Identification of a reporter strategy for functional haematopoietic stem cells during *in vitro* expansion

Fiona Bain

MSc by Research

University of York

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Abstract

Haematopoietic stem cells (HSCs) are the peak of the haematopoietic hierarchy and can both self-renew and differentiate into all mature blood cells. HSC transplantation has long been used clinically for cancer treatment, gene therapy, and, increasingly, autoimmune conditions (multiple sclerosis) and viruses (HIV). Despite decades of research, efficient HSC expansion *in vitro* has been extremely difficult to achieve. A recent breakthrough in mouse HSC expansion (allowing up to 899-fold increases in HSC numbers over 28 days) is hugely significant, yet HSCs remain the minority of cells in the culture, and significant HSC heterogeneity exists – problematic when initiating cultures with single cells. Optimising the expansion protocol to increase HSC self-renewal divisions and HSC content of the cultures will allow molecular and cellular analysis of these expanded cells. Further understanding these cells will ideally enable application of refined expansion protocols to human HSCs. The current gold-standard for HSC identification is transplantation yet this is expensive and time-intensive. Replacing this with a universal HSC reporter strategy, able to identify HSCs to the same level of efficiency and removing the need for reporter mice for HSC identification would be extremely beneficial. This would open up reporter strategies to alternative mouse strains, including disease models, and also to human HSCs. In this thesis, I found that ESAM can replace the *Fgd5*$^{+/ZsGreen}$ reporter mouse as an efficient reporter for functional HSCs in culture. We used 28 day expansion screens to test candidate molecules able to predict HSC content of clones and identified the surface marker Siglec F as a candidate for distinguishing between the LT-HSC and progenitor populations. Finally, we tested the addition of novel compounds to expansion cultures to improve HSC self-renewal divisions, and found FSTL1 as a molecule that potentially promotes more HSCs to successfully expand in culture.

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Authors declaration:

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

1.0 **Introduction**

1.1 Haematopoietic stem cells and haematopoiesis

The blood system, consisting of a liquid phase known as the plasma, and a cellular solid phase (1), is vital for survival - providing the means of transport of nutrients to cells and waste away from cells, as well as containing the cells of the immune system. The process of forming the cellular component of the blood system is known as haematopoiesis and is a hugely productive system, producing 4-5x10^{11} haematopoietic cells a day in the average adult (2). In order to maintain blood cell homeostasis and maintain the correct mature cell populations despite their constant turnover, this must be not only a continuous process, but also a tightly regulated and adaptable system.

Broadly speaking, mature blood cells can be split into myeloid and lymphoid cell lineages. The myeloid lineage contains the megakaryocytes, erythrocytes, granulocytes and macrophages, whilst the lymphoid lineage contains T, B, and innate lymphoid cells (2, 3). The major characteristics of these mature cell populations are listed in Table 1 and are essential for normal functioning of the blood system.

Cell Type		Function	Size	Life span	Number at any given time	References
Erythrocytes		- Transport oxygen from the lungs to tissues as a source for ATP synthesis - Collection of waste carbon dioxide from cells, to be removed via the lungs	6.2-8 μm	120 days	Male: 4.3-6 million/mm^3 Female: 3.5-5.5 million/mm^3	4
Leukocytes	Neutrophils	- 62% of leukocytes Kill a wide range of pathogens via phagocytosis or super oxide / cytotoxic release for more widespread targeting	10-12 μm	6 hours - 3 days	2000-7500/ mm^3	5 6
	Eosinophils	-Kill parasites via toxin release from granules -Involved in allergic reactions -Maintain the balance between T-helper and T-regulatory activity in the lungs and the intestines	10-12 μm	8-12 days	40-400/ mm^3	7 8
	Basophils	-Tissue resident cells expressing the surface high-affinity IgE receptor, and are the primary source of IL4. -When activated, release histamine to attract additional immune cells to injured site. -Release heparin and anticoagulants to prevent clotting and improve migratory ability of leukocytes to injured area. -Modulate the allergic response.	12-15 μm	1 – 2 days	0-100 / mm^3	9 10 11

	Monocytes	-Phagocytose and present antigens on their surface to activate other immune cells. -Release chemokines. -Once activated by danger signals recognised by pattern recognition receptors, they proliferate and enter tissues where they differentiate into macrophages to destroy old/damaged cells.	13-30 μm	2 hours – 3 days	200-800/ mm^3	12 13
Megakaryocyte		-Produce platelets and release them into circulation.	50-100 μm	5 days	20,000/mm^3	14 15
Platelets		-Essential in blood clotting (release of procoagulants and vasoconstrictors). -Chemoattract neutrophils and monocytes to damaged areas -Assist in removal of blood clots when no longer needed -Release of growth factors to maintain endothelium.	Maximum 2-3 μm	3-7 days	150,000-400,000/ μL of blood	16 17
Dendritic Cells		-Antigen presenting cells. Key in naïve T cell activation and maintaining immune tolerance.	15-20 μm long, 0.1-0.3 μm wide	Few days - weeks	13-37/ μL of blood	18 19
Lymphocytes		-T cells: cell mediated immunity -B cells: humoral immunity via antibody production -Able to produce memory cells to respond more rapidly upon re-exposure to an antigen. -Antigen presenting to activate other immune cells.	7-8 μm	Memory cells: years Everything else: weeks	1300-4000/ mm^3	20 21 22 23

Table 1: Key haematopoietic cell types, their functions and key characteristics.

Rare haematopoietic stem cells (HSCs), at the peak of the haematopoietic hierarchy, are the multipotent population responsible for the generation of all mature blood cells. Their ability to perform symmetric self-renewal divisions to expand their pool, whilst also being able to asymmetrically divide into more mature cells, is a trait unique to stem cells (1,3). The first wave of primitive haematopoiesis occurs on day 7 of mouse development (Day 17 in humans) in the yolk sac blood islands of the embryo (24). However, these primitive cells do not have indefinite self-renewal capacity and are unable to reconstitute the blood system following transplantation. Definitive haematopoiesis in the aorta gonad mesonephros (AGM) and placenta follows on day 8 (Day 21 in humans) and results in the production of the first HSCs with persistent self-renewal which are able to be transplanted (24). The foetal liver begins to be colonised with HSCs from day 9, these arriving from the AGM and yolk sac (24). By Day 12, the foetal liver is the primary site of definitive haematopoiesis, only changed just before birth where this switches to the bone marrow (BM). Here, the HSC population is retained to ensure life-long mature cell production (1,24), with a key developmental switch occurring between 3 and 4 weeks of age where HSCs go from being actively cycling to quiescent (25, 26)

In the adult BM, the extent of self-renewal can vary within the HSC population (2), which is what separates long-term HSCs (LT-HSCs) which have life-long self-renewal capacity, from short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs) (3). This has been confirmed in LT-HSCs from their ability to contribute >1% to both mature myeloid and lymphoid cells at 4-6 months post-transplant). ST-HSCs and MPPs typically have limited self-renewal and can therefore only contribute to mature cell production following transplantation for around 8 weeks (2, 27), without producing daughter cells that can repopulate in secondary transplantation recipients. Therefore, serial transplantation experiments in irradiated hosts are currently the gold-standard for functional LT- HSC validation. They can prove that HSC self-renewal divisions, producing cells equally undifferentiated and themselves able to self-renew, did take place. HSC numbers themselves can be influenced by both intrinsic and extrinsic signals – if the viability, stem-cell functionality or proliferation is altered, the number of HSCs present in the BM will change, meaning that this balance and the signals HSCs receive must be tightly controlled (27, 28) (Figure 1).

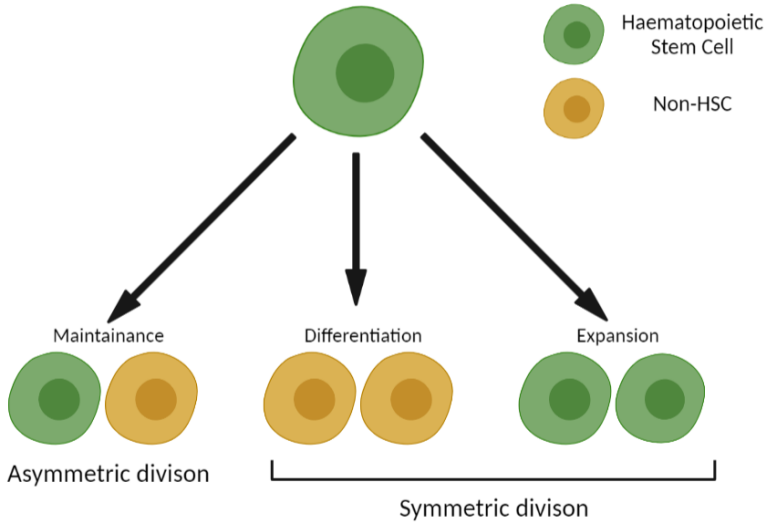

Figure 1: The balance between symmetric and asymmetric divisions in HSCs is tightly controlled and relates directly to their self-renewal and repopulation capacity

Vast proliferation is required to produce the numbers of mature blood cells required each day, and this typically occurs at the progenitor cell level, rather than at the HSC level. Despite this, progenitors have limited self-renewal ability and without replenishment from HSCs would exhaust themselves in a few weeks in transplantation assays (27). However, of interest, recent lineage tracing studies have demonstrated that transplantation assay have drastically under-estimated the durability of contribution from progenitor cells, with many non-HSCs contributing in a sustained manner (28, 29) What is not disputed is that LT-HSCs predominantly exist in a dormant state of quiescence (known as the G_0 phase of the cell cycle) which has been speculated to protect and preserve the integrity of their genetic material from genotoxic insults or the mutational stress of cellular division. This, in turn, increases their longevity, protecting from oncogenic mutations and leukemogenesis, and allowing lifelong production of blood (30, 31). One of the key contributors to mutational stress has been suggested to derive from quiescence exit which can be a direct cause of DNA damage. (32, 33).

Haematopoiesis has historically been thought to be a step-wise development from HSC to mature cell with discrete cellular compartments and associated functions. However, it has been revealed more recently to be a gradual progression of differentiation, which takes places over many cell divisions. This is matched by incremental changes in gene expression as lineage choices are made, and the differentiation potential of the cells is restricted (32) (Figure 2). Supporting this are ATAC-Seq experiments which show that slight changes in chromatin states can define subpopulations. (34, 35, 36).

Interestingly, as well as the traditional differentiation pathways, there also appear to be more direct routes from HSC to mature cell. In particular, some megakaryocytes are thought to arise directly from specific HSCs, bypassing the progenitor stage and associated lineage checkpoints (36, 37, 38, 39, 41, 41)

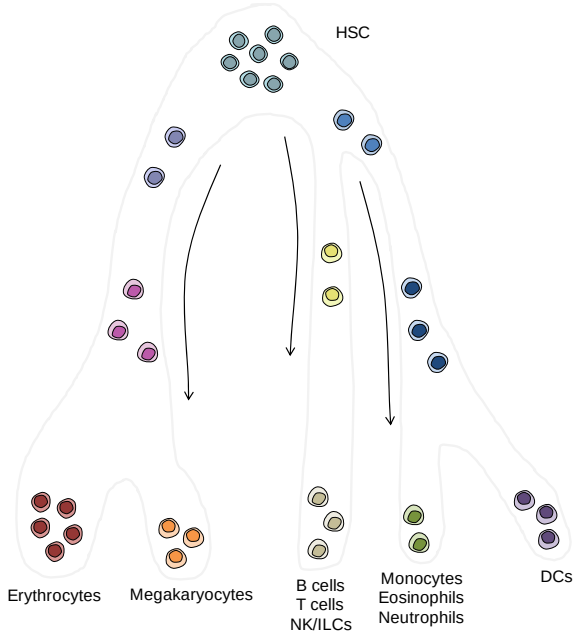

Figure 2: Haematopoietic differentiation is a continuum from immature LT-HSC at the apex to the terminal mature blood cells.

To study the haematopoietic hierarchy and distinguish between cell populations at different levels of differentiation, cell surface marker staining is often used (1, 34). Staining with fluorescent antibodies allows us to use multi-parameter Fluorescence Activated Cell Sorting (FACS) for prospectively identifying and isolating live cell populations (Table 2). This technique leaves the sorted cells alive and healthy, enabling them to be put into culture conditions and maintained *in vitro* or transplanted into recipient animals for functional assay *in vivo*.

However, this approach comes with a few key limitations, including the significant expression differences of these markers in cellular states that are different to freshly isolated quiescent HSCs (3). This limits many isolation techniques and marker combinations to freshly isolated cells, and not cells which have been in culture for example, thereby precluding the isolation of HSCs. Additionally, as of now, no strategy exists that can isolate HSCs to 100% purity, and most require 4-6 colours at least, making imaging studies challenging.

Fluorescent reporter mice such as the *Abcg2, Fgd5, Vwf, Ctnnal1, Gprc5c* and *Hoxb5* have been developed to aid HSC isolation, with these genes being expressed predominantly within the phenotypically primitive HSCs (40, 41, 43, 44). In particular, *Ctnnal1*, *Fgd5*, and *Hoxb5* enrich for the complete LT-HSC population, as opposed to a subset of HSCs as is the case for Gprc5c and *Vwf* for

example (40, 43). My project therefore utilised one of these models, the *Fgd5* ZsGreen mouse, due to its previous use on several phenotypically defined HSC populations, and the validation by several independent transplantation studies (45). FGD5 protein contains a Guanosine exchange factor, likely acting via CDC42 in the VEGF pathway (45). The knock-in *Fgd5* receptor was only expressed in 0.06-0.1% of total live cells in the BM, and showed labelling of all the phenotypic HSC population, with all HSC functional activity also limited to the BM *Fgd5*+ cells.

Cell Type	Surface Markers
Long term HSCs	Sca1^{+} CD150^{+} CD117^{+} CD48^{-} CD34^{-} CD49b^{low} CD135^{-} CD11b^{-} Gr1^{-}
Short term HSCs	Sca1^{+} CD150^{-} CD117^{+} CD48^{-} CD34^{+} CD135^{-} CD11b^{-} Gr1^{-}
Multipotent Progenitors (MPP)	Sca1^{+} CD117^{+} CD48^{-} CD34^{+} CD135$^{+/-}$ CD11b^{-} Gr1^{-}
Common Myeloid Progenitor	Sca-1^{-} CD117^{+} CD16/32^{-} CD34^{+} CD41^{high}
Common Lymphoid Progenitor	Sca-1^{+} CD117^{+} CD93^{+} CD127^{+} CD135^{+}
Granulocyte-Monocyte Progenitor	Sca-1^{-} CD117^{+} CD16/32^{+} CD34^{+} CD64^{+}
Megakaryocyte-Erythroid Progenitor	Sca-1^{-} CD117^{+} CD16/32^{-}CD34^{-} CD64^{-} CD127^{-}

Table 2: Surface markers used to identify various haematopoietic populations. Staining for these markers with fluorescent antibodies allow isolation of these populations via fluorescence activated cell sorting, or analysis via flow cytometry.

The haematopoietic system is extremely adaptable and can rapidly respond and produce the required mature cells needed to combat the stresses it may experience (46). These include, but are not limited to infection, chronic blood loss, malignant haematopoiesis, and chemotherapy. (32, 33). Previous studies of single cell HSC transplants into irradiated mice have highlighted an HSC's individual ability to reconstitute the entire system (1, 34, 48). This in itself is an example of the great proliferative and differentiation responses that HSCs can enact as a result of environmental stress. Despite their huge capacity to expand and proliferate, in order to maintain healthy haematopoiesis throughout life, the balance between self-renewal and differentiation needs to be tightly controlled. HSC self-renewal and differentiation is under the influence of layers of paracrine and autocrine regulation, influenced in turn by many cytokines, chemokines and growth factors. Excessive self-renewal accompanied by a block in differentiation can cause leukaemias, whilst excessive differentiation will lead to HSC exhaustion (49, 50, 51) (Figure 3).

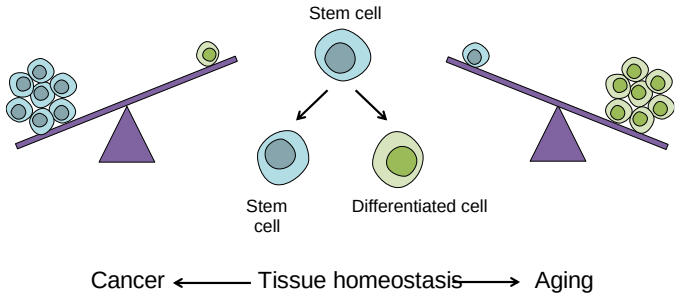

Figure 3: The balance between maintenance of the HSC pool and differentiation should be constantly kept at a homeostatic equilibrium. Excess differentiation will exhaust the stem cell pool, whilst excess self-renewal, usually coupled with a block of differentiation can cause haematopoietic malignancies and leukaemias.

1.2 The stem cell niche

In adults, the primary niche for HSCs is the BM, where the cellular microenvironment is primed to maintain the HSC pool and regulate their fate. The local niche environment also protects the HSCs from cytotoxic and mechanical damage (52, 53, 54). Cytokines, growth factors, chemokines and mechanical stimuli required to regulate HSC activity are produced not just by the HSCs themselves, but also by the network of neighbouring BM niche stromal cells including fibroblasts, macrophages and endothelial cells which are well known to be closely associated to the HSCs. (55, 56, 57).

Even within the BM itself, the interaction of the bone and the vascular system creates several subniches, each with their own characteristics and key in maintaining subpopulations of both quiescent and proliferating HSCs (58, 59)

The first, the endosteal, is closely associated to osteoblasts who exert their influence over HSC behaviour, in particular in maintaining quiescence, via secretion of TPO, SCF, angioprotein-1, C-X-C motif ligand 12 (CXCL12), and notch signalling (58, 60, 61). Alternative niches include the perivascular, located near the arterioles, and the perisinusoidal where the HSCs live in close quarters with endothelial cells covering the blood vessels. Both these niches are more associated with active HSCs – proliferating and producing more mature cells (58).

In the perisinusoidal niche, the endothelial cells influence the self-renewal and proliferation of the HSCs via cell-cell physical contacts (typically via E-selectin). Also present here are many mesenchymal stromal cells which help maintain the HSC population via paracrine and juxtracrine signalling (59).

Although typically residing long-term in the BM, HSCs can be mobilised to enter the peripheral blood. Furthermore, HSCs are also capable of homing back to the BM and engrafting. These processes rely on a complex interplay of inter- and intra- cellular communication including cytokines, chemokines and adhesion molecules (58). Of note, the SDF-1 and CXCL12 interaction is key in HSC retention in the BM (60, 61), whilst G-CSF signalling is a mainstream clinical strategy to mobilise the HSC component. Although G-CFS is handled well by patients, it is markedly less efficient at proving

the numbers of peripheral blood cells that are required compared to new alternatives such as SCF (62), GROßT (63, 64), and AMD3100 (65)

1.3 History of HSC Research

Functional studies of HSCs began in the 1950s with experiments showing that intravenously injected BM cells are able to restore long term blood formation in irradiated mice (66). Till and McColloch went on in 1961 to use spleen colony assays (CFU-S) (67). These CFU-S were a significant breakthrough as they demonstrated that single cells could give rise to multiple cell lineages as well as daughter cells that could do the same job in a secondary transplantation. CFU-S were used to reveal and understand principles we now expect to be present in the HSC population. These were followed in the 1980s by the use of retrospective models using retroviral barcoding to detect the ability of single transplanted cells to both produce mature blood cell progeny as well as self-renew and generate their own progeny that also had these abilities (41, 68, 69).

Limiting dilution assays were developed to try and quantify the cells with this self-renewal and differentiation capacity (67) and newer techniques to quantify HSCs continued to be developed allowing direct measurements of HSC frequency (70, 71) using electrophoretic detection (72), or flow cytometry initially using CD45 allotypes (73).

More recent approaches have utilised reporter transgenes (74, 75), coupled by the ever-increasing use of flow cytometry to enrich for functionally-defined HSCs. Flow cytometry was initially (and still is to a certain extent) restricted by the changing expression levels of protein markers both during HSC cell cycling, and *in vitro* vs *in vivo* expression (61, 76, 77, 78), limiting early attempts of this approach to quiescent G0 HSCs.

The SLAM phenotype ($CD150^+$, $CD48^-$. $CD244^-$) (79) was later discovered and proved especially useful as the conserved expression of these proteins throughout the cell cycle and *in vitro* makes this phenotype ideal for identifying actively cycling, and cultured, HSCs (80, 81). The addition of Endothelial Protein C Receptor (EPCR) to the SLAM panel (ESLAM) was later shown to further improve the efficiency of this HSC isolation strategy. Even by itself, EPCR removes the need for antibodies against other traditional markers such as c-Kit, Sca1 and lineage markers, when isolating primitive haematopoietic populations. (81, 82)

1.3.1 Challenges with studying HSCs

Although progress has consistently been made over the last 60 years, researching HSCs relies on detecting, quantifying and then efficiently isolating this population (1) which has proved difficult as a result of several key challenges associated with studying this cell type.

Firstly, HSCs are unable to be visually identified or distinguished morphologically from other primitive blood cells with significantly less durable self-renewal – ruling out this method of identification.

Secondly, there is an innately low frequency of HSCs – as they make up only 0.004% of viable murine BM cells, limiting experiments to isolating a maximum of 1000-2000 cells from each 8-12 week old mouse. This limits large scale approaches such as proteomics and metabolomics which typically require much higher quantities of cells. Although the HSC number slightly increases with age, this is accompanied by reduced functionality and increased heterogeneity. This reduced functionality is in terms of the accumulation of myeloid-bias HSCs with limited self-renewal and proliferation which produce fewer numbers of mature cells, as highlighted by secondary transplantation experiments (83, 84). Older HSCs also present with a 2x lower engraftment response (84). These features, acquired with age, limit their applicability to functional studies (84, 85). Interestingly, through long-term serial transplantations we can induce young HSCs to present with these characteristics too, again linking these properties to ageing and therefore likely to the accumulation of genetic mutations as well as epigenetic modifications (84). These newly acquired features likely link directly to age-related pathologies and general decline in the function of the blood tissue.

Thirdly, HSCs present with significant cell to cell heterogeneity in terms of number and type of mature cells that they produce (33, 86**)**, their proliferation and differentiation capacity, as well as their response to ageing (87). This phenomenon has been rigorously documented since being observed first by Muller-Sieberg (88, 89), and is a hallmark of this population of cells with repopulation capacity. Despite this, we still fail to fully understand the molecular mechanisms behind this variability.

In the 1960s Becker (90), McColloch and Till (67) revealed heterogeneity in size, number, and type of cells produced by HSCs in CFU-Ss, as well as variations in the size and number of secondary colonies. The use of retroviral studies of the 1980s also to highlighted self-renewal variations and lineage bias (85) and in the early 2000s single HSC transplants further confirmed the self-renewal/differentiation and repopulation kinetic variations. (34, 91)**.** The strength of single-cell HSC transplantation lies in the ability of us to track all progeny back to one starting cell. Dykstra et al., 2007, showed that of 352 mice who underwent single HSC transplantation, 93 showed donor contributions to the white blood cell compartment, for at least 4 months (34). Of interest, a single HSC transplantation study was able to divide the transplanted HSCs into four categories (alpha, beta, gamma or delta) based on the mature cell output from each HSC, in particular ratio of myeloid (granulocyte and monocyte) to

lymphoid (B and T cell) (34). Importantly, and in relation to my own studies, the classifications of HSCs shifted to the subtypes with less durable self-renewal activity (gamma and delta) when cultured *in vitro* – highlighting the significant impact of culturing on HSC activity and functionality (34) (Table 3).

Classification	Mature cell production pattern	Self-renewal activity	% of HSCs displaying without culture	% of HSCs displaying after culture
Alpha	High GM : (B+T)	High levels, and maintained self-renewal *in vivo* in secondary transplantations	65	18
Beta	Balanced GM : (B+T)			
Gamma	Low GM : (B+T)	Not linked with extensive self-renewal Unable to repopulate in secondary transplantations	35	82
Delta	B+T, but no GM			

Table 3: HSCs can be subdivided into 4 categories based on their repopulation kinetics and mature cell output. The frequency of HSCs falling within each of the sub-groups changes upon *in vitro* culture

Studying HSC heterogeneity further will bring us closer to working out if it is derived from intrinsic, pre-determined diversity (91), or if it is a result of differing environmental stimuli (92, 93, 94).
It is important to consider informative (biological variation induced changes in HSC function) and generic heterogeneity (induced by universal processes such as the cell cycle), as well as heterogeneity due to the presence of non-HSCs contaminating the population. This in particular highlights the benefit of combining molecular and functional assays (87), as well as ideally an isolation strategy that could isolate HSCs to 100% purity.

1.4 HSC expansion

1.4.1 Importance of expanding HSCs

HSCs able to produce all mature cells of the blood system and are essential in regenerative medicine. HSC transplantation can be used to treat blood-based cancers (95), but is also increasingly being used for gene therapy (96), viral infections (such as HIV) (97), and a range of autoimmune conditions including Multiple Sclerosis (98). CD34 is a surface marker for human HSCs (78). In clinical HSC transplantations, the likelihood of success is directly correlated to HSC dose (with a minimum of 3-4x10^6 $CD34^+$ cells/kg of body weight needed) (99) so expansion of these cells could revolutionise these cellular and gene therapies.

Improving the efficiency of HSC expansion by increasing the number of self-renewal divisions *in vitro* would allow derivation of large quantities of mature cells ready to meet ever increasing transfusion and transplantation demands. To date, the *in vitro* generation of red blood cells, platelets, T cells, megakaryocytes and neutrophils from HSCs has been achieved (100, 101, 102, 103)
Increasing *ex vivo* HSC self-renewal would also allow large-scale transcriptomic, epigenomic, proteomic and metabolomic approaches to be undertaken, as these methods have previously been nearly impossible due to the large numbers of mice which would be needed to reach the required cell numbers.

1.4.2 Previous progress in HSC expansion

Many approaches have been taken to refine and increase HSC expansion.
Firstly epigenetic modifiers have been a focus - of several Histone Deacetylase inhibitors tested, valproic acid (VPA) was found to be the most efficient at improving $CD34^{+}CD90^{+}$ cell expansion *in vitro*, and transplantation studies show VPA prevents the loss of human HSCs in culture (104)
Additionally, treatment with 5azaD/TSA (an alternative histone deacetylase inhibitor) resulted in human HSC expansion, and transplantation studies revealed that this treatment retained cells with serial transplantation ability. (105)
MicroRNAs can also be harnessed to improve *in vitro* expansion. Overexpression of miR125a results in the differentiation stage-specific targeting of pro-apoptotic genes in haematopoietic stem/progenitor cells (HSPCs) has been implicated in increased mouse HSC numbers in-vivo over 8-fold (105, 106)
Another approach has been transgene overexpression, in which Homeobox protein HOXB4 (107) and developmental pluripotency-associated protein 5a (108) induced overexpression have been shown to be positive regulators of expansion and improve mouse HSC self-renewal *in vitro*
Furthermore, HSCs transduced with Nucleoporin98-Homeobox presented with expansions of 1000 fold to 10,000-fold. This starkly contrasted with the HSC decline in control cultures (109) and transgenic overexpression of Notch (110) and Wnt (111) signalling also increases *in vitro* HSC self-renewal.
Cytokines and soluble factors also hold significant potential in this area.
Early work focused on cytokine concentrations and combinations. Mouse HSCs were found to survive in serum free media for 10 days with SCF, Flt3L, and IL11 (50, 100). SCF and Flt3L together are enough for HSCs to survive and proliferate, however in order to retain stem cell activity, needed GP130 pathway stimulation (often achieved in mice using IL11, and humans with IL6) (50)

In vitro, liquid media containing key cytokines alongside human or bovine serum albumin can maintain HSCs for between 1 and 2 weeks, but result in only low levels of expansion (50). Supplementing cultures of human HSCs with SR1 resulted in 50-fold increases in $CD34^+$ cells, and 17-fold increases in cells able to engraft and reconstitute the blood system once transplanted into immunodeficient mice (112). This small molecule induces these effects via antagonising the aryl hydrocarbon receptor. Alternatively, the addition of small molecule UM171 to cultures led to a 13-fold increase in human LT-HSCs compared to untreated controls (113), through promoting the detoxification of reactive oxygen species

In STEMSPAN cultures, the addition of small molecule inhibitors of Hsp90 or TRic, which activate the transcription factor Hsf1, were found to support and maintain HSC functionality and their serial reconstituting capacity *ex vivo* (114). Finally, MPL agonists, which mimic the activity of thrombopoietin, have also been shown to promote HSC expansion *in vitro* (115, 116)

The use of zwitterionic hydrogels called attention to the fact that mechanical stimuli are crucial in HSC self-renewal (117). Fibronectin-based hydrogels have also been shown to have promise in improving HSC expansion, likely via modulation of integrin signalling and associated promotion of the TPO pathway (50, 118, 119)

As shown in the pivotal Wilkinson et al., 2019 paper - replacing serum albumin, which has considerable batch to batch variability, with chemically defined polyvinyl alcohol in *in vitro* expansion media with SCF and TPO can enable up to 800-fold, and long-term expansion of mouse HSCs in culture (118).

1.4.3 Challenges of HSC expansion *in vitro*

Despite the urgent need for refinement, coupled with huge investment and research attention over the past 60 years, the expansion of fully functional HSCs over long periods of time has largely eluded researchers - with only modest expansion of 2 to 4-fold (50) reported in serum-free conditions, and expansion in culture linked to loss of self-renewal and long-term reconstitution activity (50)**.** The challenge of *in vitro* expansion further increased by the heightened protein synthesis stress and altered proteostasis linked to expansion in culture, in turn restricting HSC function and self-renewal, almost creating a negative feedback loop.

Similarly, expression differences between freshly isolated and cultured HSCs as well as between quiescent and cycling HSCs limit the use of many potential surface markers to identify HSCs across these different states (eg Tie2 (120), endoglin (121) and Mpl (122), CD49f (123) and CD38 (124).

The poor molecular understanding of the HSC expansion conditions and the expanded HSCs themselves is a major barrier to understanding the cellular and molecular mechanisms underpinning

HSC expansion. This information would help adapt existing expansion protocols to human cells, leading to further, much needed, improvements in human HSC expansion and its clinical applications.

Molecular mechanisms can begin to be understood by using linked molecular and functional assays - allowing direct comparison between the two, but this would require advanced knowledge of which cultures contained functional HSCs in order to be time and cost permissive.

1.4.4 Current state-of-the-art HSC expansion and identification

The most recent breakthrough in mouse HSC expansion came from the development of a new 28-day protocol by the Nakauchi and Yamazaki labs, which uses just two cytokines – Stem Cell Factor (SCF) and Thrombopoietin (TPO) (118) and a fully defined serum-free medium. This provides a refined environment for HSC expansion allowing long-term and substantial self-renewal of HSCs in culture (118). HSC expansion was confirmed by both limiting dilution assays before and after expansion using the purified $CD150^{+}CD34^{lo/neg}Sca1^{+}Lin^{-}$ population, and split clone transplantation assays (118).

The protocol is based on the replacement of serum albumin with a chemically defined substitute - polyvinyl alcohol (PVA) which retains the same ability to moderate and stabilise the cytokine levels, but its impact on the metabolism of the HSCs is not yet understood (125). Despite the ground-breaking 200-900-fold expansion that this system can achieve, HSCs remain the vast minority of cells at the end of the culture period, significant HSC heterogeneity exists within the clonal populations, and the process is not yet translatable into human HSCs. Currently there are also no reliable strategies for prospectively isolating cultures containing high numbers of HSCs.

Recent work in our laboratory has overcome the latter deficiency – by using the the *Fgd5*$^{+/ZsGreen}$ reporter mouse in combination with the expression of EPCR ($F^{high}E^{high}$) to isolate functional HSCs. (Unpublished, Che et al., bioRxiv). It is able to mark the functional and transplantable HSC compartment *in vitro*, and has been linked to both increased total chimerism and wide ranging contributions to T cells, B cells and Granulocyte-macrophages. A key strength of this system is the uniform use of Fgd5 and EPCR across different cellular states, as often surface markers used to identify freshly isolated HSCs can have altered expression levels *in vitro* (67 ,68). $F^{high}E^{high}$ correlates with percentage of cells being phenotypic HSCs, and these markers are lost as HSCs begin to differentiate. (45, 81, 126).

1.5 Key HSC proteins

Throughout this project %LSK will be used a parameter to mark functional HSCs. This represents the percentage of cells in the well that are Lineage negative (negative for the markers CD11b-, Ly6g-), whilst expressing the surface proteins Sca-1 and c-Kit. This identification strategy significantly enriches the HSC component; however, it has a caveat of marking the progenitor cell compartment too. (127)**.** This was elucidated by studies in the 1990s which compared whole BM transplants from young and old mice. Old BM was significantly more efficient at engrafting, which was found to result from increased HSC concentrations in the BM which accompany ageing. Despite this, a significantly decreased fraction of the HSCs isolated from old BM were functional in long term single-cell transplantations. This implies that the markers used to isolate HSCs were marking the progenitor population also, a population which is increased in older mice (84).

C-kit is a receptor tyrosine kinase, encoded by the W locus, and is expressed on HSCs as well as mast and germ cells. Knockdowns of this gene result in issues with T and B cell maintenance, and decreased mast cell count (128, 11). Mutations in the W locus, highlighted in W41/W41 mice, result in fertile mice, with reduced HSC number. This is invaluable experimentally, as using sub lethally irradiated host mice of this type allows as easy detection of transplanted HSCs as lethally irradiated WT hosts. (128) Sca-1, encoded by the SI gene, is a transmembrane growth factor which associates with GPI. It binds to and activates tyrosine kinase, KIT. Deficiencies in this gene prevent short-term HSC transplantation, and CFU-S formation – thought to be a consequence of impaired HSC self-renewal (128, 129, 130).

Both c-Kit and Sca1 have variable isoforms, each with slightly different functions.

The type 1 transmembrane surface protein Endothelial Protein C Receptor (EPCR/CD201/PROCR) can be added to the LSK classification, in order to increase purity (126). EPCR is essential in both the anti-inflammatory and endothelial protective activity of its binding partner APC via the PAR1 pathway (131, 132). The PAR1 signalling pathway is essential in the retention of the EPCR-expressing LT-HSC population in the BM through limiting both NO production and Cdc42 activity (133, 134). BM homing is vital in protecting HSCs from genetic damage and myelotoxic injury (134). Organisms genetically modified to express low levels of EPCR have resulting HSC BM homing defects alongside increased circulating HSCs (134).

Although previously shown to be a reliable marker for cultured murine HSCs, where it marks a subpopulation of CD34+ cells linked with robust reconstitution and multi-lineage repopulation, (126, 135) it has currently not been found beneficial when sorting human HSCs (125).

1.6 Aims of the thesis:

Overall, this thesis had two primary aims:

1) To identify robust markers of functional HSCs *in vitro*

Our lab has recently shown that the absolute number and proportion of expanded cells expressing the Fgd5$^{+/ZsGreen}$ reporter gene, in combination with expression of the surface marker Endothelial Protein C Receptor (EPCR) is a reliable and efficient HSC reporter strategy (81). This combination correlates strongly with HSC function - defined by cells that have increased donor chimerism and contribution to multiple blood cell lineages post-transplantation in irradiated mice. However, the strategy is not 100% efficient as although nearly 100% of functional LT-HSCs are Fgd5^{+}, not all Fgd5^{+} cells are functional LT-HSCs. Furthermore, since detecting the *Fgd5* expression is reliant on the Fgd5$^{+/ZsGreen}$ reporters and not all mice will be crossed onto this background, it became clear that a more universal, *in vitro* HSC-identification system would be required for broader use in mice and might also serve to isolate the functional human HSCs *in vitro* as well.

2) To further improve the expansion protocol using a candidate pathway approach

Increasing the efficiency of the HSC expansion protocol, by increasing the number of self-renewal divisions and purity of the culture (increasing the % phenotypic HSCs) will provide HSCs for both clinical and scientific use if successfully translated into human HSCs. For this aim, I tried to optimise the culture in two ways: first, I monitored cell surface markers throughout the culture to determine the optimal time for determining the HSC content of a clone and; second, I tested new compounds to try and enhance self-renewal expansion divisions in culture. The latter will derive primarily from our HSC gene signature identifying likely targets for improving *in vitro* expansion (81) - ESAM, Fstl1, VEGF, CD62L, Siglec F.

Preliminary data: the HSC gene set (Figure 6)

In a large body of work initiated prior to my thesis in mouse HSC expansion cultures, where single cell clones were expanded and split into matched functional and gene expression assays (81), PCA analysis of the gene expression data was able to separate cultures containing functional HSCs from those that have no HSCs. Following this the loading plots were computed to identify the top 100 genes driving these gene expression differences. Linear and logistic regression analysis was used to correlate each gene with repopulation outcome (the ability to produce blood cells in an irradiated mouse post-transplant), GM contribution (the ability to produce granulocyte/monocyte cells – 2 types of short-lived mature blood cells) and donor chimerism (binary yes/no). The genes that were significantly linked to all 3 outcomes were used for the signature and a more complete description of the whole process is in reference 81.

Esam	Fgfr1	Fstl1	Gm38066
Insyn1	Lzts1	Pim2	Ptk2
Skint3	Tgfbr3	Timp3	Arx
Dennd2a	Klhl4	Mpdz	Myof
Nrk	Palld	Prdm16	Prex2
Ryk	Slamf1	Zfp532	

Figure 6: HSC gene signature previously identified by our lab via PCA analysis. Identifies key genes associated with HSC activity – defined by repopulation outcome, GM contribution, and donor chimerism.

2.0 Materials and methods

2.1 Mice strains

This project used *Fgd5-ZsGreen* knock in/ knock out mice. Both genetic engineering methods, Knock in means refers to the insertion of a DNA sequence, coding a protein, at a particular locus in an chromosome, whilst knock out involves the targeting and in activation of a gene. This strain carries a knock-in/knock-out mutation of the *Fgd5* gene in which ZsGreen is inserted (knocked-in) to replace one of the Fgd5 alleles (which is knocked out by this process). This should have no phenotypic effect as Fgd5 is haplosufficient. The wild-type (WT) mice used were *Fgd5* WT littermates and on a CD57Bl/6 background. All mice used were aged 8-12 weeks. All animals used were kept in the BSF at University of York in specified pathogen-free conditions and were provided with sterile food, water and bedding according to the UK Home Office Regulations.

2.2 Tissue processing

Mice were culled by cervical dislocation and CO^2 asphyxiation. Femurs, tibias and pelvic bones collected from WT or *Fgd5* mice were processed via flushing in Phosphate-buffered saline with 2% foetal calf serum (PBS 2% FCS) (FCS; Sigma Aldrich, St Louis, MO, USA(Sigma), PBS; Phospho-buffered saline, Sigma).

2.3 Red blood cell lysis

Cells underwent red blood cell lysis using ammonium chloride. The cells were spun at 300 x g for 5 minutes, before gentle removal of the supernatant and resuspension in 3mL PBS 2% FCS. 5mL ammonium chloride was added for 5 minutes on ice, followed by a short mix using a vortex, and 5 more minutes on ice. The mix was then diluted with 12mL PBS 2%FCS and spun again at 300 x g for 5 minutes, supernatant removed, and resuspended fully in 500µL PBS 2%FCS/mouse, and transferred into a 5mL polystyrene tube for lineage depletion.

2.4 Lineage depletion

Using the EasySep Mouse Haematopoietic Progenitor Cell Enrichment Kit (STEMCELL Technologies, SCT), the haematopoietic stem and progenitor cells were enriched. 500µL of cell suspension was mixed with 10ul of EasySep Haematopoietic Progenitor Isolation cocktail (removing cells expressing CD5, CD11b, CD19, CD45R/B220, Ly6G/C(Gr-1), TER119) and left for 15 minutes on ice. 15µL of EasySep Streptavidin RapidSpheres was then added for another 15 minutes on ice. After the incubation, 2mL of PBS 2%FCS was added and the FACs tube put into an EasySep Magnet for a 3-minute incubation at room temperature. After incubation, whilst still in the magnet, the supernatant is poured off into a new FACS tube and the process repeated.

2.5 HSC isolation from Bone Marrow using FACS

The now enriched cells were stained for the ESLAM markers (Table 1, 79), and 7AAD at a 1:1000 dilution to act as a live-dead stain. ESLAM defines the $CD48^-$ $CD150^+$ $EPCR^+$ $CD45^+$ population, able to identify cells with HSC activity. ESLAM HSCs were isolated using FACS (24, Table 5, Figure 4), and sorted single cells per well into the inner 60 wells of a 96-well plate, each preloaded with 100µL of media. The outer wells were filled with 200µL/well of sterile PBS to limit evaporation.

Antibody	Fluorochrome	Clone	Company	Single Stain Volume (µL)	Experimental sample volume (µL)
CD45	BV421	30-F11	BD Biosciences	0.3	0.4
CD48	APC	HM48-1	Biolegend	1	0.4
CD150	PE-Cy7	TC15-12F12.2	Biolegend	1	0.4
EPCR	PE	RMEPCR1560	SCT	1	0.4
Sca1	BV605	D7	Biolegend	0.3	0.4
7AAD	PE5		Life technologies	1:1000	0.4

Table 5: Antibody staining panel used to sort ESLAM cells via Fluorescence activated call sorting (FACS)

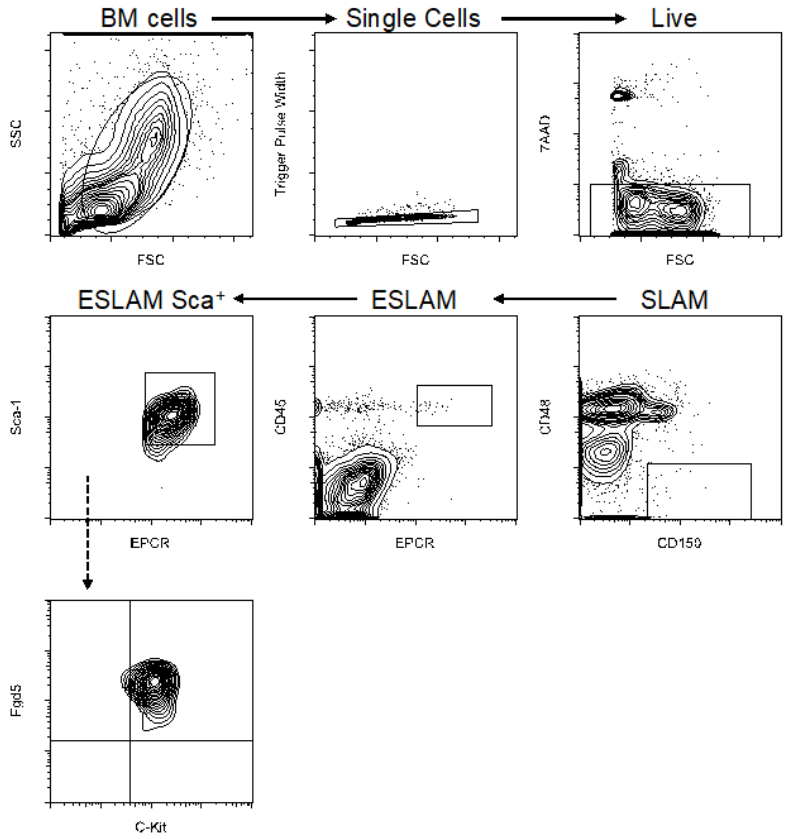

Figure 4: Representative gating strategy for ESLAM cells gated by FACS. EPCR$^+$CD45$^+$CD150$^+$CD48$^-$Sca1$^+$ HSCs are isolated. Despite not being used as part of the usual gating strategy, these cells are also shown to be c-Kit and Fgd5 positive.

2.6 28-day expansion protocol

Cultures were initiated with 1 ESLAM cell per well on a fibronectin coated 96-well plate (Corning) with 200µL/well of media (Table 6). Cells were kept at 37 degrees Celsius with 5% CO_2 fully replenished every 2-3 days using a multichannel pipette from day 5. This expansion protocol enables 200-900-fold *in vitro* expansion of HSCs (118).

Component	**Volume to make up 1ml (µl)**
Hams F12 Media (1x, Gibco)	958
ITSX (100x GIbco)	10
PVA (87-90% hydrolysed, Sigma)	10
HEPES Buffer (1M, Gibco)	10
PSG (100x, Gibco)	10
SFC (10µg/ml)	1
TPO (100µg/ml)	1

Table 6: Fully defined, serum free, 28-day expansion culture media. (118)

2.7 Flow cytometric analysis of *in vitro* cultures

Flow cytometry was used to analyse single clones at each week during the 28-day culture for presence of HSCs and mature blood cells (34). This allowed us to determine how efficient expansion has been under each tested condition (Figure 5, Table 7). When analysing Fgd5, although it is an internal protein, it's ZsGreen florescent reporter is excited by the 488nm laser, and its resulting emission at 505nm is bright enough to be able to be detected via flow cytometry.

Mid-culture samples were collected via a 220µL volume of media added post media change, before briefly mixing with a pipette to gently disturb cells, and removing the excess 20µL. The cells taken were stained with antibodies, and fluorescent count beads (TruCount Control Beads, BD) were added to calculate total cell numbers. The samples were run on the Cytoflex LX equipped with 405nm, 488nm and 638nm lasers, and analysed using FlowJov10 (Treestar, Ashland, USA).

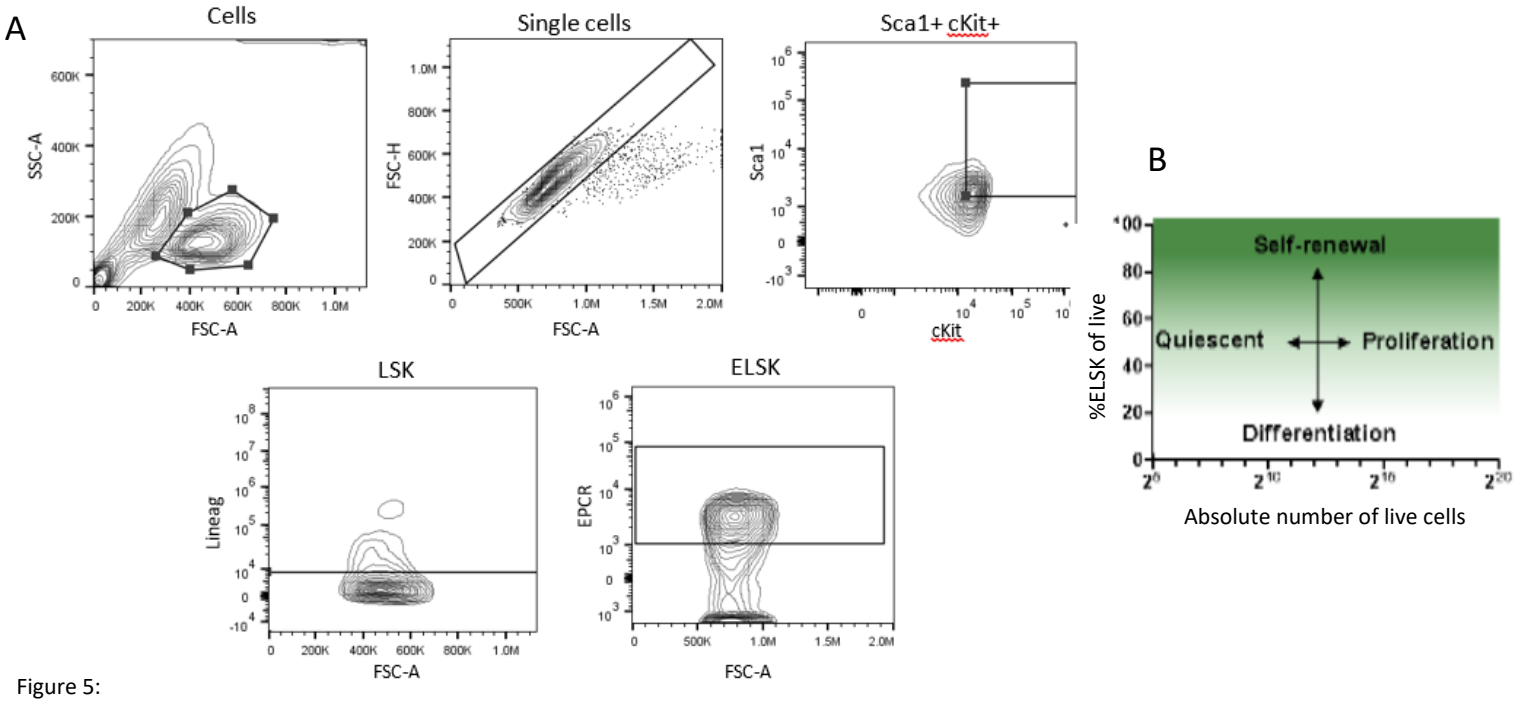

Figure 5:

A) Representative ELSK gating strategy. Single clones are analysed at weekly timepoints and/or the final 28-day timepoint to analyse ELSK content and purity of the clone. Flow cytometric analysis can allow easy comparison between clones in terms of both their proliferative state and level of differentiation. The addition of fluorescent counting beads to samples can allow back-calculation of total cell counts

B) Interpreting clonal analysis. During flow cytometric analysis, fluorescent count beads can be added to the well and ran with the sample. These beads are able to be used to back-calculate absolute cell number for each clone. By plotting cell number of the clone against percentage of phenotypic HSCs in the well, we can compare clones ability to self-renew and/or proliferate.

Antibody	Stain	Clone	Company	Experimental sample volume	Single stain volume
CD45	AF700	A20	eBioscience	0.1	0.4
Ly6g	BV421	1A8	Biolegend	0.1	0.4
Sca1	BV605	E13-161.7	Biolegend	0.1	0.4
c-Kit	APC-Cy7	2B6	Biolegend	0.1	0.4
EPCR	PE	RMEPCR1560	SCT	0.1	0.4
Mac1	BV510	M1/70	Biolegend	0.1	0.4
CD150	PE-Cy7	TC15-12F12.2	Biolegend	0.1	0.4
ESAM/Siglec F/ CD62L	APC	1G8 (ESAM) E50-2440 (Siglec F) MEL-14 (CD62L)	Biolegend BD Biosciences ThermoFisher	0.1	0.4
7AAD			Life Technologies	0.1	0.4

Table 7: Antibody staining panel for Cytoflex analysis at weeks 2, 3 and 4. Used to gate live cells, negative for 7AAD expression, as well as determining ELSK content of the well and CD150, ESAM/SiglecF/CD62L expression.

2.8 VEGF and FSTL1 culture protocols

ESLAM cells were sorted via FACS into 28-day expansion culture media already containing the proteins/signalling molecules of interest, each at 3 different concentrations (Table 8). Each media change replaced the old media with fresh media of the same concentration. This ensured the cells were exposed to consistent levels of the protein throughout the entire course of expansion.

	Catalogue Number	**Company**	**Low concentration**	**Mid Concentration**	**High Concentration**
Fstl1	1694-FN-050	R&D systems	50ng/ml	200ng/ml	500ng/ml
Neutralising VEGF	743-R3-100	R&D systems	0.1µg/ml	1 µg/ml	5 µg/ml
Recombinant VEGF	775104	BioLegend	0.02 µg/ml	0.2 µg/ml	1 µg/ml

Table 8: Signalling modifiers added to expansion cultures, and the concentrations tested

2.9 Statistical Analysis

All Pearson's correlation and ANOVA tests were calculated, and graphs plotted using GraphPad Prism 9.

3.0 Results

The 28-day expansion cultures published by Wilkinson et al (118) have gained enormous interest, and analysis of these cultures via large scale screens and molecular characterisation has been widely undertaken- both of which rely on functional validation. The current gold-standard assay for HSC validation is an *in vivo* transplantation into irradiated mice (50) – an expensive and time-intensive process which requires large numbers of recipients as well as long-term follow up of the animals (typically 6 months). This approach would ideally be reduced, and eventually replaced, by an equally, or even more, efficient *in vitro* reporter system able to identify functional HSCs in these expansion cultures. Identifying stable markers, whose expression remains unchanged upon *in vitro* culture is vital in order to determine the efficiency of expansion protocols.

3.1 A new 2-marker strategy for identifying functional, expanded HSCs in vitro

Removing the need for reporter genes in HSC identification would provide a more widely applicable reporter strategy for all mice strains and disease models, and has potential to be translated into human HSCs.

LSK (Lineage^{-}Sca1^{+}cKit^{+}) cells contain the functional HSC compartment and will be used as a key measure in the data-sets presented throughout this thesis. Importantly, LSK on its own cannot reliably predict HSC-containing clones as it also marks a wide-range of short-term progenitor cells lacking durable self-renewal. Still, the LSK phenotype remains useful as a first pass analysis for correlated high verses low HSC content in induvial clones.

3.1.1 ESAM marks high %LSK HSC expansion cultures

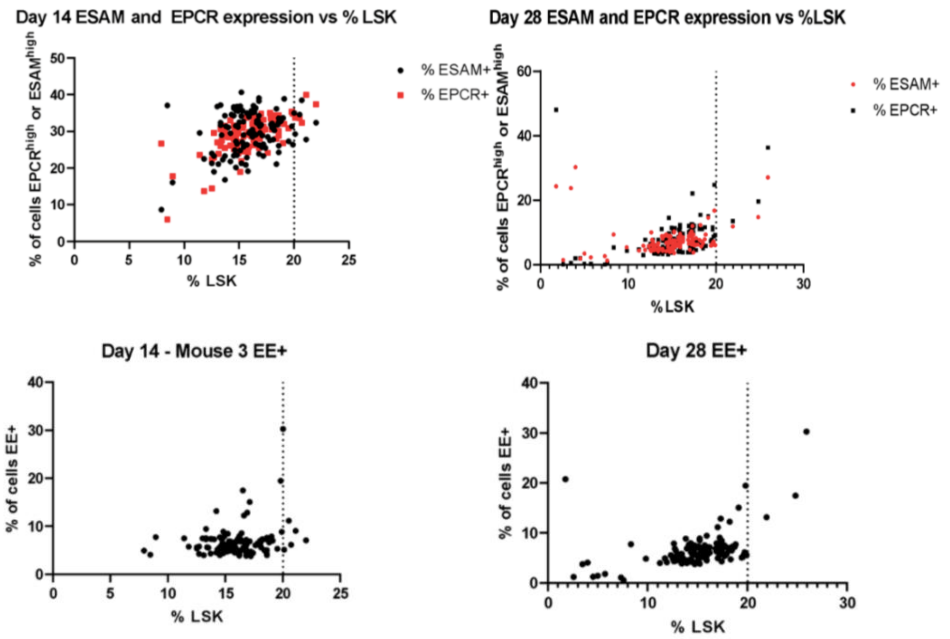

Figure 7: Data collected via flow cytometry on days 14 and 28 of 28-day expansion cultures. All cultures started with single, freshly isolated ESLAM HSCs. Correlation is shown between %LSK and EPCR/ESAM expression, as analysed by flow cytometry.

Top left: ESAM/EPCR: Pearson's R value = 0.062/0.3255, p-value = 0.497/0.000254
Top right: ESAM/EPCR: Pearson's R value = 0.31/0.6782, p-value = 0.00789/<0.0001
Bottom left: Pearson's R value = 0.2412, p-value = 0.009732
Bottom right: Pearson's R value = 0.4479, p-value = <0.00001

The transmembrane protein endothelial cell-selective adhesion molecule (ESAM), which appears in the generated HSC gene signature, has a role in the formation of Tight Junctions and Ca^{2+} dependent, homophilic adhesion between cells in the vascular wall (136). ESAM is an efficient marker for fresh HSCs, in freshly isolated foetal, adult and aged mice (137, 138, 139) where its expression is significantly increased (in terms of both ESAM levels in the membrane, and mRNA levels) in LT-HSCs compared to ST-HSCs and multipotent progenitors (136, 137). In freshly obtained BM, ESAM marks the HSC population, specifically the LT-HSCs and primitive lymphoid and myeloid-erythroid progenitors (137, 138) whilst its expression decreases throughout haematopoietic differentiation (137). Interestingly, elevated ESAM transcripts have been found in freshly isolated human HSCs (133), potentially opening it up as a universal reporter strategy for both human and mice cells.

My first thesis aim was therefore to assess whether ESAM might replace *Fgd5* as a strong *in vitro* reporter of HSC function. All experiments were initiated with single, freshly isolated $CD45^{+}EPCR^{+}CD48^{-}CD150^{+}$ (ESLAM) cells sorted via FACS into fibronectin-coated, 96-well plates. They underwent a 28-day expansion culture (118), with 10% of the clones taken post media change on day 14 for flow cytometric analysis, and the remainder harvested on day 28 for the same assessment. Over the course of 28-day expansion cultures, ESAM expression was found to correlate well with %LSK. Combining ESAM with EPCR (EE^{+}), increased the strength of correlation. (Figure 7). Importantly, the clonal populations that met these criteria had especially high levels of both ESAM and EPCR, making this marker particularly exciting (Figure 7).

Additional flow cytometry data revealed ESAM expression correlates well with $CD150^{+}$ expression, a known surface marker of HSCs, throughout the culture period (Figure 8). Further analysis reveals that at day 28 of expansion cultures initiated with single ESLAM cells, both CD150 and ESAM significantly (to $p<.05$) positively correlate with %ELSK (Figure 9). ELSK ($EPCR^{+}LSK$) is another identification strategy for the functional HSC compartment, where increasing %ELSK correlates with increased HSC self-renewal and proliferation. Cultures containing >20% ELSK contain functional HSCs as evidenced by transplantation assays. This further corroborates the ability of ESAM to mark the functional HSC compartment and implies that inclusion of ESAM could also negate the need for CD150 in the identification of cultures containing functional HSCs.

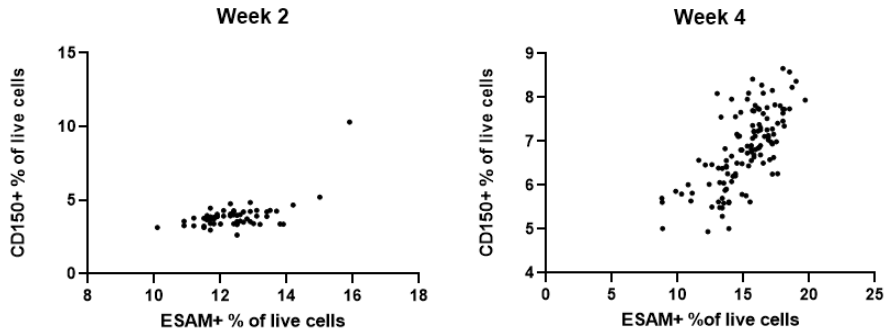

Figure 8: ESAM+ % plotted against CD150+ %. Taken at week 2 and 4 timepoints of a 28-day expansion culture. Cultures initiated with single ESLAM cells sorted via FACS.

Left graph: Pearson's R Value = 0.5975, p-value <0.00001
Right graph: Pearson's R value = 0.7365, p-value <0.0.00001

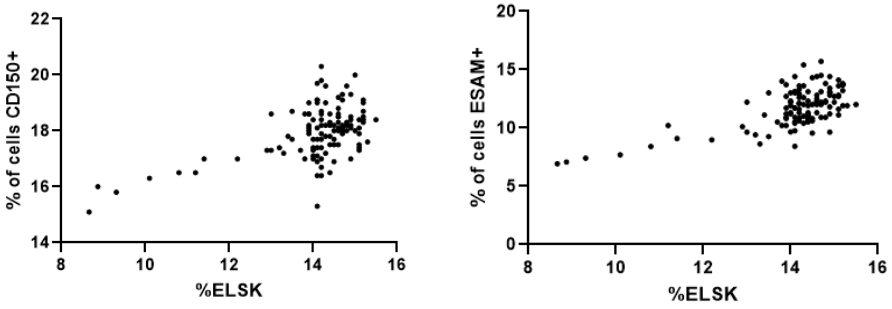

Figure 9: ESAM+ and CD150 plotted against %ELSK. Taken on day 28 of a 28-day expansion culture. Cultures initiated with single ESLAM cells. ELSK is used as a marker for the functional HSC compartment, increasing %ELSK reveals increased HSC self-renewal and proliferation

Left graph: Pearson's R Value = 0.742, p-value <0.00001
Right graph: Pearson's R value = 0.3425, p-value 0.000055

Through plotting ESAM directly against expression of the *Fgd5* reporter we see a significant positive correlation (Pearson correlation, R value = 0.367, p=.000037). These data show that ESAM marks the functional HSC population known to be identified by *Fgd5* (Figure 10).

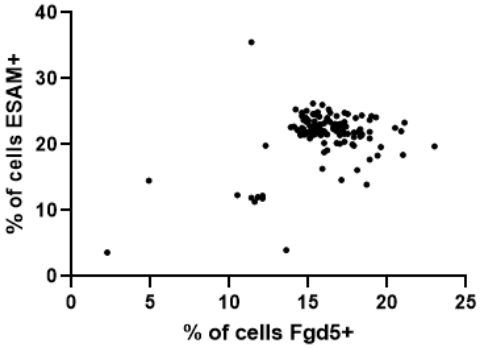

Figure 10: Direct comparison between % of cells expressing ESAM, and those expressing the reporter gene, *Fgd5* as determined by flow cytometric analysis. This data was collected on day 28 and all cultures were started with a single ESLAM cell

Additionally, plotting proportion of cells EELSK (ESAM^{+}ELSK) against FELSK (*Fgd5*$^{+}$ELSK), again reveals a strong positive correlation (Pearson's R value=0.9887, p value = <0.00001) reinforces the idea that ESAM marks the same clones containing functional HSC as *Fgd5* (Figure 11). The strong correlation between these two marker strategies justifies replacing the use of the *Fgd5*$^{+/ZsGreen}$ reporter mice with the cell surface molecule ESAM, leading to a much more widely applicable strategy for HSC biologists in the future.

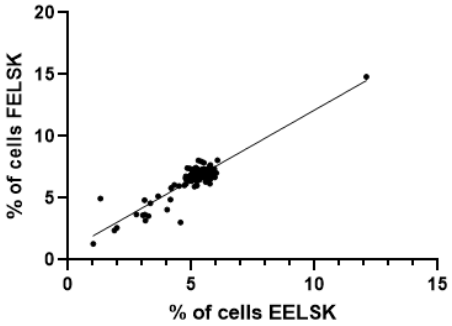

Figure 11: Plotting % of cells FELSK against % of cells EELSK reveals a significant positive correlation. This data was collected on day 28 and all cultures were started with a single ESLAM cell

3.2 Further improving the expansion protocol

In addition to refining the marker strategy described above in 3.2, this thesis also aimed to improve the amount of HSC expansion in the protocol through the use of targets generated by matched gene expression and functional transplantation data.

3.2.1 Early detection of cultures with high HSC content

From a practical perspective, we first explored whether we could predict HSC-containing clones earlier in an effort to shorten the length of the expansion culture, save reagents and time, and increase the efficiency at which we can undertake these experiments. To determine if we were efficiently able to predict high HSC-containing clones earlier than the 28-day point, 60 single E-SLAM cells were sorted via FACS into a 96-well fibronectin coated plate, and kept in 28-day expansion conditions (118). At the day 14 and 21 time points, 10% of the clone was taken via multichannel pipette after a full media change and analysed by flow cytometry. At day 28, the entirety of the clone was taken and analysed the same way. Percentage LSK of each clonal population generally increased from Day 14 through to Day 28 (From one-way repeated ANOVA, it revealed a significant difference at $p<0.05$ between day 14 and day 21, however the p-value between Day 21 and 28 was not significant at $p<0.05$, as had a value to 0.065). (Figure 12). Unfortunately, we were unable to see the early emergence and maintenance of high %LSK cultures. This prevents us using %LSK data from earlier on in the culture period to identify the clones that will end up the most productive. Moving forward, it would be interesting to try and determine whether EELSK might be more discriminatory and this will be explored in future studies.

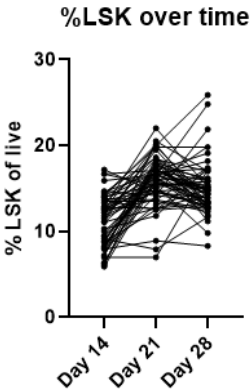

Figure 12: % LSK of a clone plotted over the course of the expansion culture – flow cytometry data taken from days 14, 21, and 28 of expansion cultures initiated with single ESLAM HSCs.

3.2.2 Utilisation of *in vitro* reporter to identify new markers of HSC differentiation in expansion cultures

In this section, I took advantage of the data that showed that >20% ELSK cells in single-cell initiated 28 day cultures had a 100% correlation with clones containing functional HSCs to undertake screening assays to identify new markers of HSC content in expansion cultures. Two such markers formally tested were CD62L and Siglec F. Siglec F was chosen as it was identified, whilst generating the HSC gene signature, to negatively correlate with HSC content of clones and so held promise as an interesting negative marker for functional HSCs. CD62L is a marker for a unique population of early lymphoid-primed cells – they retain lymphoid and monocyte potential, but lack erythroid. It is also a marker for the mature leukocyte population. Being an early marker of cells beginning to differentiate

gives reason to investigate it in the context of a being negative marker (140). Additionally, they are both surface proteins, making them easy to stain with antibodies and analyse via flow cytometry.

CD62L

CD62L (L-selectin) is a surface glycoprotein and a known marker for the leukocyte population (141, 142). It is thought to have roles in tethering to the endothelium (141, 142).

To determine its potential use as a negative marker for functional HSC content in expanded clones, 60 single ESLAM cells were plated via FACS into a fibronectin coated 96-well plate and underwent 28 days of expansion (118). At day 28, the entire clone was removed via multichannel pipette and analysed by flow cytometry.

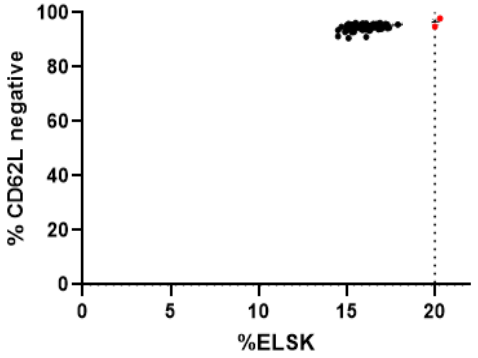

Figure 13: CD62L expression plotted against %ELSK, taken from flow cytometric analysis of 60 wells on day 28 of a 28-day expansion culture. Each culture began with a single ESLAM HSC. Cultures falling above the dotted threshold at 20% ELSK contain functional HSCs and are able to be transplanted

On day 28, all clones had more than 90% of their cells negative for this marker. Plotting %ELSK against % of cells that were negative for CD62L showed that as the expression of CD62L in the culture very slightly decreased, %ELSK slightly increased. (Pearson Correlation, p=0.000465, Figure 13). Although this may suggest that CD62L could be used as a negative predictor for %ELSK of a culture, the incredibly small difference expression between wells (90-99% negative in all samples), suggests that it is marks cells that have already left the ELSK phenotype. This makes it unusable as a marker of differentiation in 28-day expansion cultures.

Siglec F

We then moved on to test Siglec F (CD170) as a candidate marker for differentiation and HSC content of the cultures.

Siglec F is a murine sialic acid-binding immunoglobulin-like lectin F, and is a known marker for eosinophils (a mature myeloid blood cell type) (143). To determine the HSC content of clones supplemented with Siglec F we sorted 60 single E-SLAM cells via FACS into the inner 60 wells of a fibronectin-coated 96-well plate. The cells were cultured in 28-day expansion conditions (118), and the functional HSC compartment of each clone was assessed on day 28 using flow cytometry. On average there was a positive correlation with clones containing higher % ELSK cells (Pearson's R value= 0.623, p value = <0.00001), however the very best clones (right hand side of Figure

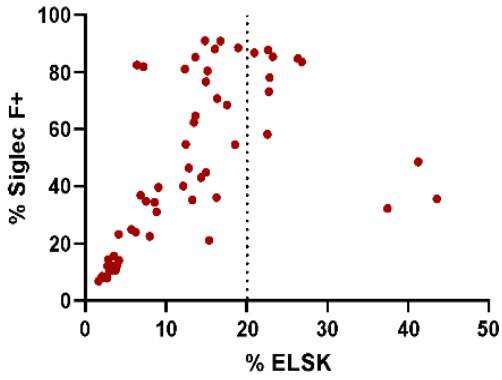

Figure 14: Siglec F expression measured by flow cytometry plotted against %ELSK. Data taken from 60 wells on day 28 of 28-day expansion protocol, cultures initiated with a single ESLAM HSC. Cultures falling above the dotted threshold at 20% ELSK contain functional HSCs and are able to be successfully transplanted

14) did not have particularly high Siglec F expression. This raises the interesting possibility that Siglec F begins to be expressed on the very first "non-HSC" progeny created by LT-HSCs. Combining Siglec F with the ELSK strategy and transplanting the positive and negative fractions would test this hypothesis and, if valid, could result in an even more accurate strategy to identify HSC-containing clones in culture.

3.2.3 Functional validation of candidate molecules

The molecules selected, VEGF and FSTL1, have previously both been linked to regulation of HSC self-renewal and/or survival (55, 144, 145, 146, 147) and so seemed choices that would likely result in a beneficial functional outcome.

VEGF

VEGF is a signalling protein present in the BM niche, and is known to be a key regulator of the vascular endothelium and haematopoiesis via an internal autocrine feedback loop (55). VEGF is co-expressed alongside its receptor on both freshly isolated and differentiated haematopoietic cells, this alone being enough to imply some level of VEGF autocrine activity is involved in haematopoiesis. Ablation of the VEGF gene and small-molecule intracellular inhibitors of the VEGFR have previously been shown to reduce HSC colony formation, size and survival (55).

To determine the effect of modifying VEGF signalling in expansion cultures, expansion cultures were initiated and supplemented with either recombinant VEGF (to activate VEGF signalling) or a neutralising VEGF antibody (to inhibit VEGF signalling). Molecules were tested at 3 different concentrations and flow cytometric analysis was undertaken at week 2, 3 and 4 timepoints (10% of clone at weeks 2 and 3, full clone at week 4). Neither activation or inhibition of VEGF significantly altered the %LSK of cultures compared to control. (Figure 15)

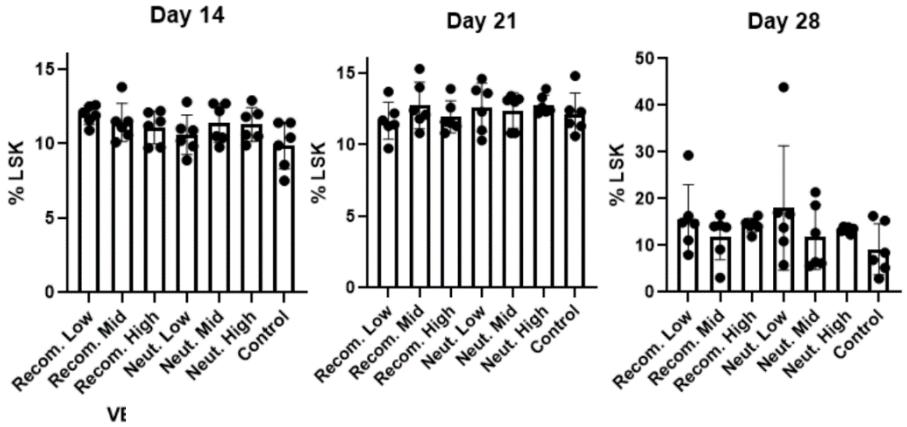

Figure 15: Inhibitors and activators of VEGF signalling were supplemented to the expansion culture media. The effect of this signalling modification on efficiency of HSC expansion is shown. This efficiency was determined using flow cytometry was used on days 14, 21, and 28 to analyse the %LSK of the clones initiated with single ESLAM cells. 6 single cell wells per condition.

Recom.	Low: 0.02ug/ml.	Mid: 0.2ug/ml.	High: 1ug/ml
Neut.	Low: 0.1ug/ml	Mid: 1ug/ml	High: 5ug/ml

FSTL1

This protein was selected to its presence in the HSC gene signature, a functional screen of regulators of HSC repopulation (146), as well as it being a secreted protein, making it convenient to add to culture media. Fstl1 is a glycoprotein key in angiogenesis (144), cell proliferation and differentiation, the immune response, and TGF- β/bone morphogenic protein signalling and has been widely researched in regards to its role in lung development (145).

120 single ESLAM cells were seeded in the expansion conditions – and Fstl1 was provided at 3 different concentrations (50, 200 and 500 ng/mL) and for the entire culture period. At day 14, (where the accuracy of the %ELSK has not been formally evaluated), the addition of *Fstl1* reduced %ELSK significantly (to the level of $p<0.05$) at low (50ng/ml) and mid (200ng/ml) concentrations (Figure 16).

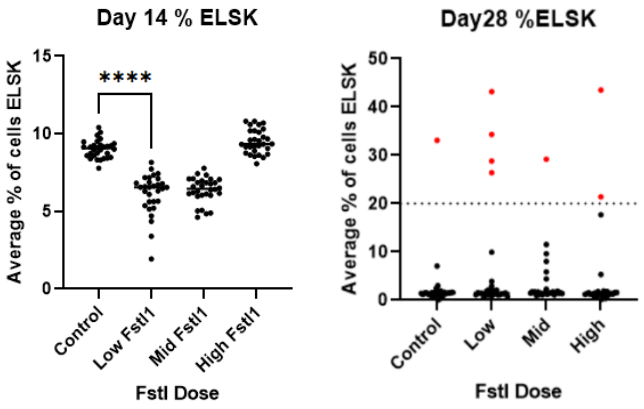

Figure 16: FSTL1 was supplemented to the expansion media for the entirety of the culture. The effect of Fstl1 treatment on the efficiency of HSC expansion was determined using flow cytometric analysis at day 14 and day 28 using ELSK as a measure for HSC content of the well . 30 single ESLAM wells per condition
Low = 50ng/mL Mid = 200ng/mL High = 500ng/mL
**** = significant to the level $p<0.05$

However, at day 28, where a minority of clones are above the 20% ELSK threshold for containing functional, transplantable HSCS, the number of cultures above the threshold was higher in cultures treated with 50ng/mL FSTL1 (4/30) than control (1/30) but a chi-squared test comparing the number of cultures >20% LSK on day 28 at each concentration gives a p-value of 0.359752. These data from Days 14 and Day 28 appear contradictory – with low concentrations of FSTL1 initially decreasing, and then increasing %ELSK. This may be because FSTL1s effects result from combating and discouraging the negative impact of differentiated cells – which would already have accumulated in the culture in the first 14 days, and wouldn't have existed in the initial population.

This suggests that while treatment with 50ng/ml FSTL1 seemed promising, further experiments would need to be undertaken to determine its mechanism, and a wider range of lower doses should be tested for determining an optimal dose of recombinant FSTL1.

Taken together, the data presented in this thesis demonstrate that ESAM expression can replace the need for the *Fgd5* to identify functional HSCs *in vitro*, Siglec F is a promising candidate for further distinguishing between LT-HSCs and ST-HSCs, and low doses (~50ng/mL) of FSTL1 might improve the proportion of single HSCs that can create a large number of daughter HSCs over the 28 day expansion period.

4.0 Conclusions and Discussion

For decades, researchers have been trying to expand the *in vitro* HSC pool, with little major success until the recent breakthrough of a 28-day expansion culture which expanded functional HSCs >200-fold (118). To approach the challenge of refining expansion, I set out to identify a new robust marker strategy for functional, expanded HSCs. Removing the need for the reporter gene *Fgd5* would be an incredibly valuable step as not all mice are crossed on this background - significantly limiting the applicability of this reporter strategy to other strains including many disease models, as well as to human HSCs. I also aimed to further improve the pre-existing expansion protocol via manipulating the culture conditions and trying to identify new predictive markers for 'good' clones. Increasing the number of self-renewal divisions and purity of the expansion cultures will allow refined production of HSCs for both clinical and scientific use.

From the series of experiments conducted in this project, several conclusions can be drawn. Firstly, ESAM is able to replace the use of the *Fgd5* reporter mouse and appears to be as efficient as CD150 at marking expanded *in vitro* HSCs. This is shown through ESAM's strong correlations with %LSK and %ELSK, CD150 and *Fgd5* expression- marking the same, HSC-rich, clonal populations. Importantly, the correlation between FELSK and EELSK is highly significant.

I next moved on to focus on the potential to recognise the clones richest for HSCs earlier in the 28-day expansion culture. However, it was found that the clones with the highest %LSK at day 14 or day 21 were not maintained as the best throughout the culture period, showing that data collected earlier than Day 28 as a predictive measure would be unreliable with the strategies tested so far. The next steps involved testing novel compounds in the 28-day expansion culture to improve HSC self-renewal divisions and purity of the resultant clones. However, neither the alteration of VEGF signalling, nor Fstl1 treatment, resulted in significantly improving existing levels of expansion. This

was inconsistent with prior studies showing that these proteins could influence HSC function and maintain HSC survival (55, 144, 145, 146, 147). In light of this, additional experiments exploring a wider range of concentrations and increased sample sizes would be valuable. FSTL1, in particular at the lower dose of 50ng/mL, produced clones containing >20% ELSK at a rate of 4 fold higher compared to control, although this was from a single biological replicate and would need further validation. A logical focus going forward would be to test a refined dose response curve (e.g. of 1ng/mL, 10ng/mL, 50ng/mL, and 100ng/mL) to help specify the optimum concentrations and determine whether provision of FSTL1 enhances HSC self-renewal *in vitro*.

Finally, I aimed to identify potential new markers indicative of high HSC content. Although the mature leukocyte marker CD62L did not appear promising as a marker able to identify successful clones, Siglec F appeared to be more promising. Seeming to mark the progenitor populations, but not the LT-HSC population, it has potential to be added to existing reporter strategies, including LSK and ELSK, to subdivide the marked cells and so identify the most primitive HSCs. This warrants functional validation via taking the highest performing clones, separating the Siglec F positive and negative populations, and transplanting these separately into irradiated mice for long term, and potentially also secondary transplantation experiments. Studying the functional consequences of Siglec F expression could further verify if it marks specifically for the progenitor population.

The work in this thesis prompts a need to further validate ESAM as a marker for the functional HSC compartment in formal transplantation assays. Our work underscores the importance coupling molecular experiments to functional transplantation studies – the gold standard in functional HSC validation. To test ESAM as a marker, one could expand new 28-day cultures and sort for clones with high or low expression of ESAM. The clones will them be split for functional assays (transplantation) and gene expression assays (RNAseq) to ensure that both the molecular and functional features of HSCs are in the ESAM^{+} population (Figure 17).

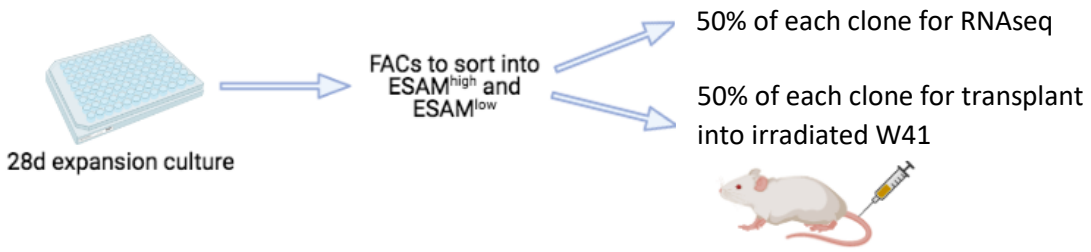

Figure 17: Schematic depicting paired molecular and functional assays following 28-day expansion. After initiating 28-day expansion cultures, the clones will be split into ESAM^{high} and ESAM^{low} fractions. 50% of each fraction will be used for transplant into irradiated W41, and 50% for RNAseq.

Removing the need for researchers to cross the *Fgd5*$^{+/ZsGreen}$ mouse into any other transgenic strains of interest would make HSC identification in expansion culture from various mouse strains increasingly time and cost efficient as well as potentially opening it up to human HSCs (i.e, ESAM may also mark human HSCs in culture). Combining increased efficiency of HSC reporting with improved *in vitro* expansion would allow studies previously restricted by HSC numbers in mice (e.g., Cabezas-Wallscheid et al. used almost 1000 mice to complete HSC proteomic analysis (43)) to take place more easily and more often.

In addition to focusing on expanding murine HSCs, the next major step will be translation into human cells - a process already started by our Japanese collaborators whose proof-of-principle experiments show a slightly adapted version of this protocol being able to modestly expand human HSCs - increasing their transplantability compared to the initial cell suspension in the process. The ability of this system to expand both human and mouse HSCs would vastly reduce the number of animals needed for HSC research, and provide a more accurate HSC *ex vivo* model for human disease. Refining *in vitro* human HSC expansion would also allow more large scale experiments and screens to take place, and would enable production of unlimited mature blood cells out of the body – vastly improving availability and efficiency of clinical blood transplants.

Lehnertz et al. (148) have very recently identified Hepatic Leukaemia Factor (HLF) as an incredibly specific surface marker for human HSCs through transcriptomic analysis of fresh and *ex vivo* cord-blood derived HSCs. Importantly, cells expressing HLF contain all HSC functional activity both *in vitro* and *in vivo* in serial transplantation settings – a property that is very rare for surface markers, as their expression often changes over time in culture (74,76). Coupled with improving *in vitro* human HSC expansion protocols, being able to better identify the HSC population of the clone using novel reporters such as HLF is incredibly powerful as both a scientific and clinical tool.

In summary, the possibility of a potentially universal HSC identification strategy, coupled with continual progress in both murine and human HSC *in vitro* expansion is exciting both in terms of the huge clinical impact this could have through increasing cell numbers for gene therapy and HSC transplantation, but also on a purely scientific front. This would enable previously unachievable experimental approaches such as metabolomics and proteomics to take place more easily, as well as creating a platform with which we can dissect the properties governing HSC self-renewal and heterogeneity efficiently and at a large scale.

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