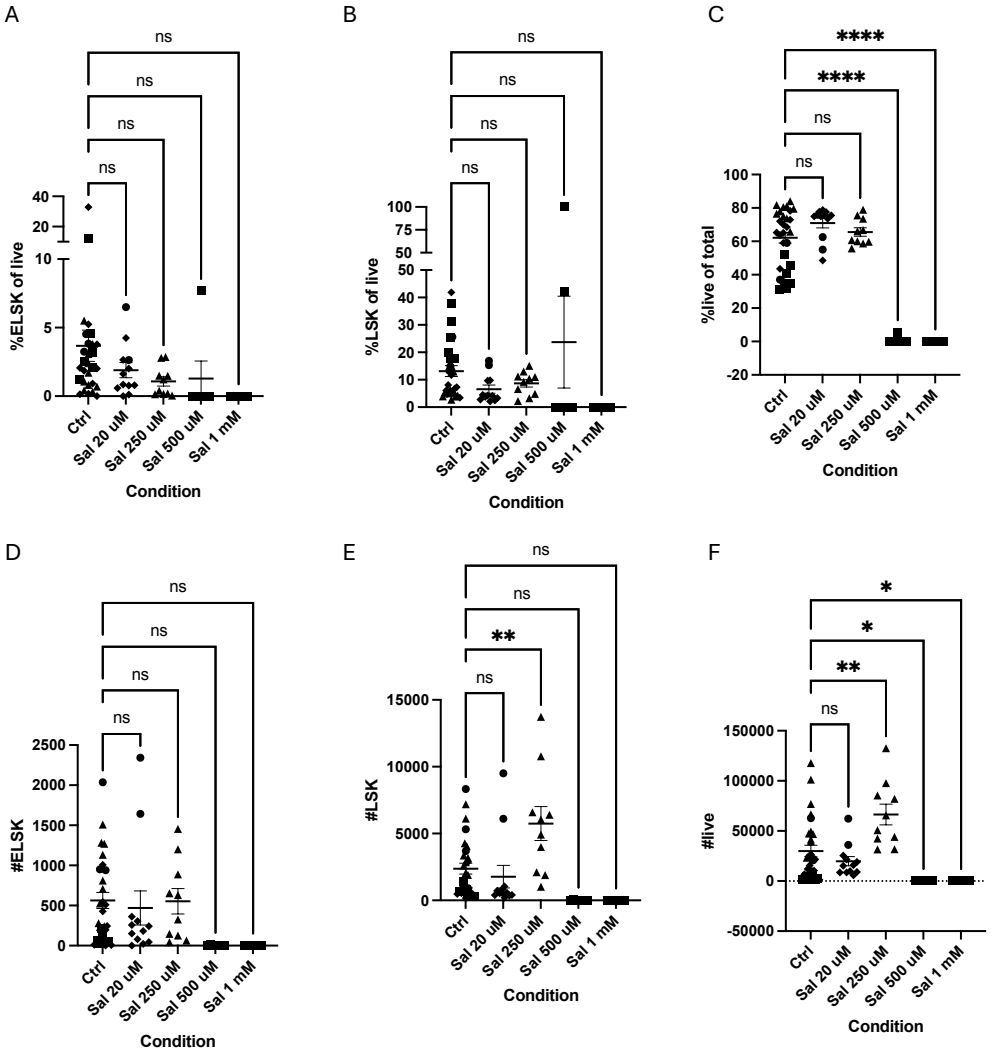

Figure 10: Salidroside may enhance HSC expansion at 250 µM. A) the %ELSK of live of total in the separate conditions. B) the %LSK of live per condition. C) %live of total per condition. D)#ELSKs per condition. E) #LSKs per condition. F) #live cells per condition. Each shape represents a separate biological repeat. Two-way ANOVA performed, ns: P > 0.05, *: ≤ 0.05, P**: P ≤ 0.01, ***: P ≤ 0.001, ****:P ≤ 0.0001

Another way to target oxidative stress is through treatment with antioxidants. N-Acetyl cysteine (NAC) is an antioxidant that targets reactive oxygen species, preventing DNA damage. Therefore, the hypothesis of these experiments was that treatment with NAC would stabilise the cultures and enhance the expansion of HSCs *in vitro* under normoxia. However, this did not appear to be the case, as no significant differences were seen at either dose, 100 µM and 500 µM, in either the number of cells, the %ELSK, or %LSK content (Figure 11), although the 100 µM treatment appeared to enhance viability through the raw number of cells seen. Again,

this was just one biological repeat with 10 technical repeats, thus more repeats are necessary to truly confirm findings.

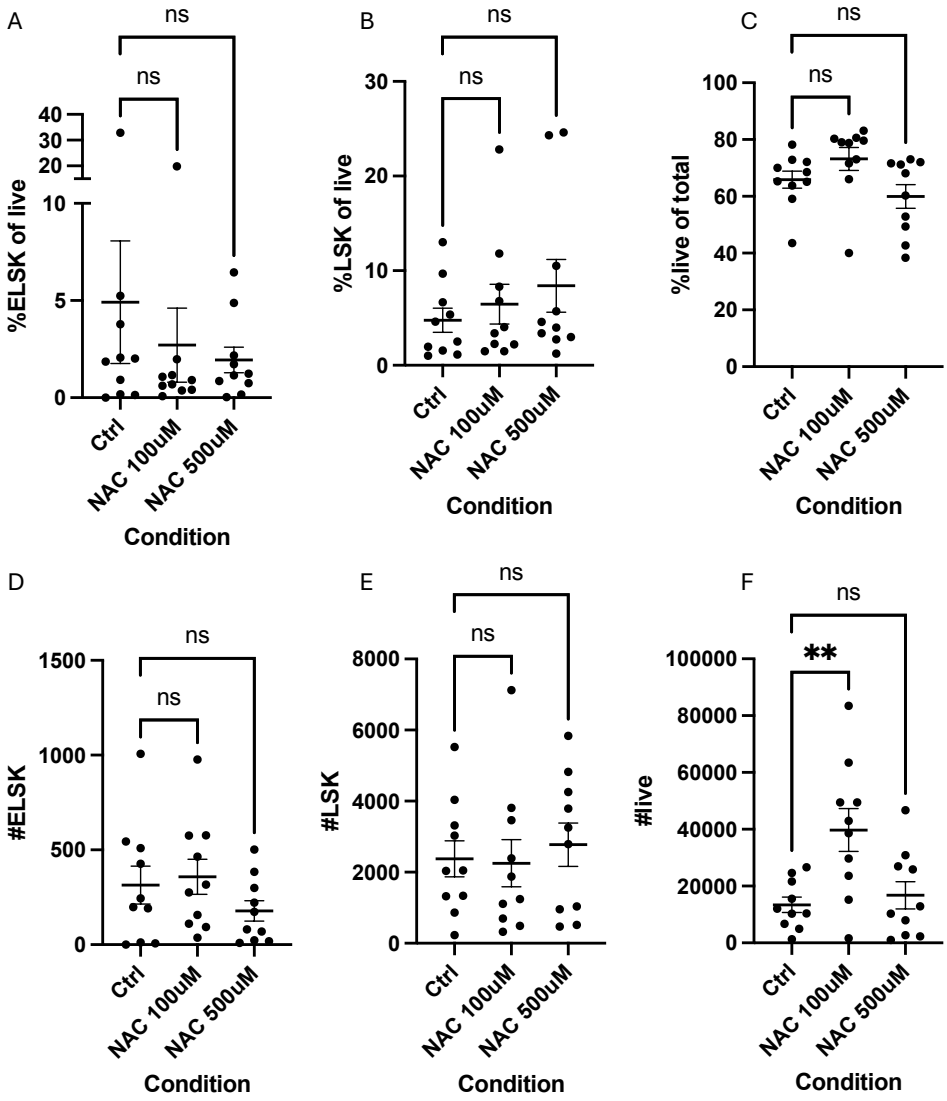

Figure 11: N-Acetyl Cysteine has no effect on bulk HSC cultures. A) %ELSK of live per condition, B) %LSK of live per condition, C) %live of total per condition, D) #ELSK of live per condition. E) #LSK cells per condition. F) #live cells per condition Two-way ANOVA performed, ns: $P > 0.05$, **: $P \leq 0.01$

Finally, a third antioxidant was investigated, resveratrol. As well as having antioxidant properties, resveratrol has anti-tumour properties and protects diabetic retinal cell death in mice (180). In terms of HSC research using resveratrol, there are some conflicting findings within the literature. In an *in vivo* study in mice, repeated feedings of resveratrol (5mg/kg) lead to an increased frequency of LSK cells (181). In human HSCs *in vitro,* one group found

that resveratrol impaired expansion as it increased the rate of cellular apoptosis and increased the erythroid commitment of the HSCs (182), whilst another group found that with 10 µM of resveratrol of human cord blood CD34+ cells expanded the cells significantly *in vitro* (183).

In the experiments performed in the serm-free PVA-based media in this project, two treatment concentrations were chosen, 10 µM and 25 µM. Both concentrations significantly impaired HSC expansion with no samples having a %ELSK of live of over 1% (Figure 12) and the raw number of ELSKs was significantly decreased. Viability was also impaired in the %live of total fraction, however this was not seen in the raw number of cells. Therefore, in this expansion system resveratrol has an inhibitory effect on murine HSC expansion.

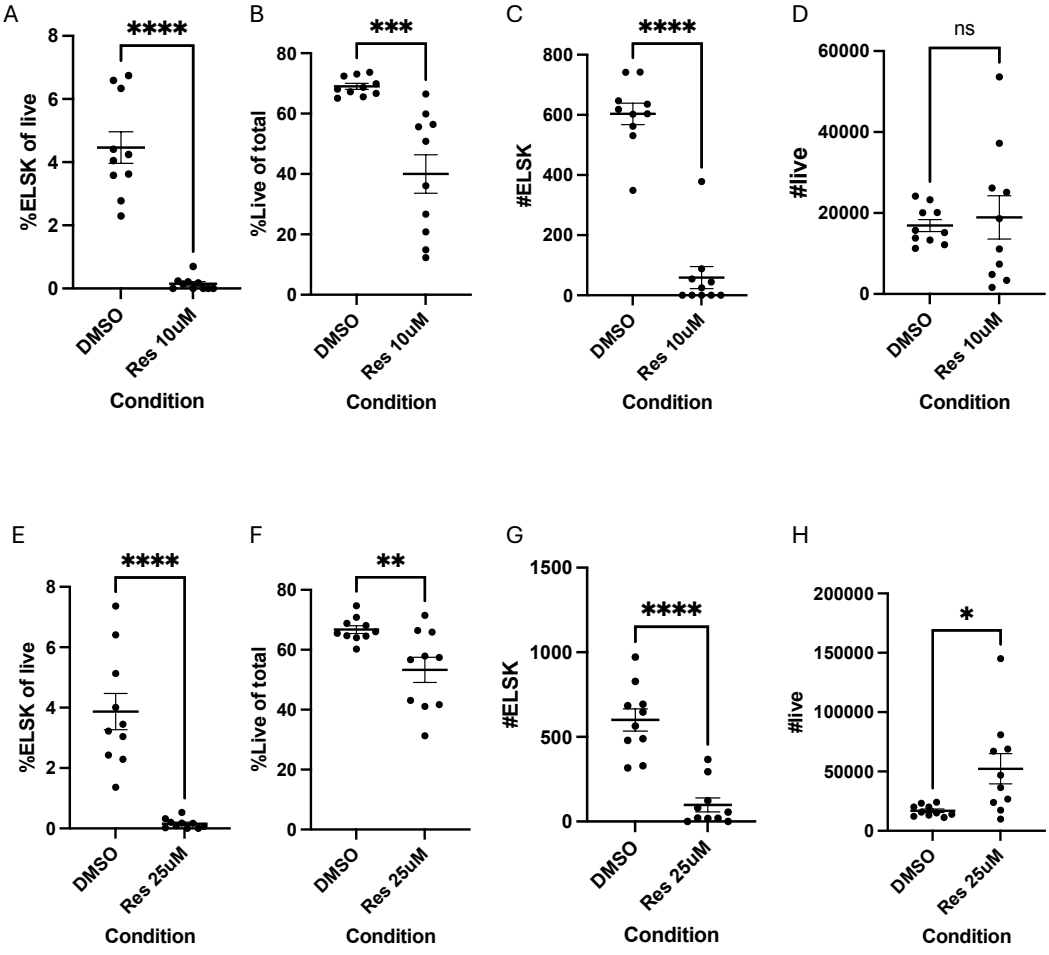

Figure 12: Resveratrol impairs cellular viability of HSCs. A+E) %ELSK of live per condition, B+F) %live of total per condition, C+G) #ELSKs per condition, D+H) #live cells per condition. Unpaired T-test performed, ns: $P > 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****:$P \leq 0.0001$

Exploring the Inhibition of transforming growth factor beta (TGF-β) to improve HSC expansion

TGF-β is the ligand for the TGF-β receptor, which upon binding of the ligand and phosphorylation of the receptor, the Smad family of transcription factors is activated allowing entry into the nucleus resulting in growth inhibition (184). TGF-β is a known inhibitor of HSC expansion *in vitro* although it has not been studied in the F12 PVA-based system before (185, 186). In this case, as expected, TGF-β was a potent inhibitor of HSC expansion and cellular viability, with almost all cells being dead by the end of the 10-day culture (Figure 13D), hence there were very few ELSKs (Figure 13D). It is important to highlight the fact that there is no significant difference between the %ELSK of live of the conditions, but this is because the number of live cells is so small: if there is one ELSK out of three live cells, that well would have a %ELSK of live of 33.3%, thus emphasising the importance of the raw numbers as well as the percentages. Also of note is the variability between experiments, as each shape in the graphs represents a biological repeat. In the #live and #ELSK cells graphs (Figure 13: C and D), the experiment represented with the squares has a larger number of cells than the other two biological repeats shown as circles and triangles. However, when looking at the %ELSK of live and %live of total, the populations between the repeats appears more mixed thus is still relevant.

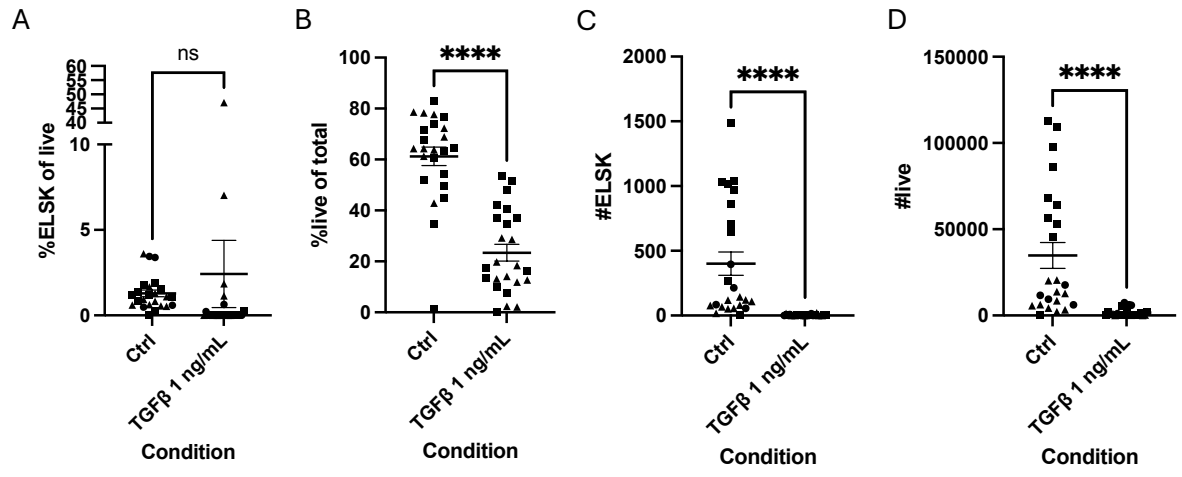

Figure 13: TGFβ inhibits cellular viability and HSC expansion in the F12 PVA system. A) %ELSK of live per condition, B) %live of total per condition, C) #ELSK of live per condition, D) #live cells per condition. Each shape represents a separate biological repeat. Unpaired T-test performed, ns: $P > 0.05$, ****:$P \leq 0.0001$

Therefore, the next hypothesis investigated was that the inhibition of TGFβ signalling may enhance HSC expansion, as suggested by the previous results. SB-431542 (SB) is a small molecule inhibitor of the TGFβ receptors activin receptor-like kinase (ALK) 4, 5 and 7 (187). In this experiment, treatment of 10 μM SB was chosen as higher concentrations were toxic to cells. Unlike the drastic effect the TGFβ had on the HSCs, SBs effect had no significance either on the viability of the cells, ELSK or LSK content, both as a percentage of a population and the raw number of cells (Figure 14). However, it does appear that the #ELSKs in the SB-treated condition is slightly elevated compared to the control. Again, much like the investigations with TGFβ treatment, there is some large variation between the biological controls with the square shapes not woven within the circles and triangles in each condition in the raw number of cells, but as a percentage the populations do appear to weave together.

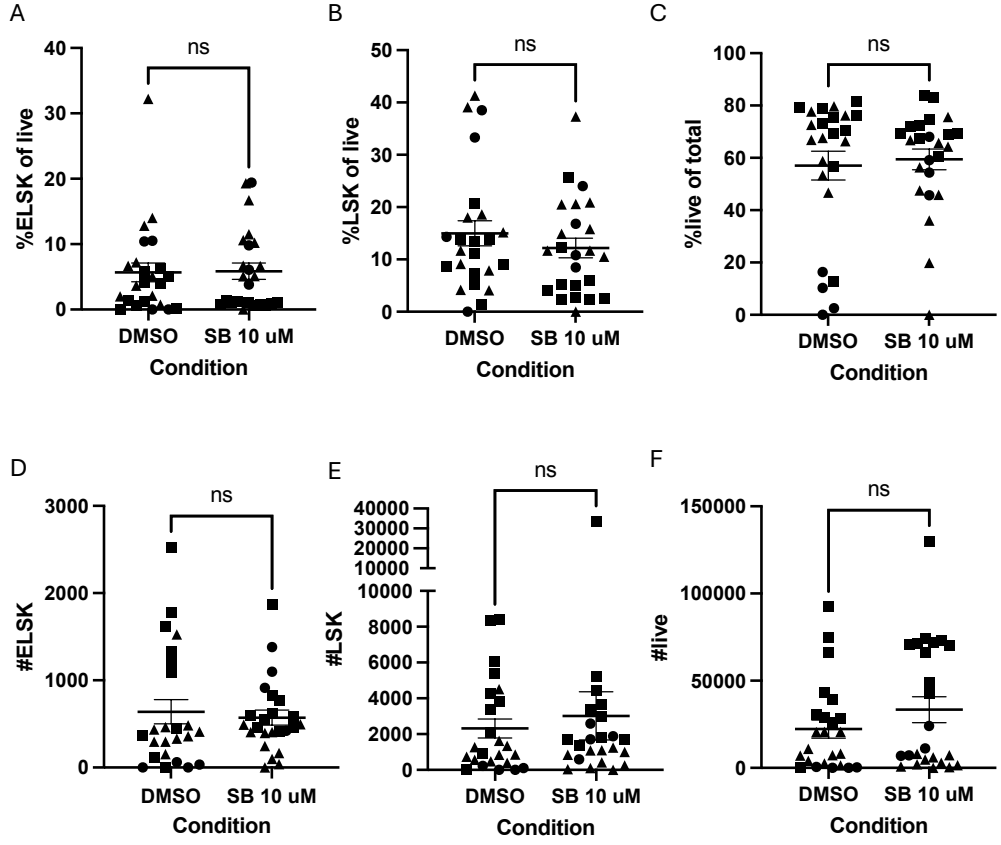

Figure 14: SB-431542 may improve HSC expansion, though not significantly. A) %ELSK of live per condition, B) %LSK of live per condition, C) %live of total per condition, D) #ELSK of live per condition. E) #LSK cells per condition. F) #live cells per condition. Each shape represents a separate biological repeat. Unpaired T-test performed, ns: $P > 0.05$.

Tumour necrosis factor alpha impairs HSC viability in the PVA-based system

Tumour necrosis factor alpha (TNFα) is a pro-inflammatory cytokine produced by macrophages. It has been reported to have a controversial role in HSC regulation (188, 189). In some studies, it has been shown to promote HSC survival and prime for myeloid differentiation (190). Its role in the F12 PVA system has not been disclosed thus it was of interest to be investigated. Two concentrations of TNFα were chosen, 10 ng/mL and 100 ng/mL, as they are within the range of another study done by Yamashita et al which used a range of concentrations from 1 ng/mL up to 10 ug/mL (190). It was found that TNFα impaired the viability of the cells as the %live of total was significantly decreased in treated wells compared to the control. However, the %ELSK and %LSK were not significantly affected from the live population nor were the raw number of LSKs (Figure 15). The raw number of ELSK cells in the wells were significantly decreased in the treated wells. This is not necessarily bad for the cultures as often the ones that have a large cell number, contain fewer progenitors than the wells with more stem cells as these cells do proliferate more slowly. However, it must be noted this experiment was only one biological sample with 10 repeats so would warrant more biological repeats to determine the true effect of TNFα.

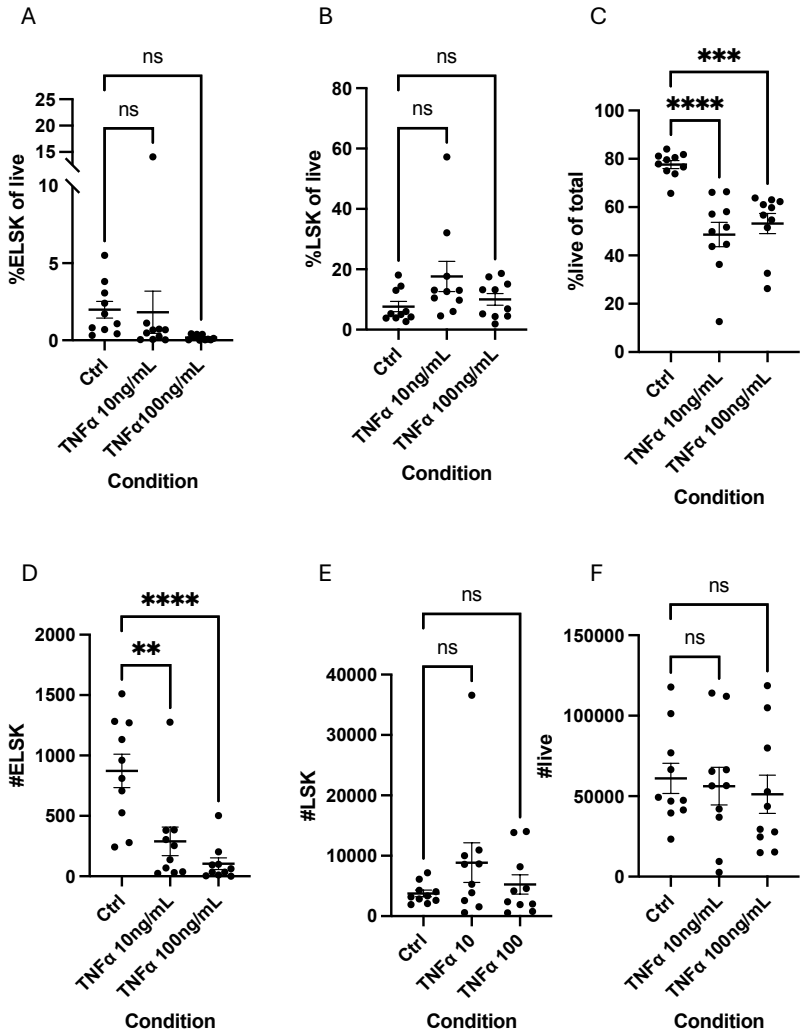

Figure 15: TNFα impairs the viability of ESLAMs in F12/PVA media. A) the %ELSK of live of total in the separate conditions. B) the %LSK of live per condition. C) %live of total per condition. D)#ELSKs per condition. E) #LSKs per condition. F) #live cells per condition. Two-way ANOVA performed, ns: $P > 0.05$, **: $P \leq 0.01$, ***: $P \leq 0.001$, ****:$P \leq 0.0001$

Exploring the use of Angiotensin-II to improve HSC expansion

Through our previous secretome investigations, fat mass and obesity associated (FTO) protein was found to be enriched in repopulating HSC clones. FTO is an mRNA editing protein and is a protein known to contribute to obesity. It has been suggested that the expression of FTO can be upregulated through the hormone, angiotensin-II (AGII) which is generally used to treat hypotension (191, 192). These investigations found that although the treatment with AGII slightly impaired the viability of the cells (Figure 16C, 16F), HSC expansion was improved as the %LSK of live was significantly increased (although %ELSK of live was not (Figure 16A), nor

the raw number of LSKs, and the raw number of ELSKs was significantly decreased) (Figure 16B). Again, there is some large variation between biological repeats as the squares are higher than the circles in the raw number of ELSKs, LSKs, and live cells, most noticeably in the control condition. This variation may be the reason for the more significant differences in the raw number of ELSKs compared to the %ELSK of live population. Due to this, another biological repeat of these conditions would be necessary to verify whether findings are true.

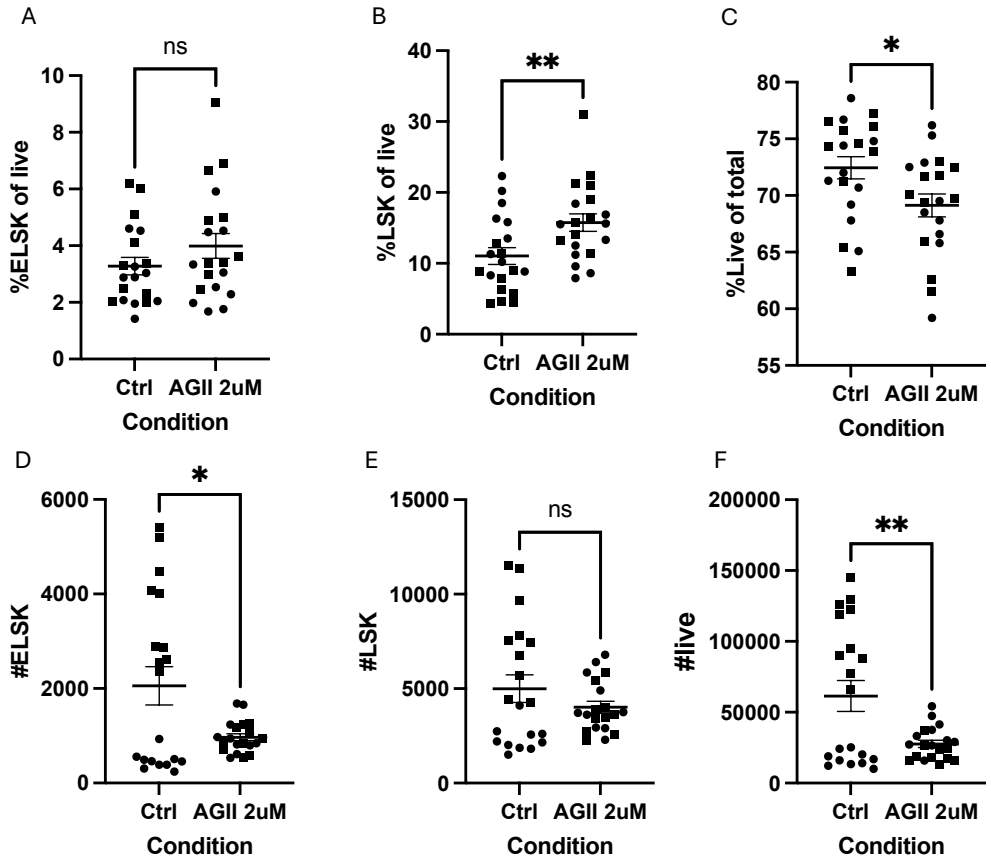

Figure 16: Angiotensin-II significantly improves HSC expansion whilst slightly impairing viability. A) %ELSK of live per condition, B) %LSK of live per condition, C) %live of total per condition, D) #ELSK of live per condition. E) #LSK cells per condition. F) #live cells per condition. Each shape represents a separate biological repeat. Unpaired T-test performed, ns: $P > 0.05$, *: $P \leq 0.05$, **: $P \leq 0.01$.

Discussion and conclusions

This project aimed to utilise the differentially expressed proteins between repopulating clones of *in vitro* expanded murine HSCs and clones that do not repopulate blood well, as well as

utilising previous data, to improve the PVA-based system already used for expanding murine HSCs *ex vivo*. Although the current system is successful and can exhibit up to 899-fold expansion in one month, of those expanded cells, only 10% of those expanded cells are ELSKs (*in vitro* expanded HSCs) and if only 50% of ELSKs are true LT-HSCs, that gives an output of up to 5% of live cells as HSCs (193). Therefore, although the method is perhaps the best available, it is not yet optimal, and any improvements made to maintain the cultures with less heterogeneity is worthwhile. If we could improve this system and translate it to the recently described polymer-based system for human HSCs this could be revolutionary for cell and gene therapy applications that use HSCs as the base cellular product (80).

Novel proteins to improve *in vitro* expansion of HSCs

Throughout the first part of the project, a number of proteins were found to have differential expression in *in vitro* HSC clones that can reconstitute blood well compared to ones that cannot. This analysis of DIA-MS data was possible through use of the limma package on R, which shows significant differences between two different populations. Although this was a detailed analysis to use, in future work with this data, together with further experimental work to identify more differentially expressed proteins, an analysis into the pathways that are involved in repopulating HSC clones could be interesting. Cytoscape is a platform available for generating networks and maps that visualise molecular interactions. For example, this could be used with the top 150 expressed proteins in both clone groups involved to highlight if different pathways are used by repopulating clones, which then could be targeted *in vitro*.

In this investigation, the criteria used to categorise the clones is one used by the lab, although usually in experiments that utilise secondary transplants. Due to time constraints of this project, secondary transplants have not yet been performed. In this case, the classification criteria appear to be an appropriate method of grouping the HSC clones as the PCA plots formed clusters around those of the categorised groups. However, in one mouse in particular, H988/23_1, the HSC clone was classified as 'good' but only had high levels of GM contribution in the first month after transplant and exhibited low levels of B cell contribution after 20 weeks of transplant. To truly know the classification of each clone, these secondary transplants

would need to take place which may change the grouping on some of the clones used in this primary study. Therefore, further experiments would be of use.

Of the analysis that was performed, 9 proteins were found to be upregulated in repopulating clones, and 4 proteins were downregulated. Some of these proteins are known to play roles in HSC regulation already or have functions slightly related to mature blood lineages thus may be interesting to investigate. For example, *Tut4*, one of the proteins found to be upregulated in repopulating clones, is known to interact with lin28b, a protein that is a known foetal HSC marker but interestingly is not an adult HSC marker. It has been reported that although there is this link between tut4 and lin28b, tut4 is also expressed broadly whilst lin28b expression is more limited to undifferentiated stem cells (such us embryonic) that will have a higher power of potency compared to HSCs, thus why we see the upregulated expression of tut4, but not lin28b (163). Within mice, it has been reported that tut4 is highly expressed within the LSK compartment of blood cells which decreases significantly as cells mature into lineage committed (lin^+) cells. Furthermore, deletion of *tut4* (and *tut7*) showed a dramatic decrease of LT-HSCs *in vivo* (194). However, it may be difficult to utilise this finding *in vitro* to try and improve the expansion media; although many tut4 inhibitors are available commercially, there are not any agonists for tut4, although it may still be interesting to investigate whether inhibiting tut4 using TS-002266, for example, impairs HSC expansion (195).

A number of the other proteins upregulated in 'good' HSC clones are known to be involved in other blood cells: Hint1 is involved in mast cell activation; Sema7a is expressed in erythrocytes and T lymphocytes. Although there is currently no link in literature between Hint1 and HSCs, there is between Sema7a and HSCs. In human progenitors, CD34+ cells that expressed high levels of Sema7a have a reduced rate of production of megakaryocytes (196). Furthermore, it has been reported that megakaryocytes can impair HSC self-renewal (154). Interestingly, the humanised version of the PVA-based media used in this project, uses the addition of UM729 (originally UM171) as a megakaryocyte inhibitor to stabilise the cultures, therefore its addition to this murine PVA-based media, would be interesting to investigate (80).

S100a9 was one of the proteins that was found to be downregulated in repopulating HSC clones. In normal haematopoiesis, S100a9 is expressed at low levels in myeloid progenitors which increases as the cells mature into monocytes and granulocytes (197), thus it could be inferred that it is not expressed highly in HSCs as they are at the apex of the haematological

hierarchy. Therefore, it could be interpreted that the non-repopulating clones express higher levels of S100a9 as they are more differentiated at transplant, which is supported by the significance between the difference between %ELSK at transplant between repopulating clones and non-repopulating.

Tet2 is a known protein to be mutated in haematological malignancies, such as myeloproliferative neoplasms, acute myeloid leukaemias, and some lymphoid malignancies, whereby its knockout leads to a myeloid skew with disrupted erythropoiesis (198-201). In *Tet2*$^{-/-}$ mice, as well as those effects seen in mature blood cells, there is also increased expansion of LSKs *in vivo* (202). Therefore, it could be inferred that the more successful HSC clones are better re-populators of blood due to the decreased expression of *Tet2*; the PCA plots from the meta-analysis of the secretome data with the transplant data of the clones, show both the %ELSK of live and the %live of total of the cells was significant in contributing to the variation between 'good' clones and 'bad' clones. If this were to be investigated further, a Tet2 inhibitor could be used, however selective inhibitors have proved elusive. For example, a novel reported small molecule inhibitor, Bobcat 339 (203), was found to have exhibited its inhibitory effect due to calcium contamination during production of the product and when this contamination was removed, there was no inhibition of Tet2 demonstrated (204).

Of the other proteins found to have differential expression profiles between re-populating HSC clones and clones that do not re-populate clones well, no evidence could be found linking them to haematology or haematopoiesis, thus this investigation may have opened new potential roles for these proteins. Another protein upregulated in repopulating clones is Drap1. No literature could be found disclosing a role of the protein in HSC function and maintenance, thus it may be intriguing to investigate further. Drap1 is a known transcriptional repressor and is significantly enriched in re-populating HSC clones. It could be inferred that these cells have more stem-ness and thus will not have genes transcribed for proteins that commit the cells to maturation. It has been reported that HSCs have fewer proteins transcribed than their more mature counterparts (92), and in the secretome data obtained in this project, the 'good' clones had fewer proteins found in the secretome data compared to the non-repopulating clones (although not significantly, not shown), further corroborating this point.

It should be noted that many of the proteins identified in the secretome of 'good' and 'bad' clones in this study, including the ones investigated from a previous study in the lab, are not proteins that are known to be secreted. Therefore, it is unclear how these proteins have been identified in the condition media where no cells should be included. It may be due to the fact HSCs are usually cells in suspension and adhering them using fibronectin may result in some cells not being adhered well resulting in their removal during the media changes where the condition media for MS analysis was collected. On the other hand, these intracellular proteins may be present in samples due to cell lysis as a result of cell death. Although this is may mean some of the data does not directly show proteins from the secretome, the abundance of these proteins may still be relevant to be researched further. Furthermore, although no direct comparison to the data previously generated in the lab has been performed, it is of note that this MS study did not outline Parp1 or FTO, two proteins that were enriched in good clones in a previous study, were not significantly enriched in this study. It would be worthwhile to perform more repeats and perform analysis of the combined data to see which proteins are up/down regulated in different biological/technical repeats to allow for a higher level of reliability of the data. This study found genes similar to *FTO*, involved in mRNA editing and transcription/translation regulation e.g. *Drap1*, *Tut4* and *Cpsf6* thus is still relevant.

Improving current HSC expansion techniques is difficult

The second part of the project involved using our labs previous secretome analysis and previous literature on proteins that effect HSCs to see if the current media for expanding HSCs *in vitro* could be improved. A range of molecules were examined on their effect on bulk ESLAM cultures over a 10-day period.

It has been well reported that HSCs are vulnerable to DNA damage *ex vivo* through the movement of the cells from a hypoxic environment to a normoxic one. Therefore, attempting to prevent oxidative stress and DNA damage was an attractive avenue to investigate in the serum-free expansion system to see if HSC expansion could be improved. In this case, salidroside, an agonist to Parp1, a DNA repair protein, was investigated alongside olaparibol, a Parp1 inhibitor. Olaparibol had no effect on HSC expansive or cell number, whilst salidroside had the potential to improve expansion at 250 μM whilst not impairing viability, although

more repeats are required to truly determine the effect. For more detailed research in the future, it would be useful to directly assess whether Salidroside or Olaparibol were sufficient to modulate Parp1 activity. This could be achieved through a fluorometric assay that measures the directly the activity of Parp1 through the production of nicotinamide.

NAC was an antioxidant that was investigated which functions through targeting ROS. Unfortunately, this protein had no significant effect on the ESLAM cultures in terms of %ELSK or %LSK of live or the raw number of ELSKs or LSKs from one biological repeat. It is a well believed that NAC is an effective ROS scavenger, especially to superoxide and peroxides (205). However, the reaction rates are particularly slow by NAC, compared to other ROS scavengers such as thiol peroxidases (up to 10^{8}-fold slower to reduce peroxide), therefore it may not have exerted any effect on the ESLAM cultures due to this (205, 206).

Thus, another antioxidant was investigated, resveratrol. As previously mentioned, there have been conflicting studies of the result of resveratrol on HSC expansion (181-183). Although, in this case, the protein impaired viability and as a result, HSC expansion.

To continue the research into the effect of hypoxia and combatting that effect of growing HSCs *in vitro*, more detailed research could certainly be achieved with more time. Of the nucleic acids, guanine is the most vulnerable to ROS of becoming Oxo-Guanine (8-Oxo-dG, in DNA) (207), and its abundance is able to be measured by ELISA or liquid chromatography tandem mass spectroscopy (208).The compounds investigated in this study related to oxidative stress (e.g. NAC, Salidroside etc) could be used in the HSC cultures and the amount of 8-Oxo-dG could be measured, to determine the level of rescue demonstrated by the compounds, and whether they could enhance *in vitro* HSC cultures to increase the chance of having more 'good' clones found in culture. Furthermore, hypoxic chambers could be utilised for true negative/positive controls, as well as compounds such as hydrogen peroxide or superoxides that are known to induce 8-Oxo-dG damage.

Next, TGF-β was investigated as it is a known inhibitor of HSC expansion but had not been explored in this expansion system before. Expectedly, TGF-β impaired both cellular viability and HSC expansion completely. To try and target TGF-β signalling to attempt to enhance expansion, a TGF-β receptor inhibitor, SB-431542, was used. This small molecule had no significant effect on HSC expansion although the HSC populations did seem to be pushed up slightly by SB compared to the DMSO control. From reviewing the literature, it appears that

the TGF-β receptors inhibited by SB are not highly expressed in the HSC pool. In *in vivo* conditions, HSCs expressing ALK5 total ~12% of the stem cell population whilst after 24 hr growth in vitro, HSCs expressing ALK5 make up ~44% of the stem cell pool (209). This may explain why the effects seen with the SB treatment are not significant. Along with the further research into the suitability of using Olaparibol or Salidroside, the same could be researched into SBs capability to modulate TGF-β activity. This could be measured through quantitative PCR or Western blot investigations into the Smad family of proteins that are downstream of TGF-β.

TNFα was another protein examined as it has been shown to have conflicting effects on HSCs in literature (188, 189). In the PVA-based system used in our experiments, TNFα impaired cellular viability but did not affect the HSC populations (LSK) both in raw number and as a percentage of the live gate. As just one biological repeat was performed, it would benefit from more to determine the true effect. Often, as previously mentioned, an impairment of viability is not necessarily a bad thing, as the wells that have a large cell number, contain fewer progenitors, and wells with a lower cell number have more stem cells with less differentiation as stem cells proliferate at a slower rate. However, with the impairment of the raw number of ELSKs it may be of use to take this experiment into a single cell 28-day culture to see if with longer periods of time a starker difference is seen between the controls and the TNFα treated samples.

Finally, AG-II was used to upregulate FTO expression, after FTO was found to be upregulated in 'good' HSC clones in M. Jassinskaja's previous secretome work. In our experiments, AG-II was found to upregulate HSC expansion whilst impairing viability slightly. This is similar to what has been seen in human HSCs with AG-II upregulating FTO which enhanced homing and engraftment of HSCs (210). As a potential enhancer of HSC expansion, it would be useful to take this study from short-term bulk HSC cultures to single-cell 28-day cultures to determine the effect on single clones. However, dosage may have to be adjusted slightly as the dose may be too high for a single HSC or perhaps delaying the first dose for after the first media change.

This project has discovered potential additions to improve the current gold-standard for expanding murine HSCs *in vitro*. Angiotensin-II is the most successful attempt so far, but the secretome analysis has highlighted other pathways and proteins that could be targeted in future experiments, some of which have been known to affect HSCs and some that have had

no link to haematology before. These and others should be investigated in the future for their role in aiding (or inhibiting) HSC expansion.

Recently, a novel serum-free, chemically defined media was published by Sakurai et al. for human HSC expansion, which was based on the murine media used in these experiments (80). Compared to the standard StemSpan media, there was an increased frequency of LT-HSCs after a 10-day culture. The media itself, referred to 3a in the paper, is serum-free (much like the murine system) and contains few cytokines as they contribute to variability between batches, and promote the HSCs to differentiate. The lack of serum is key for human expansion studies as it would allow the expanded HSCs to be used for transplants as no animal products would be used in the process of expansion. HSC transplants are a key therapy in many blood malignancies, but often there are not enough cells for reconstitution, in cord blood for example. Thus, expanding the cells *ex vivo,* without serum in the media, would allow the cells to be transplanted, demonstrating the need for clinical-based practices in labs. The 3a media itself replaces serum albumin with Soluplus (PCL-PVAc-PEG) which is a polymer much like the PVA used in the media in this project. Furthermore, TPO is replaced by the small molecule butyzamide, and SCF is replaced with 740-YP. The same lab that developed the PVA-based media for expanding murine HSCs also explored the use of Soluplus as a replacement for PVA. They found that Soluplus supported murine HSC expansion up to 18,000 fold, demonstrating that refinement to the serum—free system is possible (159, 211). The clear similarities between optimal methods for expanding human and murine HSCs show promise that the proteins discovered here, could be translatable to humans. Furthermore, improved *in vitro* techniques for expanding HSCs will remove the need for the vast number (hundreds) of mice used for proteomics studies (112) allowing research to be undertaken quicker and with less need for ethical considerations and costs necessary with large animal studies.

To conclude, HSC research is currently a very exciting area of study with recent advancements in expanding both murine and human HSCs being discovered and explored. If these methods were pushed further to become more successful through studies like the one performed under this project, new avenues could be opened. Firstly, in terms of in research moving away from animal studies, and secondly in terms of clinical applications for gene therapies and HSC transplants becoming more widely available for patients.

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