Extrinsic regulation of fate choice in mouse haematopoietic stem cells

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September 2018

This dissertation is submitted for the degree of Doctor of Philosophy

Abstract

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The mechanisms regulating stem cell self-renewal, proliferation, and differentiation are still not fully understood. Improving our knowledge of these processes will not only provide greater insight into stem cell biology but will also have major implications in the understanding of cancer development, since numerous cancers can trace their origins back to single stem cells.

It has previously been shown that variations in culture conditions can alter fate choice in haematopoietic stem cells (HSCs). For decades cytokines have been used to maintain and expand mouse and human HSCs *in vitro*, with a number of studies demonstrating that cytokines directly influence HSCs fate choice. In this thesis, I explored the extrinsic regulation of mouse HSCs fate choice using three different approaches:

1) Modulation of cytokine concentration
2) Establishment of minimal conditions to retain HSCs function *in vitro*
3) Development of 3D matrices to provide physical support beyond liquid culture

The first results chapter (3.1) identifies that the amount of Stem Cell Factor (SCF) signalling does not alter the number of functional HSCs retained, but may alter the degree of clonal expansion post transplantation. Chapter 3.2 demonstrates that minimal cell culture conditions depending solely on gp130 signalling can maintain HSCs as single cells for an extended period of time. These cells retain full functional repopulation potential but present with a myeloid differentiation bias. Finally, Chapter 3.3 represents a first proof-of-principle series of experiments showing that HSCs are better supported on soft substrates, implicating physical forces in influencing HSCs maintenance *ex vivo*.

In conclusion, these findings further confirm that SCF is a key regulator of HSCs fate, but is not essential for the retention of HSCs function. The newly established minimal cell culture medium allows the specific investigation of various molecules affecting HSCs fate choice at the single cell level. Furthermore, it offers a new platform for studying exit from quiescence in a controlled manner over several days. This latter aspect could have major implications for the delivery of gene therapy and for HSCs expansion efforts in the future.

Declaration

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

It does not exceed the prescribed word limit for the relevant Degree Committee.

Preface

Chapter 3.1

All work carried out was supervised and guided by Dr. David Kent. I carried out all animal experiments after receiving training from David Kent, Tina Hamilton and Dean Pask. Miriam Belmonte aided in animal sample preparation as well as cell culture on several occasions. Dr. Winnie Lau, Dr. Nicola Wilson and Sonia Nestorowa advised me on the single-cell RNA sequencing and developed the modified protocol used in this thesis. I was involved in the bioinformatic analysis of the scRNAseq data, which was mainly carried out by Dr. Fiona Hamey, Lila Diamanti, Dr. Hugo Bastos and Rebecca Hannah. All single cell sorts were carried out at the Flow Cytometry Core Facility at the Cambridge Institute for Medical Research (CIMR) by Dr. Reiner Schulte, Dr. Chiara Cossetti, and Gabriela Grondys-Kotarba.

Chapter 3.2

All work carried out was supervised and guided by Dr. David Kent. I carried out all animal experiments as stated above. Miriam Belmonte aided me in animal sample preparation as well as cell culture on several occasions. Adam Wilkinson provided me with the medium make up for the serum-free culture. Daniel Bode normalised the single-cell index data. Serena Belluschi isolated the human HSCs at the NIHR Cambridge BRC Cell Phenotyping Hub facility. RNA library preparation was carried out at the Genomics Core facility of the Cambridge Stem Cell Institute. I was involved in the bioinformatic analysis of the RNAseq data, which was carried out by Dr. Fiona Hamey and Hugo Bastos. All single cell sorts were carried out at the Flow Cytometry Core Facility at the Cambridge Institute for Medical Research (CIMR) by Dr. Reiner Schulte, Dr. Chiara Cossetti, and Gabriela Grondys-Kotarba.

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All work carried out was supervised and guided by Dr. David Kent. I carried out all animal experiments as stated above. Atomic force macroscopy was carried out by Alejandro Carnicer-Lombarte. Dr. Carla Mulas provided me with matrix gels and guided me when I produced them myself. All single cell sorts were carried out at the Flow Cytometry Core Facility at the Cambridge Institute for Medical Research (CIMR) by Dr. Reiner Schulte and Dr. Chiara Cossetti.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor Dr. David Kent for giving me the opportunity to carry out my PhD in his lab, for his guidance and support at any stage of this experience, and for giving me the freedom to pursue research questions in my own way. Through his knowledge, encouragement and remarkable positive attitude, I truly developed as a scientist and person, and feel prepared to take the next steps in my research career.

I would very much like to thank my second supervisor Dr. Elisa Laurenti for her insightful comments and encouragement to pursue hard questions.

I thank my fellow lab mates for stimulating discussions, for all the fun we had in the last years and for refilling the snack drawer. I am especially grateful to Miriam Belmonte for never saying no to helping me out and to Mairi Shepherd for all the guidance she gave me on my presentation styles. I would furthermore like to thank all the members of the Göttgens lab, the Green lab, the Laurenti lab, the Hodson lab, and the Ringshausen lab for useful discussions and advice and especially for creating a stimulating environment to conduct my PhD in.

My sincere thanks also goes to Tina Hamilton and Dean Pask, who trained and supported me in animal handling.

I am very thankful to Dr. Reiner Schulte, Dr. Chiara Cossetti and Gabriela Grondys-Kotarba for long hours at FACS, for fun conversations and helpful comments to improve my experiments.

I will never forget my fellow Wellcome Trust girls: Fiona Hamey, Livvi Harris and Lucia Cordero Espinoza. I am so glad I have met you, I could not have asked for a more interesting and diverse bunch of students to go through this experience with together.

A special thanks goes to my partner Phillip Smith for his love and support throughout the entire process of finishing my PhD, especially when writing up.

Last but not least, I would like to thank my parents for supporting me throughout the last 9 years that led up to finally submitting my PhD.

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°C	Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
µm	Micrometre
7-AAD	7-amino-actinomycin D
AF	Alexa Fluor
AFM	Atomic Force Microscopy
AGM	Aorta-gonad-mesonephros
AKT	Protein kinase B
Ang-1	Angiopoietin 1
AP-1	Activator Protein-1
APC	Allophycocyanin
Appx	Appendix
ASPA	Animals Scientific Procedures Act 1986
Bala	Balanced
BD	BD Biosciences
BIT	Bovine serum albumin, insulin, and transferrin
BM	Bone marrow
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
BV	Brilliant violet
CAR cells	CXCL12 abundant reticular cells
CB	Cord blood
CD	Cluster of differentiation
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CFC	Colony forming cell
CFSE	Carboxyfluorescein succinimidyl ester
CFU-S	Colony forming unit - spleen
cGy	centi-Gray
CLP	Common lymphocyte progenitor
CMP	Common myeloid progenitor
Col I	Collagen Type I
Cre	Cre recombinase
CRU	Competitive repopulating units
DAPI	4′,6-diamidino-2-phenylindole
DEX	Differentially expressed

DMSO	Dimethyl sulfoxide
Dox	Doxycyclin
E	Embryonic day
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELDA	Extreme limiting dilution analysis
Elf-4	E74-like factor
EPO	Erythropoietin
ERK	Extracellular signal–regulated kinase
Exp	Experiment
F	Force
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
Fig	Figure
FITC	Fluorescein isothiocyanate
FL	Flt3 ligand
FoxO	Forkhead box O
G	Granulocytes
GFP	Green fluorescent protein
GO	Gene ontology
G phase	Gap phase
GCSF	Granulocyte colony stimulating factor
GMLP	Granulocyte/Monocyte/Lymphoid progenitors
GMP	Granulocyte/Monocyte progenitor
h	hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSA	Human serum albumin
HSCs	Haematopoietic stem cell
HSPC	Haematopoietic stem and progenitor cell
IFN	Interferon
Ig-like	Immunoglobin-like
IL	Interleukin
Ile	Isoleucine
IMDM	Iscove's Modified Dulbecco's Medium
IT	Intermediate term
ITS-X	Insulin-Transferrin-Selenium-Ethanolamine
KD	Knock-down
Kd	Dissociation constant
KL	Kit ligand
KO	Knock-out

LDA	Limiting dilution analysis
Lepr	Leptin receptor
Leu	Leucine
Lin	Lineage
LMPP	Lymphoid-primed multipotent progenitor
LPS	Lipopolysaccharide
LSK	Lineage^{neg} Sca-1^{+} c-Kit^{+}
LT	Long term
LTC-IC	Long term culture initiating cell
LTRC	Long term repopulating cell
Ly-bi	Lymphoid-biased
M	Monocytes
mg	Milligram
M phase	Mitosis phase
MAPK	Mitogen-activated protein kinase
MEF	Myeloid Elf-1-like factor
MEK	MAPK/ERK Kinase
MEP	Megakaryocyte/Erythrocyte progenitor
MGF	Mast cell growth factor
min	Minutes
miRNA	Micro-RNA
MkP	Megakaryocyte progenitor
mL	Millilitre
mM	Millimolar
MolO	Molecular overlapping
MPP	Multipotent progenitor
Mx1	Mx dynamin-like GTPase
My-bi	Myeloid-biased
MyoII	Myosin II
N/m	Newton per metre
N/A	Not applicable
Nes	Nestin
ng	Nanogram
NGF	Nerve growth factor
NH_4CL	Ammonium chloride
NK	Natural Killer cell
nN	Nanonewton
NOD/SCID	Nonobese diabetic/severe combined immune deficient
p.	page
p-adj.	Adjusted P-value

P	P-value
PB	Peripheral blood
PBS	Phosphate-buffered saline
PBSFE	PBS supplemented with 2% FCS & 50mM EDTA
PCA	Principle component analysis
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PGE2	Prostaglandin E2
Phe	Phenylalanine
pHSCs	Phenotypic HSCs
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
pI-pC	Polyinosinic-polycytidylic acid
Pos	Positive
Pro	Proline
PTEN	Phosphatase and tensin homolog
R	Restriction point
r	Radius
Rb	Retinoblastoma
Rho	Rhodamine123
RPS6	Ribosomal protein S6
RSK1	Ribosomal protein S6 kinase alpha-1
RT	Room temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
s	Second
S phase	Synthesis phase
SAV	Streptavidin
SCF	Stem cell factor
scRNA seq	Single cell RNA sequencing
SD	Standard derivation
SDF1	Stromal cell derived factor 1
SE	Standard error
SEM	Standard error of the mean
Ser	Serine
SF	Steel factor
SFEM	Serum-free expansion medium
Sl	Steel gene
SP	Side population
ST	Short term
STAT	Signal transducer and activator of transcription
TGF beta	Tumour growth factor beta

TKO	Triple knock-out
TNF	Tumour necrosis factor
TPO	Thrombopoietin
Tyr	Tyrosine
U/mL	Units per millilitre
Val	Valine
W	White-spotting gene
W41	C57BL/6$^{W41/W41}$-Ly5.1 mouse
WBM	Whole bone marrow
WT	Wild type

1 Introduction

1.1 Haematopoiesis and haematopoietic stem cells

Haematopoiesis is the process by which all mature blood cells are produced[1]. In the bone marrow of a healthy adult, an estimated 10^{11}–10^{12} blood cells are produced every day[2,3].

Maintaining the balance between continuous blood cell production and depletion is essential, as disruption of homeostasis can result in, among others, anaemia, myeloproliferative neoplasms, and leukaemia[4].

Blood is a tissue consisting of an aqueous plasma and a cellular component[5]. Blood plasma makes up 55% of the total blood volume. It consist mainly of water (95%) and contains soluble proteins, circulating nucleic acids, electrolytes as well as metabolites such as glucose, vitamins, and amino acids[6]. The cellular component is composed of erythrocytes, thrombocytes, and white blood cells (leukocytes) such as lymphocytes (B and T cells), monocytes/macrophages, and granulocytes. There are four types of granulocytes, which are neutrophils, eosinophils, basophils and mast cells. Erythrocytes are involved in gas exchange between tissue and lungs, mainly transporting oxygen to the tissues. Megakaryocytes in the bone marrow produce thrombocytes (platelets), which play an important role in haemostasis by mediating blood coagulation and tissue repair. Leukocytes function in the defence of the immune system against infectious agents[1,5].

All cellular blood components are derived from a very rare subset of haematopoietic stem cells (HSCs) at the apex of the haematopoietic hierarchy. HSCs are multipotent, therefore can give rise to cells of all blood lineages, and have the ability to self-renew, thus give rise to one or two equally potent daughter cells upon division[2,7].

In human, it is estimated that 1 in $3x10^6$ bone marrow cells is an HSCs, which is > 2-fold more frequent than in peripheral blood. In cord blood 1 in $0.93x10^6$ cells is an HSCs, as determined by limiting dilution analysis in a xenograft mouse model[8]. This differs from mouse, in which functional studies of whole bone marrow have shown, that approximately $1x10^5$ of total bone marrow cells are HSCs[9,10].

1.1.1 Developmental haematopoiesis

The majority of studies defining vertebrate developmental haematopoiesis have used mouse, chicken, and zebrafish as model organisms. However, the underlying processes are broadly applicable to other vertebrates including humans[11,12].

In vertebrates, blood cell development occurs in two separate waves; the primitive wave and the definitive wave. In mice, during the primitive wave, erythroid progenitors appear in so-called blood islands that line the extra-embryonic yolk sac at embryonic day E7.5 (E17 in human)[11,13-15]. Their main function is to oxygenate the surrounding tissue in the rapidly developing embryo[11]. These haemangioblast-derived primitive cells form large nucleated erythrocytes that lack self-renewal capacity and as a consequence, the primitive wave is of a transient nature[11,13,14].

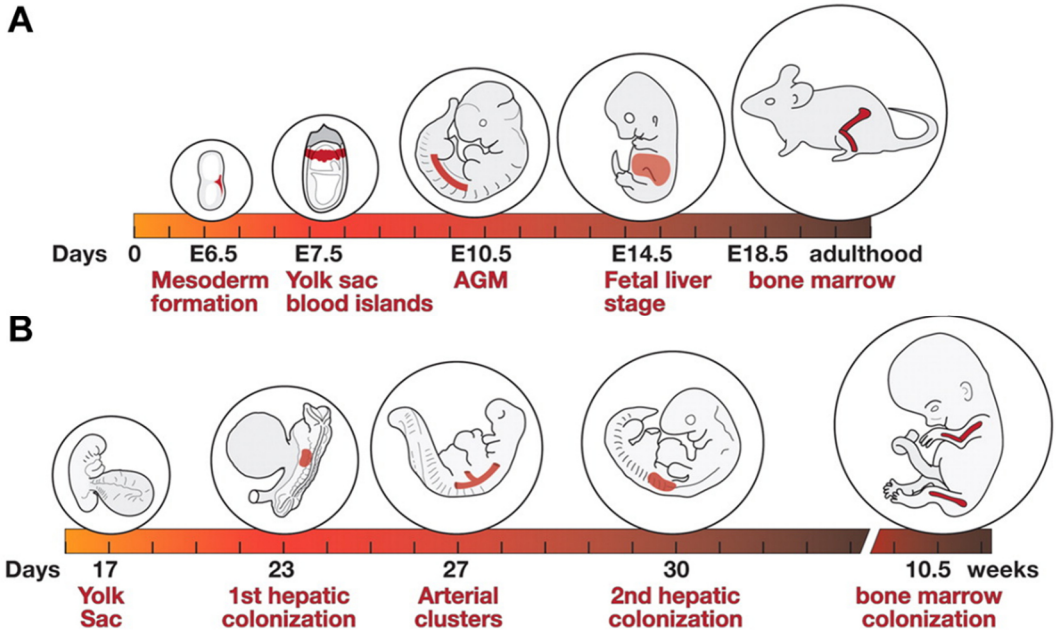


Figure 1: Comparison of developmental stages in haematopoiesis between the mouse and human embryo[15].

A) Mouse haematopoietic development. Mesoderm forms during gastrulation at E6.5, followed by the development of blood islands in the yolk sac at E7.5. At 10.5 HSCs emerge in the AGM region, placenta and arteries (only AGM shown). At E14.5 haematopoiesis has moved to the liver, followed by bone marrow colonisation shortly before birth at E18.5.
B) Human haematopoietic development. At embryonic day 17, haematopoiesis originates in the yolk sack. The liver is first colonised around day 23. Arterial clusters appear between day 27 and day 40, 3 days before a second hepatic colonisation by $CD34^+$ progenitors. At 10.5 weeks the bone marrow is colonised. Figure taken from Baron et al. *Blood* (2012)

The definitive wave begins at E8.25 in mouse and E21 in human with the establishment of circulation[15,16]. Definitive erythromyeloid progenitors first arise in the extraembryonic yolk sac followed by the placenta at E9.5. The aorta-gonad-mesonephros (AGM) region is the first intraembryonic region to harbour haematopoietic progenitors at E10 but vitelline and umbilical arteries are also sites of development[11,13,14,16].

Starting at E9 in mice the foetal liver (FL) is colonised by haematopoietic cells that were generated in other tissues such as the placenta, yolk sac and AGM[13,14,16]. Between E11 and E12, the foetal liver becomes the main region for definitive haematopoiesis until the formation of bone marrow (BM) cavities shortly before birth around E15. In human, the first hepatic colonisation occurs at E23, followed by a second colonisation at E30. At around 10.5 weeks, the bone marrow is colonised[11,15]. Throughout adulthood the bone marrow is the primary haematopoietic organ[13,14,16].

1.1.2 The bone marrow niche

In 1978, Raymond Schofield coined the term "niche" for the unique environment in which interactions of the stem cell with other cells ensures its ability to self-renew and stay in a largely dormant state[17]. As discussed above, shortly before birth, HSCs begin to migrate from the foetal liver to the bone marrow where they remain and continue to proliferate during adulthood[13,14,16].

The bone marrow is a highly vascularised organ comprised of a medullary cavity encased in cortical bone. The endosteum lines the inside of the bone. Trabecular bone, also referred to as spongy bone, forms the internal bone tissue. Protrusions create a honeycomb or sponge-like structure enlarging the bone surface and ensuring that cells in this area are in close proximity to the bone in this area[18]. Bone itself is produced by osteoblasts which are highly active in the trabecular region[19,20]. Longitudinal arteries in the cortical bone branch into radial arteries and arterioles in the endosteum, which in turn drain into sinusoids and eventually coalesce into a central sinus forming the venous circulation, which allows entry and exit of HSCs and other haematopoietic cells into and from the blood circulation[18,21,22].
Historically, it was thought that the endosteal zone was home to HSCs[23,24], which was supported by findings that phenotypic HSCs associate with the bone surface *in vitro*[25] and *in vivo*[26], in addition to observed functional changes in HSCs upon ablation of osteoblasts or other bone components[27-29]. This view of the endosteal stem cell niche was first called into question by findings that showed that deletion of key cytokines such as CXCL12 and SCF in osteoblasts did not alter HSCs function[30,31]. In more recent years the potential existence of a vascular niche has been investigated.

Besides osteoblasts[20,27,32], the bone marrow contains a variety of different cell types such as, endothelial cells[31,33], CXCL12-abendunt reticular (CAR) cells[34], Leptin receptor-expressing

(LepR$^+$) perivascular cells[35], Nestin-expressing mesenchymal cells (Nes$^+$)[36], and NG2$^+$ pericytes which overlap in their expression with LepR$^+$ and Nes$^+$ stromal cells[37] (Figure 2).
It has been shown that these cells are involved in the regulation of HSCs quiescence, proliferation, and differentiation through the production of cytokines and other molecules including stem cell factor (SCF)[31,33,37], osteopontin[20,27], stromal cell derived factor 1 (SDF1 or CXCL12)[34-37] G-CSF (granulocyte colony stimulating factor)[38], interleukin-6[39], and angiopoietin[25,36].

Immunophenotypic HSCs have been found to be in close proximity to sinusoids which are surrounded by perivascular cells that show high expression of SCF and CXCL12. A common observation is that upon ablation of these cells or disruption of their cytokine production, HSCs numbers are greatly reduced. This supports the notion that these cells play an important role in HSCs maintenance and challenges the view of specific and restricted geographical space for HSCs along the endosteal surface of the bone[31,34-36,40,41].

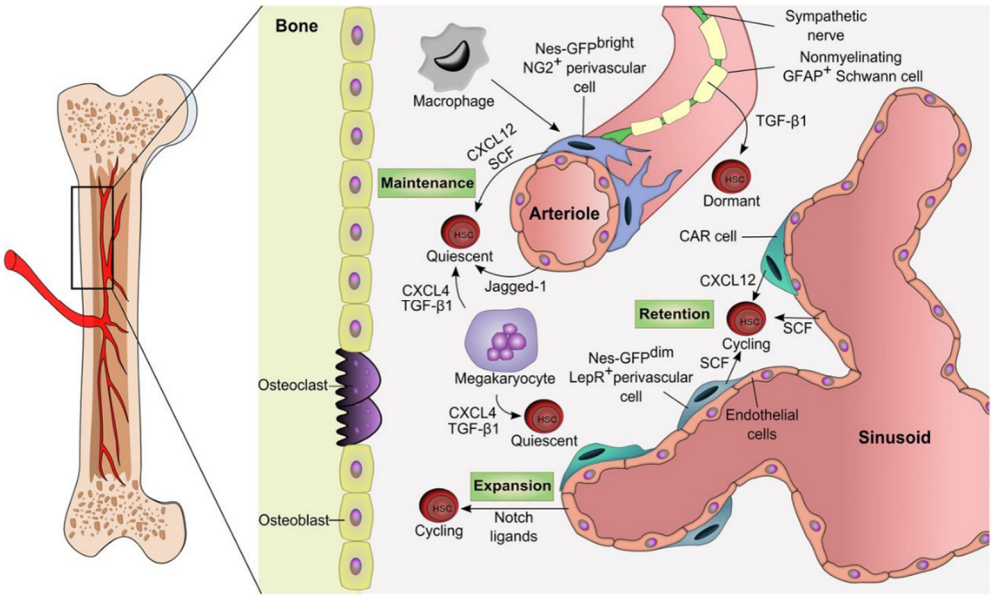


Figure 2: The adult bone marrow niche[42].
HSCs in the bone marrow can be found around arterioles as well as sinusoids receiving cytokine signals such as SCF and CXCL12 secreted by endothelial cells and perivascular cells as well as TGF-β1 produced by Schwann cells and megakaryocytes.

It remains unclear if there is one particular bone marrow niche location or if different niches can support quiescent and/or actively cycling HSCs[32,43-45]. A recent report suggests that phenotypically, HSCs seem to be randomly localised among the bone marrow with sinusoids being the sole site of egression into circulation[46]. Therefore, live *in vivo* imaging supported by

distinct and reliable HSCs markers is necessary to gain further insight into where HSCs localise under homeostatic conditions. Several reporters have recently been described (α-catulin[47], Hoxb5[48], and Fgd5[49]) with varying levels of success, however none of these mark HSCs exclusively.

1.1.3 Early evidence for the existence of a haematopoietic stem cell

In the early 1950s it was discovered that the intravenous injection of whole bone marrow cells could recover the irradiated haematopoietic system of a recipient mouse[50,51]. This research inspired the first successful human bone marrow transplant for the treatment of a leukaemic patient in 1956, where multipotency and self-renewal capacity of HSCs were formally demonstrated for the first time in a human[7,50,51].

In 1961, pioneering experiments by Till and McCulloch described the formation of colonies consisting of haematopoietic tissue in the spleens of mice that had previously been transplanted with isolated bone marrow. These colonies were termed colony forming unit-spleen (CFU-S), and each colony was suggested to be derived from a small number of cells or even one single cell. This represented the first formal evidence for the existence of an HSCs in the bone marrow[52]. Shortly after that, the same group established that the haematopoietic system consists of three compartments: i) the stem cell compartment with multipotent cells of extensive proliferative capacity, ii) the differentiated cell compartment containing cells with limited proliferative capacity and iii) a mature cell compartment consisting of specialised cells.
Additionally, tritiated thymidine uptake experiments provided early evidence that colony-forming cells are quiescent[53], which will be further be discussed in 1.2.
The clonality and self-renewal ability of CFU-S was further shown by serial transplantation of CFU-S. Interestingly, colony-forming cells gave rise to colonies with widely differing characteristics as judged by morphology and the distribution of colony-forming cells among colonies. This was early evidence for heterogeneity in the stem cell compartment[54], which will be discussed at length in section 1.3.
Complementing these *in vivo* studies, clonal *in vitro* assays were established to analyse function and differentiation capacity of single cells. Short-term semi-solid culture, based on agar or methylcellulose medium mixtures, were used to analyse single cell clonality and differentiation capacity[55,56]. Later, long-term cell initiating culture (LTC-IC) assays were developed to measure self-renewal potential and differentiation capacity of the input cell by their ability to produce myeloid and or lymphoid progeny for a minimum period of 5 weeks[56-60].

For many decades since, the haematopoietic system has been intensely studied through *in vitro* limiting dilution colony formation assays as well as *in vivo* tracking of haematopoietic clones, using retroviral marking, cellular barcoding, fluorescently tagged cells or single cell transplantation[7,61,62]. Ultimately, this has resulted in the identification and characterisation of the HSCs, which is to date, the best characterised adult mammalian tissue specific stem cell[2,7].

1.1.4 Isolation of adult mouse and human HSCs

The gold standard to definitively prove that a purified HSCs is indeed a long-term repopulating cell with durable self-renewal activity is serial transplantation into irradiated recipient mice with long-term multi-lineage reconstitution in primary (≥16 weeks) and secondary (≥16 weeks) recipient animals. This contribution is usually measured by the presence of granulocytes (G), monocytes (M), B- and T-cells in the peripheral blood. To be considered successfully reconstituted, the contribution of donor cells typically must account for at least 1% of the total whole blood count at the later stages of this assay[63]. Interestingly, some HSCs do not contribute to >1% of lymphoid lineages in primary transplants but can generate an adequate amount of GM, B- and T-cells in secondary recipients[64]. These findings, and the late appearance of some single cell-derived clones[64,65] call the above mentioned gold standard into question.

HSCs with different self-renewal potential and repopulating kinetics can be identified and isolated based on size, granularity, cell surface antigen profile and different enzymatic activity, such as the cells' ability to efflux Rhodamine123 (Rho123) or Hoechst 33342 (Side population, SP)[7,66]. Antibodies most commonly used for HSCs identification include those raised against CD117 (c-Kit), Stem cell antigen-1 (Sca-1), CD201 (EPCR), the SLAM markers CD48 (negative) and CD150, CD135 (negative, fms like tyrosine kinase 3 (Flt3) or Foetal liver kinase 2 (Flk2)), CD49b (negative) and CD34 (negative)[7,67,68]. Notably, relatively few of the surface markers used for identification are required for HSCs function[7].

In the mouse system, several groups have reported HSCs purities of >50% through different sorting strategies[10,41,69], with a recent report showing that single cell transplantation using HSCs defined by the surface marker combination $CD45^{+}CD150^{+}CD48^{-}EPCR^{higHSCs}a\text{-}1^{high}$ yields a purity of 67% in primary recipients[68] and 50% in secondary transplantation (unpublished, Kent lab).

As mentioned above, novel gene reporter mice such as α-catulin[47], Hoxb5[48], and Fgd5[49] have been generated to aid in the FACS (fluorescent activated cell sorting) isolation of HSCs and the detection of HSCs in their putative niche *in vivo*. HSCs are enriched in the positive population of each of these reporters, with estimated HSCs frequencies of 14.9%, 47.6% (in combination with other HSCs surface markers), and 31.2% for *α-catulin*, *Hoxb5* and *Fgd5* respectively, in primary transplantation. Only α-catulin$^+$ cells were transplanted as single cells with 15.4% of recipients showing multi-lineage reconstitution, which could be improved by co-staining with c-Kit to 28.6%[48]. Recent single cell expression profiling of the HSPC compartment confirms that *Hoxb5*, *Fgd5*, and *α-catulin* are indeed predominantly expressed in the long-term HSCs cluster[70].

Even though these sorting strategies result in a relatively pure population of HSCs, they each exclude cells that are functional stem cells because some HSCs are detectable in the non-phenotypic cell fraction – a striking example of which was described for CD150[41,71,72]. It therefore remains unclear whether HSCs transit between phenotypes, but irrespective of this, it is evident that there are less rigorous phenotypes that include more HSCs at lower purities. Differences in these contaminating cell fractions has a significant impact on the interpretation of gene expression studies, especially those at the single cell level.

Mouse HSCs are more extensively characterised than their human counterparts. While both systems share many conceptual similarities (e.g. rare HSCs, hierarchically organised, stepwise differentiation into mature cells), there are a number of differences which make it challenging to translate all of the research findings. In particular, there are major differences in the cell surface marker combinations used for purification and the governing signalling pathway[3]. One such example is the expression of CD34 (absent on mouse HSCs), which is the primary marker for enriching human HSCs. CD34 expression enriches for LTC-IC, which for human cells is a reliable read out for multipotency but not necessarily *in vivo* repopulation potential[3,73]. However, similar to CD150^{neg} in mouse[71], it has been shown that cells lacking CD34 expression are capable of multi-lineage, long-term repopulation in recipient animals[74,75]. Interestingly, engraftment in mice with CD34^{neg}CD38^{neg} HSCs, is detectable at later stages than that of CD34$^+$CD38^{neg} HSCs[75], suggesting that the former represents a specific HSCs subtype similar to mouse alpha-HSCs, which will be discussed in 1.3.

In addition to the absence of lineage markers, another potent marker that enriches for human HSCs is CD90 (Thy-1), which is expressed on human foetal liver, cord blood, and bone marrow HSCs[76-78]. Absence of CD38 expression further enriches for LTC-ICs as well as cells capable of multi-lineage repopulation in nonobese diabetic/severe combined immuno-deficient (NOD/SCID) mice. CD38 expression is also correlated with increased differentiation and $CD34^+CD38^+$ contain a higher proportion of actively cycling cells than $CD34^+CD38^{neg}$ Haematopoietic Stem and Progenitor cells (HSPCs)[78-80].

To date, the combined expression of $Lin^-CD34^+CD38^-CD45RA^-CD90^+CD49f^+$ is used to obtain a population of HSCs from umbilical cord blood, which are capable of reconstituting the haematopoietic lineages in nearly 10% of recipient immuno-deficient mice[81]. Notably, the xenograft setting of these experiments may restrict the ability of all LT-HSCs to successfully repopulate, thereby potentially under-estimating the frequency in various cell preparations. This is supported by the higher percentage observed in long-term multi-lineage *in vitro* assays[81].

1.2 The cell cycle and quiescence in haematopoietic stem cells

1.2.1 Mammalian cell cycle regulation

The mammalian cell cycle is tightly regulated through a network of cyclins, cyclin dependent kinases (CDKs), as well as their antagonists, the cyclin-dependent kinase inhibitors (CDKIs)[82,83]. Cyclins bind to CDKs leading to the phosphorylation of downstream target proteins involved in the progression of one cell cycle phase to the next[83]. The cell cycle can be subdivided into 4 phases: i) A Gap phase (G_1) which proceeds to the ii) DNA synthesis (S) phase, followed by a iii) second Gap phase (G_2), which in turn progresses to the iv) mitosis (M) phase[84]. Each phase is characterised by the specific families of cyclins and their corresponding CDKs, with different cyclin D family members playing a pivotal role in G_1 to S phase progression[84]. Importantly, in each phase cell cycle checkpoints are in place to ensure proper cell division[84,85]. These checkpoints are mediated by CDKIs, which inhibit CDKs and in doing so cause cell cycle arrest. Depending on their target specificity CDKIs can be subdivided into two families, the Cip/Kip family and the Ink4 family. The former is able to inhibit several different CDKs, whereas the latter inhibits CDK4 and CDK6 specifically[82,84].

The checkpoint in G_1 that needs to be overcome to progress into S phase and thus into the mitotic cell cycle, is referred to as the Restriction point (R)[84,85]. Here, the tumour-suppressor Retinoblastoma (Rb) becomes fully phosphorylated by CDK4/6. In its hyper-phosphorylated form, Rb no longer binds and inhibits, members of the E2F transcription factor family which in turn activate gene transcription[84,86]. Upon exit from M phase, Rb becomes hypo-phosphorylated

and regains its inhibitory role in G_1[84]. Differential levels of CDK6 in human HSCs have been implicated in the accelerated exit from quiescence in ST-HSCs compared to LT-HSCs[87].

1.2.2 Quiescence is a distinct protective cell cycle state

If conditions are unfavourable, cells may remain in G_1 prior to R and become non-cycling[85]. Upon exiting the cell cycle, the cell is considered to reside in a state termed G_0. In case of senescent or terminally differentiated cells this state is irreversible. Quiescent cells however, reside in G_0 and are able to re-enter the cell cycle[84,85]. Quiescence is suggested to be a protective mechanism for the cells to prevent potential mutagenic events occurring during DNA replication and cell division[2,88-90], the accumulation of which in HSCs, could lead to haematological malignancies such as leukaemia[91] or myeloproliferative neoplasms[92,93]. Nonetheless, due to the longevity of HSCs and a prolonged G_0 phase, DNA damage still accumulates. Therefore, quiescent HSCs require distinct DNA damage responses, a specialised DNA repair machinery, and a high drugs efflux capacity[90,94].

Thus, the vast majority of HSCs remain in the metabolically inactive G_0 phase of the cell cycle and in doing so preserves a rare population of quiescent HSCs under homeostatic conditions[2,69,95-98].

1.2.3 Loss of function mouse models allow analysis of cell cycle regulators

Various mouse models have been generated to investigate key regulatory molecules that control HSCs cell cycle and quiescence[88,99-104].

p53 Is a key regulator of cellular responses to stress stimuli, including DNA damage, culminating in senescence, apoptosis or cell cycle arrest[83,105]. A knock-out study in mice has demonstrated that p53 also plays an important role in maintaining HSCs self-renewal and quiescence and p53 has been shown to be highly expressed in the HSCs-enriched fraction (LSK, Lineage^{neg}Sca-1^{+}c-Kit^{+}) compartment[99,106,107]. Mice lacking p53 expression have 2-fold more phenotypic HSCs with a higher proliferation rate than their WT counterparts. *p53*$^{-/-}$ LSK cells have the ability to reconstitute an irradiated recipient mouse; however, recipients develop and die of lymphoma within 4 months[99]. It has previously been shown that KO of the ETS transcription factor MEF increases HSCs quiescence and number but does not affect their repopulation capacity[108]. To assess if p53 regulates the increased quiescence in *Mef*$^{-/-}$ double knock-out (DKO) *p53*$^{-/-}$*Mef*$^{-/-}$ were generated. DKO LSK outcompeted WT bone marrow cells in transplantation assays similar to *Mef*$^{-/-}$, thus demonstrating that p53 does not give *Mef*$^{-/-}$ cells

a self-renewal advantage. DKO mice do show a higher percentage of SP cells, pointing to the role of p53 in maintaining quiescence in *Mef*$^{-/-}$ mice[99].

One of p53's downstream target is p21[99,105]. Ablation of p21 results in the expansion of the HSPC pool but subsequent exhaustion of mutant HSCs, as cell cycle entry is no longer inhibited[88]. However, under homeostatic conditions, no accelerated turn-over of labelled HSCs was seen in *p21*$^{-/-}$ HSCs which calls into question the importance of p21 as a key regulator of quiescence[96]. Similar results to these *p21*$^{-/-}$ experiments were obtained in mice lacking expression of the CDKI p57, which under homeostatic conditions is highly expressed in LT-HSCs and to a lesser extent in ST-HSCs and MPPs. Lack of p57 reduced HSCs repopulation ability in serial transplantation indicating impaired self-renewal capacity[100,101]. *p57*$^{-/-}$ LSK cells show an increase in the cell fraction that has entered the cell cycle[100], however no alteration in cell cycle was observed in the LT-HSCs compartment[101].

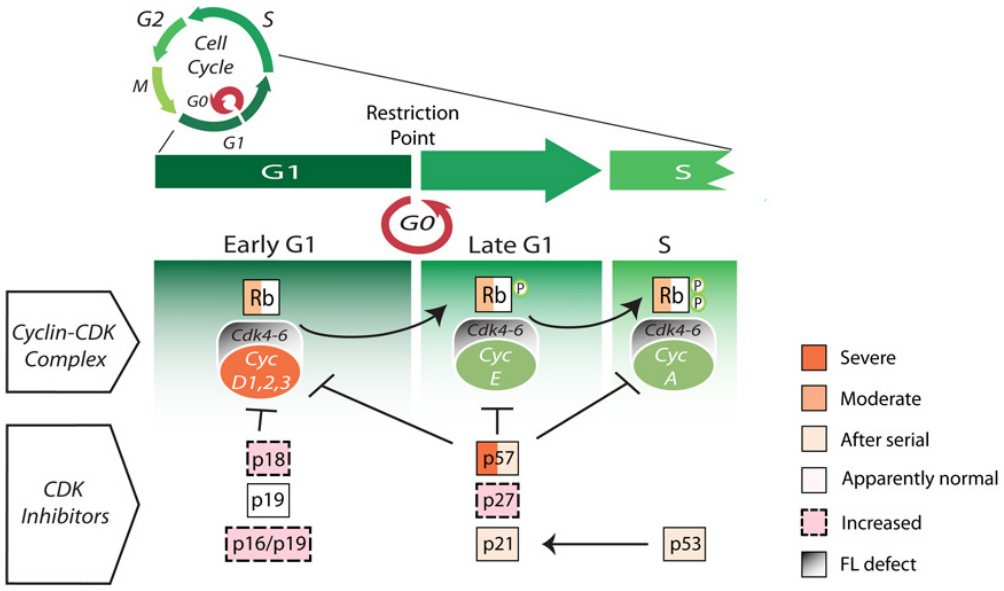


Figure 3: Regulation of the cell cycle in HSCs.
Adapted from Rossi et al., *Cell stem cell* (2012).

Both p21[100] and p27[101] expression are increased upon *p57* deletion, indicating a compensatory role for these CDKIs. Indeed, p57 knock-down (KD) in the LSK fraction of *p27*$^{-/-}$ mice results in an increase of cycling LSK, reduced repopulation activity in primary transplants accompanied by a reduction of the LSK pool in primary recipients[101]. Thus, both p27 and p57 play an important role in the maintenance of quiescence and self-renewal. Co-immunoprecipitation experiments have shown that this is mediated by the binding of p27 and/or

p57 to HSCs70, which is a member of the heat shock protein 70 family. HSCs70 acts as a chaperon for cyclin D1 nuclear translocation and accelerates its binding to CDK4/6. p27/p57 binding to HSCs70 inhibit nuclear import of cyclin D1 and, in doing so, prevents phosphorylation of Rb in the nucleus[101].
In contrast to loss of *p21* and *p57*, ablation of the INK4 family member p18 leads to a competitive repopulation advantage of mutant HSCs over WT without HSCs exhaustion in serial transplantation[102]. This suggest that extrinsic modulation of p18, possibly using a specific inhibitor, may be key for HSCs expansion *in vitro*.

Other members of the INK4 family are p16 and p19[82,109]. Ectopic expression of p16 in HSCs largely inhibits proliferation, whereas p19 overexpression completely abrogates proliferation. Both *p16* and *p19* expression levels are elevated in *Bmi-1*$^{-/-}$ mice, which present with reduced phenotypic HSCs number and hypocellular bone marrow. *Bmi-1*$^{-/-}$ bone marrow cells fail to reconstitute recipient mice, suggesting that suppression of p16 and p19 by Bmi-1 is necessary for HSCs to enter the cell cycle[109].

Interestingly, disruption of expression of single members of the Rb family (Rb, p107, and p130) does not result in severely dysfunctional HSCs. Viatour et al. therefore generated triple knock-out mice (TKO) to assess their role in haematopoiesis[103]. TKO mice died within 12 weeks of Cre-induction. Animals analysed displayed splenomegaly with extramedullary haematopoiesis, as well as increased numbers of HSCs, LSKs and GMPs accompanied by a substantial expansion of mature myeloid cells. Transplantation of TKO unfractionated BM resulted in short-term reconstitution (4 weeks), outcompeting all WT competitors. However, TKO HSCs were not capable of long-term reconstitution, with recipients dying within 5 weeks post-transplantation. This indicates that TKO HSCs are primed to differentiate into cells of the myeloid lineage but are accompanied by loss of HSCs self-renewal. Interestingly, reintroduction of one WT allele of p107 repressed the excessive myeloproliferation seen in TKO but did not compensate for deficient lymphoid cell production[103]. This shows that members of the Rb family can partially compensate for the loss of another member, but also underscore the need to better understand the molecular mechanism driving cell cycle progression and differentiation.

Functional redundancy can also be observed in *cyclin D1*$^{-/-}$, *D2*$^{-/-}$, and *D3*$^{-/-}$ mice which exhibit only a mild haematopoietic phenotype. Combined knock-out of all three cyclin D molecules is

embryonic lethal after E13.5. Conditional TKO mice present with reduction of HSCs and impaired repopulating ability, showing the importance of cyclin D for normal haematopoiesis[104]. Presence of cyclin D marks the G_1 phase of the cell cycle, where cyclin A2 – cyclin A1 is testes specific – is present in S and G_2 phase[84,110]. Double knock-out of cyclin A1 and A2 in an $A1^{-/-}A2^{fl/fl}$ mouse results in death within 30 days of Cre-induction. Administration of pI-pC after bone marrow transplantation of $A1^{-/-}A2^{fl/fl}$ BM results in reduction of cells among all haematopoietic lineages, showing that cyclin A2 is required for normal proliferation in haematopoietic cells[110].

Aside from the molecules directly involved in the cell cycle progression with well-described roles in model organisms, a number of pathways have been implicated in modulating HSCs quiescence and proliferation[83]. These include, but are not limited to, the TGF-β pathway, the PI3K/PTEN/AKT pathway, and the Wnt pathway[83,111-116].

The Transforming Growth Factor-β (TGF-β) superfamily signals through binding of the ligands to TGF-β type receptor II which forms a complex with TGF-β receptor I. This is followed by the phosphorylation of SMADs (SMAD-1, -3, -5, and -8), which then form a heterodimer with SMAD4 before translocating into the nucleus, where the complex recruits transcriptional cofactors to activate gene expression[111,117].
Yamazaki et al. showed that that pre-treatment of HSCs with TGF-β1 *in vitro* inhibits lipid raft clustering and in doing so keeps the majority of HSCs in a hibernating state[111,118]. Upon removal of TGF-β1, the cells were able to generate colonies and 20% of the surviving cells were able to reconstitute recipient mice in single cell transplants. Interestingly, SMAD2 and SMAD3 are phosphorylated in HSCs but become dephosphorylated upon cytokine stimulation. Pre-treatment with TGF-β seems to protect SMAD2/3 from dephosphorylation, indicating that SMAD2/3 regulate quiescence in HSCs[111]. The same group later showed that non-myelinating Schwann cells in the bone marrow secrete TGF-β and that HSCs lacking the TGF-β type II receptor have impaired long-term repopulation ability. Interestingly, $Tgfbr2^{\Delta/-}$ show a decrease in Smad2/3 phosphorylation and increased cell cycling, suggesting that pSmad2/3 is important for HSCs self-renewal and quiescence[119].
Other Smad molecules have been investigated as well, with conditional KO of *Smad5* failing to result in a haematopoietic phenotype with HSCs that have reconstitution ability and lineage output comparable with wild type, suggesting that SMAD5 is functionally redundant[117].

Deletion of *Smad4* on the other hand results in a mild haematopoietic phenotype with impaired self-renewal activity but normal lineage differentiation and cell cycle regulation[120].
Altogether, these findings suggest that some, but not all SMADs, are functionally redundant in HSCs, activating the same downstream pathways.

The PI3K (phosphatidylinositide 3-kinases)/AKT (Protein kinase B) pathway has been implicated in the regulation of cell proliferation, differentiation, resistance to stress, survival, and migration[83,113]. PTEN (Phosphatase and tensin homologue) is a negative regulator of PI3K/AKT[112,113]. Ablation of PTEN results in a reduction of phenotypic LT-HSCs, whereas the ST-HSCs pool is not significantly affected[113]. *PTEN*$^{-/-}$ LT-HSCs are more proliferative compared to WT control and can only transiently repopulate irradiated recipients[112,113]. Interestingly, multi-lineage reconstitution capacity of *PTEN*$^{-/-}$ HSCs can be restored using the drug Rapamycin, which selectively inhibits mTOR kinase activity, which is a downstream target of PI3K[112]. *PTEN*$^{-/-}$ does not affect the homing ability of HSCs but it does lead to an increase in mobilisation to the peripheral blood and spleen[113]. Loss of PTEN in HSCs results in decreased numbers of lymphoid progenitors and B220^{+} B lymphocytes, whereas the myeloid lineage is not affected[113]. These findings show that PTEN expression is necessary to maintain self-renewal in HSCs and plays a role in directing lineage choice.

Downstream genes of PTEN include those encoding for AKT and Forkhead O (FoxO) proteins[83]. *Akt1*$^{-/-}$ and *Akt2*$^{-/-}$ mice only show a mild haematopoietic phenotype, suggesting functional redundancy. In contrast, DKO *Akt1*$^{-/-}$*Akt2*$^{-/-}$ mice have defective haematopoiesis with mutant HSCs entering cell cycle less frequently and impaired differentiation potential[114].
AKT directly phosphorylates FOXO1, O3 and O4 and inhibits their activity resulting in their exclusion from the nucleus, where they can no longer act as transcription factors leading to cell cycle arrest through activation of p27, p130 and p21 as well as inhibition of cyclin D expression[83,115,116]. As seen before, single knock-out of *FoxO1/3/4* does not yield a severe haematopoietic phenotype, suggesting functional redundancy[116]. However, upon serial transplantation of *FoxO3a*$^{-/-}$ bone marrow, an HSCs self-renewal defect becomes apparent[115]. Double knock-out of *FoxO1/3* results in an increase in apoptosis[116]. *FoxO3a*$^{-/-}$ HSCs have elevated ROS levels, likely driving the observed increase in phosphorylation of p38MAPK[115]. FOXO1/3/4 deficiency increases myeloid cell production concomitant with a reduction in BM HSCs and LSK[116]. Mutant HSCs have impaired long–term repopulating capacity and proliferate

faster. This cell cycle phenotype is further reflected in the reduced expression levels of *p21*, *p27*, and *p130* in these TKO HSCs, whereas *Cyclin E1* and *D2* expression is elevated[116].

1.2.4 Upstream regulators of proliferation

Activation of above mentioned pathways is mediated by receptor binding of growth factors, insulin, or cytokines such as SCF (stem cell factor)[121,122], TPO (thrombopoietin)[121,123], Ang-1 (Angiopoietin)[101], and G-CSF[124] each of which have been shown to promote HSCs proliferation *in vivo* and *in vitro*. *In vitro*, these are often used in conjunction with Interleukin (IL)-3[125-127], IL-6[126,128] and IL-11[125,129] which have synergistic mitogenic effects on HSCs proliferation[125,130].

Furthermore, inflammatory signalling has been shown to stimulate HSCs proliferation[131]. These can involve type I[132] or type II[133] interferons (interferon-α and interferon-γ respectively), Tumor Necrosis Factor (TNF)[134,135], and lipopolysaccharide (LPS)[136].

More recently, posttranscriptional regulation of genes through micro-RNA (miRNA) have been shown to alter gene expression through mRNA destabilisation and translational inhibition[137]. Ablation of *miR-126* expands human and mouse HSCs and improves engraftment and homing ability[137]. In contrast, deletion of the global miRNA regulator Dicer results in the loss of functional HSCs[138]. Overexpression of *miR-125a* drives an increase in stem cell frequency and gives mouse HSCs a competitive advantage in reconstitution assay compared to wild-type[139]. This observation is in accordance with the finding that *miR-125a* deficient mice have reduced self-renewal capacity[138]. Inhibition of the micro-RNA *Let7* leads to an increase in HMGA2 expression concomitant with in an increased frequency of HSCs self-renewal as well breast cancer initiating cells[140,141]. In HSCs, it has been shown that this is regulated via the *Let7* inhibitor LIN28b and the *Let7* negative target *HMGA2*, overexpression of both these molecules increase donor cell contribution in irradiated recipients over WT[141].

The above discussed regulatory molecules and pathways are only a small sample of the complex machinery that is the cell cycle. Much is still not understood and even less is known about cell type-specific mechanisms of action. Further gene perturbation studies are therefore necessary to gain a better understanding of how the cell cycle, and in particular the process of G_0 exit, is regulated in both mouse and human HSCs.

1.3 HSCs heterogeneity

The existence of heterogeneity within the HSCs compartment was shown as early as 1964, when Till et al. reported highly variable spleen-colony formation with respect to number and types of daughter cells within the colonies[54,62]. Subsequently, haematopoietic progenitor heterogeneity was also noted in *in vitro* colony forming assays[62]. In the 1980s, retroviral marking of HSCs derived from different haematopoietic tissues allowed the identification and tracking of clones *in vivo* over extended periods of time[61,62]. Through clonal tracking of transplanted single HSCs it was shown that HSCs are heterogeneous with respect to self-renewal activity, life span, repopulation pattern, and fate commitment[63,142]. An HSCs can self-renew through symmetric or asymmetric cell division[143,144], it can undergo apoptosis or it can exit quiescence and differentiate, the latter two both leading to a decline in the HSCs pool (Figure 4)[7].

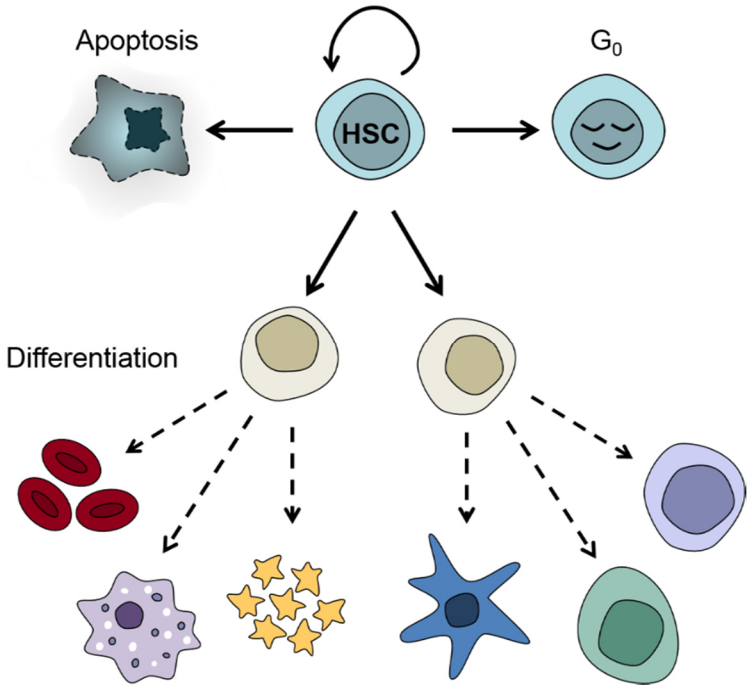


Figure 4: Putative HSCs fate choices.
HSCs can make different fate choices throughout their lifetime. An HSCs can remain in the G_0 phase of the cell cycle or it can self-renew symmetrically leading to an expansion of the HSCs pool. Additionally, the HSCs can proliferate and give rise to a differentiated daughter cell and another HSCs through asymmetric cell division, with the HSCs returning to a quiescent state. Alternatively, the stem cell can produce two differentiated cells or undergo apoptosis leading to a decline in the HSCs pool.

1.3.1 HSCs heterogeneity in self-renewal activity and life span

The HSCs population is commonly subdivided into long-term HSCs (LT-HSCs), also referred to as long-term repopulating cells (LTRCs), and short-term HSCs (ST-HSCs)[7]. LT-HSCs are

considered to be more primitive, giving rise to larger clones with greater lineage potential and often associated with delayed detection of mature progeny in peripheral blood upon transplantation[1,145].
Most importantly, LT-HSCs but not ST-HSCs can repopulate mice in a serial transplantation setting[68], a formal demonstration of their ability to create daughter HSCs.

The proliferative stress exerted on LT-HSCs would be tremendous if all mature blood cells were directly derived from this rare population of cells. HSCs are relieved from this burden by multipotent, oligo-potent and lineage-restricted progenitor cells, which are highly proliferative and possess extensive developmental potential[2,146]. A recent study using *in situ* Doxycyclin (Dox) induced transposon labelling, has attempted to quantify the dynamics of lineage replacement by multipotent progenitors (MPPs) over time. Nearly 20% of erythromyeloid clones were derived from an MPP population at 8 weeks post Dox administration. However, the caveat of this tracking method is that not all progenitor populations are labelled, thus the actual number of contributing MPP may be underestimated. In addition, cell recovery and sequencing depths may not be equal among populations, introducing technical bias[146].

Another study estimated that that LT-HSCs divide approximately once every 145 days based on the computational modelling of BrdU (Bromodeoxyuridine) label retention[95]. Studies using doxycycline induced histone 2B-GFP reporter mice have shown 80% of HSCs have divided within a 24 week chase period; however 5% of stem cells retain the label past 72 weeks. Using a model in which 80% of HSCs cycle fast and 20% of cells divide less often, it was estimated that 5.3–11.1% of fast proliferating HSCs divide daily and only 0.8–1.8% per day of the slow population divide[96]. The former observation is in agreement with findings from other groups in which 4.7%[147] or 6%[148] of BrdU-labelled HSCs divided daily. However, as BrdU incorporation over the 10-day treatment time was linear, the authors concluded that all HSCs proliferate at a similar rate. This conclusion however, does not consider the extent of functional heterogeneity in the HSCs population. Of note, only 45.9% of phenotypic LT-HSCs (LSK SLAM CD41^{neg}) incorporated the label at 10 days[148]. Another report found 87.8% BrdU-labelled HSCs (LSK cKit^{+}) at day 10 and >99% labelling after 180 days of continuous BrdU administration[147]. Thus, label incorporation in the HSCs pool is dependent on the different purities of the HSCs isolation phenotype.

The proliferation rate of the slow population mentioned above would be equivalent to once every 55–125 days in the slow cycling HSCs population, which is consistent with the lower end estimate of the previously reported 145 days[95,96]. In contrast, ST-HSCs exhibit finite self-renewal and cannot repopulate a mouse past the primary transplantation. ST-HSCs divide approximately 4 times more often than LT-HSCs and exit cell cycle faster[87,95,142].

Since BrdU has been shown to have a mitogenic effect on HSCs[95,148], another study employed CFSE (carboxyfluorescein succinimidyl ester) labelling, which is diluted to undetectable levels after 5 cell divisions. Using this method, transplanted cells were tracked for 3–21 weeks. The authors found that only few cells had not divided by at the end of the chase period. Serial transplantation of non-divided LSK or LSK that had divided >5x between 12–14 weeks post primary transplantation were able to engraft secondary recipients. Interestingly, cells that had divided >5x as early as 3 weeks post primary transplantation failed to repopulate. These findings show that serially transplantable LT-HSCs are present in both the cycling and quiescent fraction. Extrapolating from their data the authors estimate that HSCs divide every 39 days on average totalling in 18 cell divisions during the lifetime of a mouse[149]. It is important to note that this estimation relies on the reliable and comprehensive labelling of all stem cells prior to the primary transplant. Therefore, it is currently impossible to conclude with absolute certainty what the average life span of an HSCs is and how many cell division it will undergo before exhaustion of its progeny as labelling and recovery of all HSCs is not feasible with the presently available technology.

1.3.2 HSCs heterogeneity in numbers and cell types produced post-transplantation

The spleen colony formation assays described above inferred heterogeneity, but in the 21st century, clonal and single cell approaches in purified stem and progenitor cell populations revolutionised the field by formally documenting HSCs heterogeneity. These started with the work of Muller-Sieburg et al., who showed through serial transplantations of HSCs clones from limiting dilution assays (LDA) that daughter HSCs behave similarly to their parents with regard to self-renewal, primitiveness and lineage contribution[142]. Interestingly, it was observed that approximately 30% of all clones expressed a lineage bias towards the myeloid or lymphoid lineage, which was maintained through serial transplantation. These findings led to the conclusion that the lineage dominance is due to an "inherited", cell intrinsic mechanism[142,150]. The HSCs clones were classified as myeloid biased (my-bi), lymphoid biased (ly-bi) or balanced (bala) based on the ratio of lymphoid to myeloid lineage[142].

Similar findings were classified differently in a large study of single HSCs transplantations[64] where the inaccurate term of "myeloid-biased" was avoided since it which incorrectly implies that the myeloid output from the my-bi clones differed from that of balanced clones. Rather, these cells produce the same amount of myeloid cells but are deficient in lymphoid potential[7,64]. Dykstra et al. present this unbiased categorisation of HSCs based on the relative amounts of mature cell production, with HSCs giving rise to primarily myeloid cells as alpha-HSCs and those clones that have a balanced lymphoid and myeloid lineage output as beta-HSCs. Both populations are characterised by robust self-renewal and a constant repopulation pattern in primary and secondary transplantations[64]. The lymphoid progenitor cells (CLPs) derived from alpha-HSCs are quantitatively and qualitatively deficient when compared to CLPs generated from beta-HSCs[64,151]. This is consistent with accounts from Muller-Sieburg et al., which reported that lymphoid progeny generated from my-bi HSCs have impaired responses to IL-7, a cytokine important for B- and T-cell development[150]. Single HSCs with finite self-renewal activity (e.g., those that could not repopulate secondary recipients) were classified as gamma and delta-HSCs. Gamma-HSCs are largely lymphoid biased but can produce myeloid cells, whereas delta-HSCs only produce lymphoid cells 16–20 weeks post transplantation[64,151]. Benveniste et al. suggested that these should be categorized as intermediate HSCs (IT-HSCs), a transitional HSCs between LT- and ST-HSCs, since these cells show prolonged (>8 months) reconstitution after transplantation[152]. There is significant overlap between the HSCs subcategorisation used by different labs as summarised by Ema et al. and shown in Table 1 and Figure 5, emphasising the need of a unified standard to detect and describe HSCs subtypes[63].

Table 1: Variations in HSCs subcategorisation

HSCs subtype	Repopulation in weeks post transplantation	Ratios of mature progeny
Long-term	>52 weeks	not assessed
intermediate-term	>32 weeks <52	not assessed
short-term	<24 weeks	not assessed
myeloid-biased	20 weeks	Lymphoid:Myeloid: <3
balanced	20 weeks	Lymphoid:Myeloid: <10, >3
lymphoid-biased	20 weeks	Lymphoid:Myeloid: >10
alpha	16 weeks	Myeloid:Lymphoid: >2
beta	16 weeks	Myeloid:Lymphoid: >0.25, <2
gamma	16 weeks	Myeloid:Lymphoid: <0.25

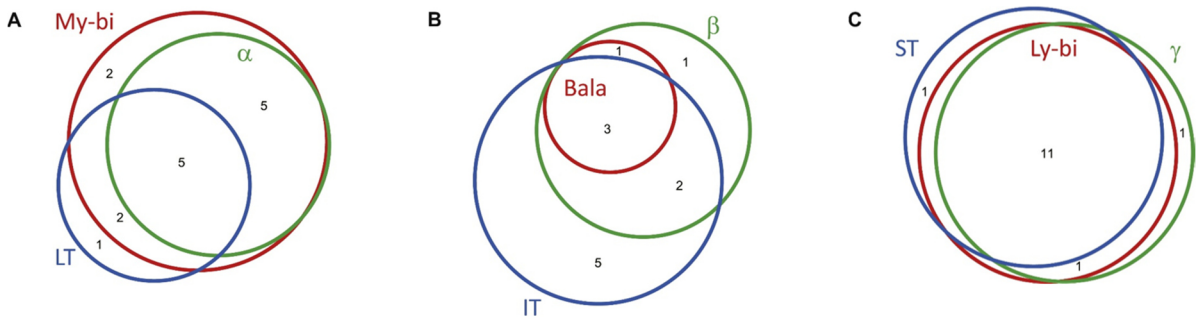


Figure 5: HSCs subtypes classified by different labs largely overlap.
Adapted from Ema et al., *Exp Hematol* (2014). Venn diagram shows the relationship between the different classified HSCs subtypes. Data was used from 30 single cell transplantations. A) 14 My-bi HSCs, 8 LT-HSCs, and 10 α-HSCs were identified. B) 4 Bala-HSCs, 10 IT-HSCs, and 7 β-HSCs were categorised. C) 12 ST-HSCs, 12 Ly-bi HSCs, and 13 γ-HSCs were identified. My-bi=myeloid biased. Ly-bi=lymphoid biased. Bala=balances. LT=Long-term HSCs. IT=Intermediate-term HSCs. ST=Short-term HSCs. α=alpha-HSCs. β=beta-HSCs. γ=gamma-HSCs. Numbers indicate positive transplant outcomes categorised in accordance to the criteria displayed in Table 1.

Interestingly, the relative proportion of alpha and beta-HSCs changes throughout mouse development. In the mouse foetal liver (E14.5) beta-HSCs account for the majority of HSCs, with only 5−10% being alpha-HSCs. Both populations increase at similar rates until E18.5 with alpha-HSCs becoming more frequent in the postnatal and young bone marrow. In the older adult bone marrow (>1 year) alpha-HSCs (ESLAM) are the dominant cell type[151] and this accumulation continues into old age. This is consistent with previous findings that my-bi HSCs are enriched in aged mice[89,153-155] and that myeloid chimaerism is increased in mice transplanted with donor HSCs derived from aged (18 months) mice[155,156]. The phenotypic HSCs (pHSCs) frequency increases with age in the mouse bone marrow[89,151,155,157]. However when this population is further subdivided into $CD150^{+}CD41^{neg}$, the HSCs compartment is significantly decreased[157], whereas a vast increase is observed in the $CD150^{+}CD41^{+}$ population[154,157,158]. This is also reflected in the recent finding by Yamamoto et al. where aged HSCs produced a significantly lower amount of T lymphoid cells in both primary and secondary transplantation when compared to young HSCs indicating that aged HSCs may become lymphoid-deficient[157].

1.3.3 HSCs heterogeneity in fate commitment

Currently available methods for HSCs isolation are unable to prospectively discriminate between distinct HSCs subtypes, which makes the study of their differences at the molecular level particularly difficult[62]. With emerging tools in the field of single cell biology, many studies have now attempted to uncover what mechanisms and molecules link transcriptional and phenotypic variations in HSCs[159-161].

In a high-throughput single cell gene expression analysis of 18 key haematopoietic transcription factors, Moignard et al. found that transcription factor expression is heterogeneous in HSCs and progenitors, with some genes exhibiting a bimodal expression, thus potentially generating three different expression states: i) high, ii) medium or iii) no expression[162]. This confirmed previous findings by Glotzbach et al. who used microfluidic-based high-throughput single cell qPCR to analyse the gene expression state of LT-HSCs[163]. The authors reported that some genes displayed asymmetric transcriptional gene distribution, which may be due to transcriptional bursts. Using their approach, Glotzbach et al. could provide evidence for subpopulations within the LT-HSCs compartment based on their transcriptional fingerprint[163]. However, a unique molecular signature that identifies a distinct functional HSCs state, such as an alpha or beta-HSCs, has yet to be identified[68,164].

1.4 Refining the haematopoietic hierarchy

Historically, it was thought that the differentiation into mature blood cells was achieved in a stepwise fashion through multiple rounds of differentiation via a large number of increasingly more committed progenitor cells[63]. The classical bifurcation model proposed that apical LT-HSCs give rise to ST-HSCs, which in turn give rise to restricted progenitor cells of the lymphoid or myeloid lineages via common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) respectively (Figure 6A). The latter progenitor pool then bifurcates into granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs). CLPs give rise to dendritic cells (DCs), pro-B, pro-T, and pro-natural killer (NK) cells which then mature into various specialised B-cells, T-cells and NK cells[91,165,166].

This classical model was first challenged by Adolfsson et al. who reported a multipotent progenitor population that failed to produce cells of the erythrocytic/megakaryocytic lineage. These were termed lymphoid-primed multipotent progenitors (LMPP)[167]. The revised model proposed that HSCs give rise to a megakaryocyte/erythroid progenitor (MkEP) cell and alternatively, loss of the Mk and E potential would generate an LMPP. Another possibility is that the ST-HSCs give rise to CMPs and LMPPs followed by bifurcation of CMPs into MkEPs and GMPs, while the LMPPs would give rise to GMPs and CLPs (Figure 6B)[63,167].
This model received further input by Arinobu et al., who suggested that lineage commitment takes place at the MPP stage through the differential expression and mutual inhibition of PU.1, and GATA-1, a transcription factor required for megakaryocyte and erythrocyte development,

thereby regulating the generation of CMPs and progenitors or both myeloid and lymphoid lineage (GMLPs). Multipotent progenitor cells (MPPs) which upregulated PU.1 would give rise to GMLPs which then in turn bifurcate into CLPs and GMPs, while MPPs with increased GATA-1 expression generate exclusively CMPs that then generate MEP and GMP (Figure 6D)[168]. The observation that dendritic cells can be derived from both myeloid and lymphoid origin implied that they originate from both GMPs and CLPs, supporting the hypothesis that a progenitor exists with both lymphoid and myeloid differentiation potential such as the GMLP[91,169].

However, second generation cellular barcoding experiments have shown that the LMPP compartment is in fact also heterogeneous and is largely comprised of unipotent progenitors generating predominantly dendritic cells (DC, ~50%), and to a lesser extent B-lymphocytes and myeloid cells. Only 3% of LMPP were observed to have a multi-lineage outcome, although barcoding experiments are always limited by their detection ability, due to low sampling. Paired daughter transplantations of LMPPs have shown that the heterogeneity in lineage output is largely maintained suggesting a cell intrinsic or "imprinted" mechanism[170]. Using a Gata1-GFP reporter mouse line, Drissen et al. investigated lineage potential in both the phenotypic LMPP and preGM/GMP compartment. Gata1-GFP$^+$ pre-GMs do not give rise to B- and T-lymphocytes and only produce few monocytes but have high mast cell out and generate Mks, erythrocytes and eosinophils[171]. Interestingly, 2–3% of LMPP do express *Gata1* which corresponds to a similar percentage of Mks generated from LMPPs[167,171]. The authors concluded that lineage segregation occurs as early as the HSCs/MPP stage based on *Gata1* expression[171]. In contrast to this finding, recent work using long-term, continuous single cell quantification of GATA1 and PU.1 expression has shown that cells that differentiated into the GM lineage increased *PU.1* expression over time but never expressed *GATA-1* during the differentiation process[172]. This implies that GATA-1 does not play a significant role in GM lineage commitment. It was furthermore shown that cells differentiating into the MegE lineage always expressed GATA-1 independent of PU.1 levels, leading to the conclusion that lineage choice is reinforced by transcription factor expression but not initiated[172] and disputing the mutual inhibition hypothesis previously stated by Arinobu et al[168].

Using a combination of index-FACS and MARS-seq, Paul et al. aimed to unravel the heterogeneity within the myeloid progenitor compartment. Transcriptional profiling showed that the CMP pool could be subdivided into subpopulations primed to adopt one of 7 myeloid

cell fates, namely erythrocytes, megakaryocytes, dendritic cells, monocytes, neutrophil, eosinophil or basophil. Surface marker expression of CD135 and CD115 (both positive) identified cells that did not give rise to cells of the Mk/E lineage[173]. This is partially consistent with previous single cell qPCR data generated by Guo et al., showing that phenotypic CMPs are shared between the Mk/E and lymphomyeloid differentiation trajectory. CD55 is a potent marker to enrich for cells differentiating towards the Mk/E lineages, with CD115 subdividing the $CD55^+$ CMP compartment further. CD115 also subdivides the GMP compartment but no functional studies on these subpopulations were performed so it is unclear whether the molecular subsets described relate to functionally heterogeneous cell populations[174].
Overall, the generally accepted model has been that restricted progenitors are derived from more multipotent progenitors, which originate from the HSCs compartment. However, recent experiments have also called this concept into question, suggesting that the differentiation of HSCs into certain lineages does not always occur via stages of progenitors, but that HSCs could give rise to lineage-restricted progenitors directly, such as repopulating common megakaryocyte progenitors (rCMP), which retain limited self-renewing potential (Figure 6C)[175,176]. Of late, the existence of a long-term repopulating megakaryocyte restricted stem cell (LT-MkSC) has been discussed. However, the frequency of this putative cell is lower than 10^{-6} and its reconstitution never exceeds 0.1% donor chimaerism, i.e. 50 cells if $5x10^4$ platelets were acquired[157]. Thus, it is challenging to draw firm conclusions from such low numbers of cells. This putative LT-MkSC was not detected in aged animals[157], even though >80% of single aged HSCs show a platelet bias[177]. This is also reflected in increased Mk/E lineage specific genes compared to HSCs obtained from young mice[177]. Yet, no secondary transplants on these supposed platelet-biased aged HSCs were performed. Thus, it is unclear if these putative HSCs were in fact HSCs or were contaminated with MkP[177]. The latter possibility is supported by an *in vitro* single cell tracking experiment, which showed that ~15% of phenotypic LT-HSCs give rise to megakaryocytes largely without prior cell division, suggesting that the input cells were in fact MkP[178].

To address the existence of a purely megakaryocyte restricted long-term self-renewing HSCs *in situ*, Rodriguez-Fraticelli et al. made use of a Sleeping Beauty lineage tracing model combined with TARIS, an improved transposon integration sequencing technique. Eight weeks after *in situ* labelling, cells of different lineages were isolated and their transposon tags analysed. Only ~10% of tags found in megakaryocyte progenitor (MkPs) were shared with multiple other lineages, suggesting that the vast majority of the megakaryocyte lineage arises

independently from other blood lineages. Interestingly, none of the isolated MkP clones shared tags solely with erythroblasts, suggesting that the MEP lies downstream of a progenitor with erythromyeloid and/or lympho-erythromyeloid potential. However, it needs to be taken into account that MkPs may be far more numerous than other cell types, thus detection limits need to be adjusted accordingly and conclusions are difficult to make without deeper sampling.
To assess long-term repopulation potential of Mk-restricted HSCs, cells were transplanted and their progeny assessed at four and 30 weeks post transplantation. Six out of eight recipients contained donor-derived multi-lineage progeny. This suggests that the majority of megakaryocyte producing clones within the phenotypic LT-HSCs compartment are not MkP lineage restricted. However, the findings of a Mk-primed cluster within the phenotypic LT-HSCs population as well as the detection of *in situ* labelled Mk clones lacking overlapping labels with other lineages, suggest that the megakaryocyte lineage is the earliest to branch off. Notably, MPP2 also produce Mks suggesting that there may be two distinct pathways for Mk production[146].

Distinct CD45 isotypes are commonly used to distinguish between donor and recipient cells in peripheral blood, however this does not allow for the assessment of erythrocytes and platelets as these do not express this surface marker. Carrelha et al. have overcome this limitation by using transgenic mice co-expressing *Gata1*-eGFP and *Vwf*-tdTomato, which fluorescently label erythrocytes and platelets respectively, as donor mice. Following single cell transplantation ~11% of mice showed only platelet reconstitution. These donor cells were termed platelet-restricted (P-restricted). Of note, few subsequent secondary transplants were carried out, with recipients showing low levels of erythrocyte, myeloid and lymphocyte lineage reconstitution. In addition, isolated Vwf^{+} HSCs (LSK SLAM) from primary recipients showed multi-lineage potential in *in vitro* assays[179]. This argues against an exclusive platelet-restricted HSCs but is in accordance with previously discussed findings[146]. However, it is possible that these tools simply track platelets and erythrocytes in alpha-HSCs. In addition to P-restricted donor cells, the authors also found platelet+erythrocyte, platelet+erythrocyte+myeloid, and platelet+ erythrocyte+myeloid+B cell restricted repopulation patterns. This indicates that megakaryocytes are situated higher up in the haematopoietic hierarchy than previously thought and may indeed be derived via two separate developmental pathways[146,179].

Transcriptional overlap between the megakaryocyte and the LT-HSCs population is a consistent feature arising in single cell transcriptomic analysis, through multiplexed qPCR or single cell

RNA sequencing (RNA seq) profiling of the HSPC compartment. Different clustering methods suggest a close relationship between the Mk/E and long-term HSCs branch[70,146,174,180]. It is important to note however, that many genes are shared between megakaryocytes and HSCs, again highlighting their close connection[70]. Yet, the possibility cannot be excluded that shared gene expression between MKs and HSCs may be due to contaminating MkPs within the HSCs branch.

Interestingly, similar observations of an early exclusive Mk differentiation branch separating from the HSCs pool were also made within the human system[181,182]. Human haematopoiesis is considered to follow a similar developmental pattern as seen in mouse, with MPPs ($CD34^{+}CD38^{neg}CD90^{neg}CD45RA^{neg}CD49f^{neg}$) giving rise to multi-lymphoid progenitors (MLP) that have both lymphoid and myeloid potential[171]. Notta et al. aimed to improve resolution by subdividing the MPP pool using the surface markers CD71 (Transferrin receptor) and CD110 (Thrombopoietin receptor, MPL). Only MPPs negative for these markers (termed MPP1) and long-term HSCs ($CD34^{+}CD38^{neg}CD90^{neg}CD45RA^{neg}CD49f^{+}$) were able to give rise to multi-lineage progeny. By comparing lineage output from progenitors derived from foetal liver, cord blood and adult bone marrow, the authors were able to show that the ratio of progenitors with multi-lineage or uni-lineage capacity changes throughout development, with more cells adopting a uni-lineage fate in adult bone marrow (82% uni-lineage) compared to foetal liver and cord blood (60% and 73%, respectively). Additionally, within the putatively unprimed HSCs/MPP compartment, a subset of cells expressing both CD71 and CD110 was detected identifying cells seemingly primed to adopt megakaryocyte/erythroid fate. These findings suggest an early branching point for the Mk/E lineage separating from the multipotent cell compartment[181].

This is also in line with recent finding by Velten et al. where human bone marrow cells from two individuals were index-sorted and RNA sequencing was performed. Clustering of the libraries showed that progression from a multi-lineage state to a distinct primed population is concurrent with the upregulation of CD38 on the cell surface. Functionally, the majority of phenotypic oligopotent progenitor populations gave rise to uni-lineage progeny, which was also reflected in their gene expression profiles. Based on gene expression clustering, the authors suggest that priming for the earliest lineage segregation occurs within the HSCs compartment between the lymphoid/myeloid and megakaryocyte/erythrocyte lineage. The cells then

transition through a CMP stage and bifurcate into GMP and MEP with CD71 enriching for cells of the erythroid lineage[182].

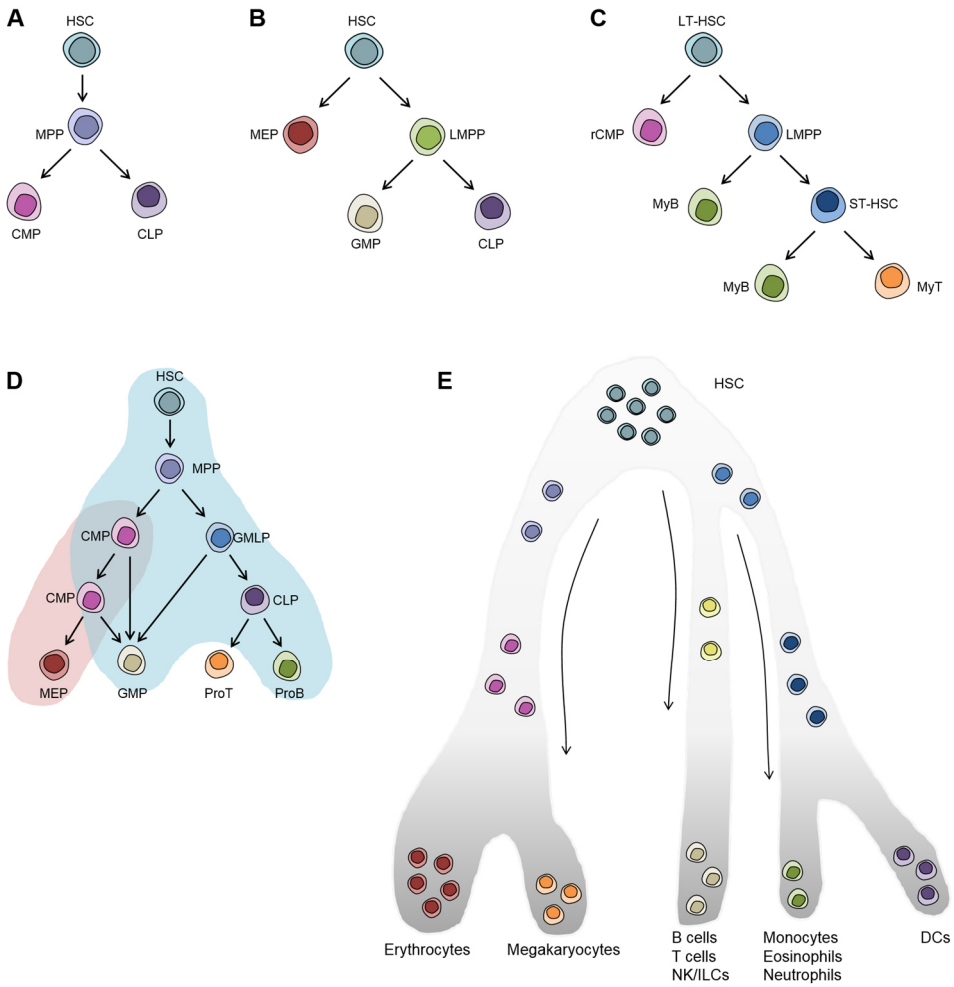


Figure 6: Different models depicting the haematopoietic hierarchy.
A) Bifurcation model. B) LMPP model. C) Direct lineage differentiation model. A-C) modified from Ema et al., *Exp Hematol* (2014). D) Lineage differentiation into lymphoid or myeloid lineage is dependent on reciprocal activation of Gata-1 and PU.1. Pink background reflects increase in Gata-1 expression, while blue depicts the increase of PU.1 in progenitors. Overlapping region represents progenitor priming. Modified from Arinobu et al., *Cell stem cell* (2007). E) Continuum of differentiation model. Modified from Laurenti, E. & Göttgens, *Nature* (2018)[183]. MPP = multipotent progenitor; CMP = common myeloid progenitor; CLP = common lymphoid progenitor; MEP = megakaryocyte and erythrocyte progenitor; LMPP = lymphoid-primed multipotent progenitor; GMP = granulocyte and macrophage progenitor; IT-HSCs= intermediate HSCs; MyB = myeloid progenitor with B cell potential; ST-HSCs= short-term HSCs; MyT = myeloid progenitor with T cell potential; DC = Dendritic cell; NK = Natural Killer cell; ILC = Innate Lymphoid cells.

In contrast to Velten et al. who used an unbiased sorting approach with retrospective lineage assignment built on gene expression data, Karamitros et al., immuno-phenotypically isolated HSCs, MPPs and several oligopotent progenitor populations from both human cord blood and bone marrow. As observed by others, most progenitors had only uni-lineage progeny, although

bi-lineage and multi-lineage outcomes were observed. GMP and MLP lacked erythroid output and MLP only generating unilineage lymphoid cells. LMPP had the capacity to generate monocytes, granulocytes and lymphoid cells but failed to generate erythrocytes and megakaryocytes, with high expression of CD10 enriching for LMPPs with lymphoid potential. Single cells of the LMPP, MLP and GMP compartment were sequenced and, like Velten et al., the authors found that cells typically defined as the above mentioned progenitor populations do not segregate from each other but rather are part of a continuum of lympho-myeloid differentiation (Figure 6E)[184]. However, in the interpretation of this data it is important to remember that due to the greater resolution of inter-cell differences and technical drop-outs, single cell RNA seq data is likely to present as a continuum in contrast to data obtained from bulk RNA seq.

Since cell surface marker expression does not faithfully reflect the transcriptional state of a given cell[174], unbiased approaches capturing vast numbers of cells without predefining them by phenotype, with possible retrospective lineage assignment through either functional outcome or gene expression profile, may aid in the understanding of lineage priming and differentiation[70,185]. For instance, Dahlin et al. has shown that single cell sequencing of > 44,000 mouse HSPC can determine the entry point of 8 individual lineage trajectories[186].

With recent findings in mind and the advances of single cell profiling technologies, several groups have proposed a more fluent continuum of transitional stages in blood cell development to replace the binary commitment model. Major amendments to the old models are that lineage priming occurs earlier than previously thought in both the mouse and human system[70,171,181,182,184,187], and that the hitherto believed oligopotent progenitor pools in fact give rise to mainly uni-potent progeny[146,173,182,184]. However, as shown for seemingly Mk-primed HSCs, when submitted to the stress of transplantations, these cells did reveal multi-lineage potential[146,179]. It is therefore necessary to keep in mind that just because a progenitor is observed to produce a single lineage, it is not necessarily true that this cell does not have oligo-lineage potential.

In this context, “potential” encompasses several lineage outcome possibilities, whereas “fate” insinuates that the cell is irreversibly committed to differentiate into a particular lineage guided by external stimuli. Hence, it is important to consider if a terminally differentiated blood cell has adopted its fate because it only possessed uni-potential capacity or because it was stimulated

to do so by extrinsic factors. *In vivo*, it still needs to be determined if fate commitment is a stochastic event or if location within the niche is an essential factor. In particular because many external stimuli are soluble factors, which may make niche location partially irrelevant. One of the external stimuli known to be produced by cells from the bone marrow niche and extensively studied for its effects on HSCs is the cytokine stem cell factor, further described in 1.5.

1.5 Stem cell factor, a cytokine involved in regulating haematopoiesis

Stem Cell Factor (SCF), also known as Mast Cell Growth Factor (MGF)[188], Kit-Ligand (KL)[189], and Steel Factor (SF)[190], is a cytokine encoded by the Steel (Sl) locus on mouse chromosome 10 and human chromosome 12[191]. In the bone marrow, SCF is produced by endothelial and mesenchymal stromal cells, including LEPR-expressing perivascular cells and adipocytes[31,192].

SCF is the ligand of the proto-oncogene c-Kit, a transmembrane receptor tyrosine kinase and member of the Platelet Derived Growth Factor (PDGF) receptor subfamily[188,189,193,194]. In 1956, it was first hypothesised that SCF and c-Kit may be complementary to each other based on the striking similarities between the phenotypes of both mutant Steel (*Sl*) and white-spotting (*W*) mice[195], the latter locus encoding *Kit*[196,197]. Mutant *Steel* mice are characterised by anaemia, reduced gonad size, and diluted hair pigmentation including white spotting. The majority of homozygous mutant mice die perinatally due to macrocytic anemia[195]. Similarly, the few surviving *W* homozygous mutant mice have white coats with black eyes, are severely anaemic, have a reduced number of mast cells and are sterile[198,199]. These findings implicate that both c-kit and SCF play an important role in the regulation of haematopoiesis, gametogenesis as well as melanogenesis[195]. SCF has moreover been found to be important for development of the interstitial cells of Cajal in the intestine where it is produced by smooth muscle cells[200,201]. Furthermore, research suggests a role for secreted SCF in neurogenesis by stimulating the proliferation of neuronal precursor cells *in vitro* and *in vivo*[202].

1.5.1 The SCF-KIT complex

SCF is encoded by 9 exons in human, mouse and rat[191]. It exists in both a membrane-anchored and a soluble form as the result of alternative splicing of exon 6 and proteolytic processing[191,203-205]. SCF binds Kit with high affinity and in a species-specific manner. Human SCF (HSCsF) does not bind mouse Kit (dissociation constant K_d >700nM), while rat SCF binds to human Kit (hKit) with 100-fold lower affinity than HSCsF to hKit (K_d 10-50nM vs. 0.5-1nM respectively)[206]. This is likely due to structural discrepancies between human and murine SCF.

SCF is a noncovalent homodimeric glycoprotein consisting of four-helix bundles, or protomers[206,207]. Soluble SCF contains 165 amino acids, with the first 141 residues being essential for receptor binding[207]. SCF forms a dimer through polar and nonpolar head-to-head interactions between the protomers[207,208]. SCF binding to Kit is driven by charge complementarity, and both human SCF/KIT and mouse SCF/KIT are expected to display the same overall binding configuration. However, the interspecies incompatibility is possibly due to the highly hydrophobic patches in mouse SCF (Ile50, Leu54, Val87, and Leu88), binding to the hydrophobic mouse KIT (Pro124 and Phe126), not being complementary to the less hydrophobic Ser123 and Tyr125 in human KIT[208]. Another report has argued that the substitution of Tyr125 by phenylalanine in mouse substituted may have resulted in the loss of a hydrogen bond leading to diminished binding affinity[209].

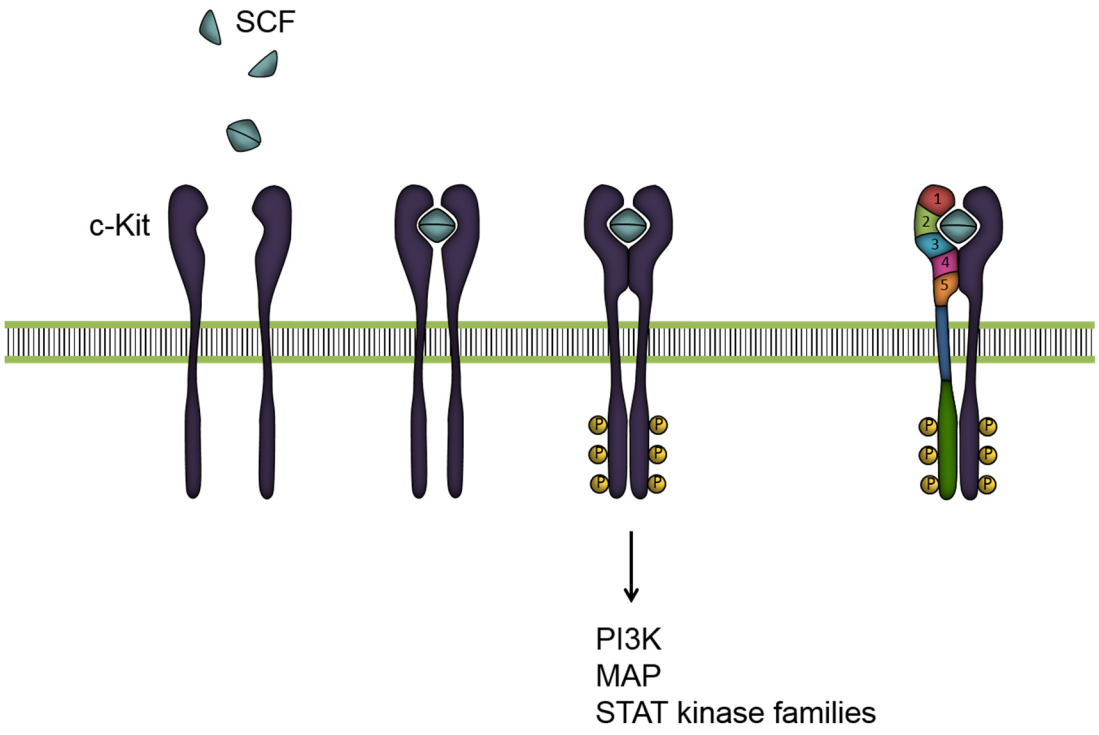


Figure 7: Stem cell factor binding to its receptor c-Kit.
Stem cell factor monomers form a homodimer and bind as such to extracellular domains 1-3 of two Kit monomers, bringing domain 4 and 5 in close proximity and causing a conformational change. Both Kit monomers bind bivalently to each other leading, enabling auto-phosphorylation on Kit.

The KIT protein is a type III tyrosine kinase receptor, which are characterised by a split tyrosine kinase domain[210]. Its glycosylated extracellular region contains five immunoglobulin-like (Ig-like) domains. KIT is anchored in the cell membrane by a single transmembrane domain, flanking the first of the bisected tyrosine kinase domains, which is separated from the second

domain by a kinase-insert region (Figure 7)[209,210]. *In vitro* binding analysis of SCF to a truncated KIT lacking both the transmembrane and cytoplasmic domains, have shown that KIT receptor binding is dependent on the ectodomain alone[210].

1.5.2 The SCF-KIT signalling pathway

Upon ligand binding, individual SCF protomers bind to the immunoglobulin-like domains D1, D2 and D3 of an individual KIT protomer[208,209]. In doing so, the monomeric KIT receptor undergoes a conformational change, allowing the interaction between the Ig-like domains D4-D4 and D5-D5 to allow dimerisation with another KIT monomer[209], followed by the bivalent binding of the ligand and in doing so stabilising the conformation (Figure 7)[209,210]. This dimerisation facilitates auto-phosphorylation of KIT [206,209]. This is followed by ubiquitination of the receptor, marking it for internalisation and lysosomal degradation[211]. Binding of soluble SCF causes a rapid and transient activation of c-KIT, while the membrane-bound isoform activates the receptor in a more sustained manner. This might be because the complex cannot be internalised as rapidly in this case[211,212].

Upon activation of the receptor, intracellular proteins bind the kinase domains of Kit directly and undergo tyrosine phosphorylation. Several groups have demonstrated that members of the STAT (Signal Transducer and Activator of Transcription) family (STAT1, STAT3, STAT5), the PI3K family (AKT, RSK1, RPS6) as well as the MAP (mitogen-activated protein) kinase family (ERK, MEK, p38, c-RAF) are phosphorylated as a result of SCF/KIT complex formation[191,206,210,213,214].

1.5.3 SCF directly affects HSCs and can act synergistically with a variety of other growth factors

Transplantation experiments performed by McCulloch, Siminovitch and Till in the early 1960's provided the earliest evidence that SCF may act directly on HSCs. Bone marrow and spleen derived haematopoietic cells of the genotype *Sl/Sld or Sld/Sld,* carrying heterozygous or homozygous mutations in the region encoding stem cell factor, were transplanted into wild type mice and vice versa and their spleens were examined shortly after for colony forming units (CFU-S). It was found that upon transplantation into wild type recipients no differences in colony forming efficiency could be observed. However, the opposite was true for the transplantation of wild-type (WT) cells into mutant recipients. Upon examination, spleens were significantly smaller and had only few colonies when compared to a wild-type recipient. Thus,

the defect exerted by the *Sl* mutation is not intrinsic to the colony-forming cells but affects the haematopoietic microenvironment[215].

Transplantation of WT foetal liver cells into the c-Kit mutant *W/Wv* recipients can partially or entirely cure the anaemic phenotype, with red blood cell counts returning to normal levels. Changes of the haemoglobin pattern to that of the donor cells, proves that the implanted cells are indeed the source of the newly generated erythrocytes[216]. Transplantation of *Sl/Sld* bone marrow cells into *W* recipients also restored blood values to normal[215].
McCulloch and Till also showed that *W/Wv* recipient mice do not require irradiation prior to transplanting haematopoietic cells derived from non-mutant litter mates (*ww*). They hypothesized that the *WWv* cells either are deficient in their colony-forming ability or require a stimulus for colony formation[217]. It is now known that the loss of Kit function, thus the inability to respond to environmental stimuli, is the cause for *WWv* cells' inability to compete with wild type cells[218]. These results strongly suggest that SCF, produced by cells of the haematopoietic microenvironment, directly acts on HSCs, whereas the *W* mutation affects the stem cells directly[215,216].

SCF has been shown to stimulate proliferation of human, non-human primate, and mouse haematopoietic stem and progenitor cells[219-221]. When SCF stimulated bone marrow cells are transferred into medium lacking SCF, clones that have already begun to proliferate will stop proliferating and die. This suggests that SCF has a direct effect on the colony initiating cells in the bone marrow[128]. In both humans and mice, SCF acts as a radioprotective agent when administered *in vivo* prior to chemotherapy (human) or irradiation (mice)[213,222-224]. However, due to its functional pleiotropy, SCF also activates melanocytes and mast cells leading to hyperpigmentation and severe allergic or anaphylactic responses, respectively[199,213,225].
Colony formation is greatly enhanced when SCF is used synergistically with a number of other growth factors and cytokines, such as IL-3, IL-6, IL-11, Erythropoietin (EPO), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF)[125-128,219,220].

When SCF is administered *in vivo*, peripheral blood and bone marrow cellularity increases within the first 7 days in both mice[126] and baboons[220] but returns to baseline levels by week three of treatment. This effect is enhanced in mice when G-CSF is used synergistically but not with G-CSF alone[126]. Contrary to these findings, it has also been reported that SCF does not

synergise with GM-CSF, macrophage colony stimulating factor (M-CSF), IL-1, IL-4, Il-5, tumour necrosis factor (TNF), and leukaemia inhibitory factor (LIF)[226]. Colony formation assays performed on HSCs ($Lin^{-}Rho123^{lo}Sca\text{-}1^{+}$) only stimulated with SCF failed to generate any colonies. However, in combination with IL-3, IL-6 or G-CSF, colonies could be obtained[226]. When used alone, SCF increased survival compared to medium without any cytokines. SCF in synergy with other cytokines stimulates cell proliferation and retains HSCs repopulating ability *in vitro* for up to 7 days. However, when competitively transplanted with fresh bone marrow, stimulated cells repopulated a significantly lower amount of recipient mice[125,226]. Thus, SCF clearly affects haematopoietic cells *in vivo* and *in vitro*, although its effects on cell division, survival and differentiation needs to be teased apart and further investigated.

Since these experiments (which spanned several decades), it has been of great interest to establish cytokine cocktails that could selectively expand HSCs by stimulating self-renewal, proliferation, and survival whilst inhibiting differentiation *ex vivo*. Several groups have shown that a combination of IL-3, IL-6 and SCF expands HSPCs in culture and maintains engrafting capability for up to 14 days[227-230]. Similar results have been published for IL-11 and SCF[129,231-233]. Interestingly, IL-3 has also been reported to have a negative effect on proliferation and the reconstitution capacity of HSCs when used without IL-6[229,230]. Conflicting reports have been published whether or not SCF synergizes with Flt3-ligand (FL)[231,232]. Failure to reconstitute the haematopoietic system after 14 day culture of LSK cells with SCF and FL alone[232] suggests that IL-6 or IL-11 is necessary to retain HSCs stemness. Contrary to this, observations have been made that stimulation of single cells (LSK) with FL and SCF leads to colony formation, which was not enhanced by IL-6 addition. However, transplantation data to confirm engraftment capabilities of these cells are missing from this study[231]. Subsequent research by the same group has shown that IL-11 and SCF in combination have a significant positive effects on the number of colony forming cells (CFC), long-term culture initiating cells (LTC-ICs), total cellular output, and most importantly competitive repopulating units (CRU). Addition of FL to these two cytokines does not change CRU expansion[124], suggesting that FL is not essential, which is consistent with the absence of the FLT3 receptor (CD135) on the surface of mouse LT-HSCs.

Yet, all studies listed above mainly have used bulk population of haematopoietic stem and progenitor cells (i.e. LSK or $Lin^{neg}Sca\text{-}1^{+}CD34^{neg}$). Thus, any effect SCF may have on HSCs

cannot necessarily be distinguished from its effect on progenitor cells. Single cell *in vitro* studies have addressed this problem. While Audet et al. and Miller et al. both used very low cell numbers to initiate colonies, the sorted populations of LS^+ and LSK only contain approximately 0.053% and 0.397% stem cells respectively[124,234].

Single cell studies with LT-HSCs have shown that the concentration of SCF differentially affects HSCs ($Lin^{neg}CD45^{mid}Rho^{neg}SP$) in their engrafting capabilities. A high concentration promotes HSCs self-renewal with 25% of cells being able to engraft, whereas HSCs cultured in 30-fold lower amount of SCF show a 3-fold reduction in reconstitution capacity from as early as 16h in culture. This reduction is maintained in 4-day clones. Interestingly, cell cycle kinetics and survival were not affected[10]. Another study showed that inhibition of lipid raft clustering of c-Kit results in the abrogation of SCF/KIT signalling, with HSCs (LSK $CD34^{neg}$) remaining as single cells *in vitro* for up to 10 days. Interestingly, some of these cells retained the ability to reconstitute all lineages in primary transplantation recipients[121]. These finding suggest that SCF may not be necessary for the maintenance of "stemness" per se, but rather it is required for HSCs proliferation and self-renewal.

1.6 *In vitro* expansion of haematopoietic stem cells

The ability to self-renew while retaining multipotency makes HSCs an ideal candidate for the treatment of haematologic disorders such as leukaemia. Since the patient's own haematopoietic system has been severely compromised by chemo- or radiotherapy, in an effort to eliminate all malignant cells, a bone marrow transplant is necessary to rebuild the blood system. Common sources of HSCs for transplantation are bone marrow (BM), mobilised peripheral blood (mPB) and umbilical cord blood (UCB) from human leukocyte antigen (HLA) matched donors. However the availability of HLA compatible donors is limited, with only 60–70% of patients finding a suitable donor and UCBs do not always contain sufficient numbers of HSCs for transplantation into an adult patient[235,236]. Developing a quality- controlled protocol for the *in vitro* expansion of (autologous) HSCs would guarantee on-demand availability of HSCs for the treatment of haematological disorders. In order to do this, we need to understand the mechanisms driving symmetric self-renewal divisions of HSCs and apply them outside the body[236].

Extensive research has been conducted into intrinsic and extrinsic regulators of HSCs expansion[236,237] (some of which were described above). The strongest intrinsic determinants to

date include Hox genes, epigenetic regulators such as the Polycomb-group proteins and miRNAs[139,236,237]. However, introduction of genetic material is undesirable in clinical protocols since the potential of malignant transformations or stem cell exhaustion cannot be excluded[236]. Therefore, much research in HSCs expansion has focused on extrinsic regulators including cytokines and developmental regulators such as known players in the Wnt signalling pathway, Shh signalling pathway, and the Notch pathway[236-238]. In addition, small molecules have recently been shown to modulate *in vitro* HSCs expansion[236,239], most notably are StemRegenin1 (SR1)[123,235] and UM171 which lead to a 2-fold and 13-fold increase in human LT-HSCs respectively after 7 day culture and in combination lead to a nearly 30-fold expansion of LT-HSCs measured by their frequency at 20 weeks following transplantation compared to uncultured BM[235]. Another promising molecule for the expansion of human HSCs is Prostaglandin E2 (PGE2). Pretreatment of cord blood cells with PGE2 prior to transplantation into patients has been shown to improve multi-lineage engraftment over those transplanted with untreated cord blood. However, its efficacy on *in vitro* expansion on human HSCs has not yet been assessed[240,241].

One of the major reasons for this may be that most HSCs expansion efforts take place in isolation, ignoring any potential contribution of the physical niche in which they typically reside. Adult HSCs reside in the bone marrow microenvironment, where they encounter cytokines, growth factors and chemokines, which are largely but not exclusively produced by niche cells[18,242]. These factors can be present in either soluble form or as insoluble, tethered transmembrane receptor ligands and extracellular matrix (ECM) molecules. The microenvironment therefore is a combination of differentiated cells, key soluble molecules and a tissue specific matrix, all potentially influencing stem cell fate choice in a complex spatially and temporally controlled manner[243,244]. Whereas the effects of soluble factors on HSCs self-renewal and differentiation have been extensively studied, very little is known about how mechanical forces within the stem cell niche instruct HSCs fate choice and only a few studies have attempted to answer these questions[242,244]. What is clear, is that HSCs express surface adhesion receptors (e.g., integrins) and are able to sense mechanical forces through cytoskeletal components[244]. Moreover, the bone marrow microenvironment is relatively soft with an elasticity measured at approximately 300 Pa[245] compared to tissue culture plastic which ranges in stiffness between 2–4 GPa, several orders of magnitude stiffer[246]. One study has suggested that stiffness affects HSCs fate choice as measured by HSCs (LSK $CD150^{+}$) to progenitor (LSK) ratio, which was maintained or increased on soft tropoelastin coated plates when

compared to non-coated controls[247]. Another pioneering study in human HSCs reported that Myosin II (MyoII) is involved in adhesion and matrix sensing, as its polarisation is supressed in $CD34^+$ stem and progenitor cells cultured on soft substrate but promoted on stiff matrices. MyoII is asymmetrically segregated upon cell division and remains high in $CD34^+$ cells whereas the MyoII low daughter cells differentiates[245]. Overall however, this remains a significantly under-explored area of HSCs expansion efforts.

This is in contrast to a number of other stem and progenitor cell systems where substrate stiffness has been shown to influence lineage differentiation[243,244]. Mesenchymal stromal cells (MSCs) are multipotent with osteogenic, chondrogenic and adipogenic potential. On stiff substrate MSCs preferentially differentiate into cells of the osteogenic lineage, whereas culture on a soft matrix leads to adipocyte differentiation[244,248]. In the liver, hepatocyte differentiation has been shown to be regulated by the composition of the extracellular matrix[249] and reports show that hepatic stellate cells remain quiescent on soft matrices and differentiate into myofibroblasts when cultured on stiff substrates similar to that of a fibrotic liver[243]. In skin, keratinocytes grown on small, circular ECM islands, which restrict cell spreading, terminally differentiated, whereas those seeded on larger island remained immature. This was due to impaired actin disassembly and remodelling, indicating cell shape is the key factor determining cell fate[250]. The same group later reported that keratinocyte morphology, spreading and differentiation was influenced by substrate stiffness. Keratinocytes on soft matrices failed to form focal adhesions necessary for the activation of downstream pathways such as the ERK pathway[251]. However, it is important to note that the anchoring points for collagen were the main determinants for these differences as opposed to matrix stiffness itself. The authors found that differentially spaced anchoring points for collagen lead to changes in cell behaviour comparable to changes when cultured on matrices of varying stiffness[251]. It is therefore necessary to consider not only the composition of the matrix itself but also the geometry (two dimensional or three-dimensional (3D)) and elasticity, all of which impact cytoskeletal tension[242,244].

HSCs traditionally have been cultured in a two-dimensional fashion on rigid tissue-culture plastic sometimes coated with ECM proteins such as collagen or laminin, on feeder cell layers or on ECM derived hydrogels such as Matrigel[242]. To tease apart the mechanical effects from the biochemical stimulus on HSCs fate choice, cells need to be grown in a 3D matrix of defined composition mimicking the natural tissue as closely as possible. Thus, naturally derived

hydrogels such as Matrigel and co-culture on feeder layers are undesirable due to the undefined nature of the material itself[242,244]. In addition, the substrate needs to allow for sufficient gas exchange and nutrient supply to guarantee maximal cell viability and the matrix must not be physically constraining thus inhibiting proliferation, migration and morphogenesis[242]. The development of scalable, artificial 3D niches would allow for high-throughput screening of optimal conditions supporting HSCs self-renewal *in vitro* leading to the expansion of human HSCs for clinical applications.

1.7 The aims of this PhD thesis

Haematopoietic stem cells have been studied for more than half a century but only in the last two decades have major advances in technology allowed us to begin studying HSCs at the single cell level in extreme detail. These advances include index-FACS, single cell RNA sequencing, single cell *in vivo* imaging, single cell transplantation, and the construction of microfluidic devices.

Yet, much of what has been described on the population level has never been verified on the single cell level and at the purity level that we can investigate today. For instance, it has been shown on the population level that SCF increases cell survival but does not stimulate self-renewal when not used in synergy with other cytokines. Yet, virtually all culture conditions aimed at the maintenance and expansion of HSCs include SCF under the premise that it enhances self-renewal.

Therefore, the first aim of this PhD thesis was to investigate if short-term SCF stimulation alters HSCs fate choice on the functional as well as the transcriptional level in single HSCs and if any changes occur in a dose dependent fashion. The second objective was to assess how the absence of SCF/KIT signalling affects HSCs functionality *in vitro*. Finally, I explored how alterations in matrix stiffness affect HSCs survival *in vitro*.

2 Methods

2.1 Mice

C57BL/6-Ly5.2 (WT) were purchased from Charles River Laboratories (Saffron Walden, Essex, UK) and maintained at the University of Cambridge animal facility. Congenic C57BL/6-Ly5.1 (WT-CD45.1) and C57BL/6$^{w41/w41}$-Ly5.1 (W41) were bred and maintained at the University of Cambridge. All mice were housed in specified pathogen-free microisolator cages and were continuously provided with sterile food, water, and bedding. All procedures performed in this thesis were in compliance with the guidance on the operation of ASPA (Animals Scientific Procedures Act 1986) and performed under project license PPL 70/8406.

2.2 Phenotypic mouse haematopoietic stem cell isolation

2.2.1 Bone marrow harvest

Mice were sacrificed by cervical dislocation. Bone marrow was harvested from femur and tibiae of both hind legs, pelvic bones, sternum and the spine. All of the following procedures were carried out under sterile conditions. Bone marrow (BM) was obtained by crushing the bones using mortar and pestle in PBS (phosphate-buffered saline, Sigma-Aldrich, St. Louis, MO, USA (Sigma)) supplemented with 2% foetal calf serum (FCS, Sigma or STEMCELL Technologies, Vancouver, CA (SCT)) and 50mM EDTA (Ethylenediaminetetraacetic acid, Sigma), from now on referred to as PBSFE.

2.2.2 Erythrocyte depletion

Erythrocyte depletion was achieved using ammonium chloride (NH_4Cl, SCT). 3 mL cell suspension in PBSFE was treated with 5 mL NH_4Cl and incubated on ice for 10 minutes (min), vortexing the sample after 5 min. Cells were then washed with 7 mL PBSFE and resuspended in 500 µL PBSFE per mouse in preparation for lineage depletion.

2.2.3 HSPC enrichment

The red blood cell depleted cell suspension was subsequently enriched using either the discontinued 3-step or the currently available 2-step negative EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit (1976A SCT) with the following modifications:
Per mouse, cells were resuspended in 500 µL PBSFE and incubated with 10 µL (1/50) EasySep Mouse Hematopoietic Progenitor Cell (HSPC) Isolation cocktail for 15 min. on ice.

20 µL (1/25) EasySep Streptavidin RapidSpheres 50001 were added and the cell suspension incubated for 15 min. on ice.

For the discontinued 3-step kit, EasySep Biotin Selection Cocktail was added (1/10), followed by a 15 min. incubation on ice. Subsequently, magnetic particles were added (1/20) and the mix incubated on ice for another 10 min. PBSFE was added to a total of 2500 µL followed by a 3 min. incubation in the EasySep Magnet at room temperature (RT). The supernatant was transferred into a new tube and the magnet incubation step was repeated one additional time.

2.2.4 Fluorescence-activated cell isolation

HSCs were isolated from the HSPC enriched cell suspension using fluorescence-activated cell sorting (FACS) following antibody staining. The cell suspension was incubated with antibody solution for 30 min. on ice, followed by a 20 min. incubation on ice with streptavidin (SAV) labelled secondary antibodies when necessary. The DNA intercalating dye 7-AAD (7-amino-actinomycin D, Life Technologies, Carlsbad, CA, USA, (Life Technologies)) was used to exclude non-viable cells. Antibodies used for HSCs isolation are listed in Table 2, p. 39. HSCs were defined as $EPCR^{high}$, $CD45^{+}$, $Sca\text{-}1^{high}$, $CD48^{low/neg}$, $CD150^{+}$. HSCs were sorted on a BD Influx cell sorter (BD Biosciences, San Jose, CA, USA (BD)) using the following filter sets 488 530/40 (for FITC), 561 585/29 (for PE), 405 460/50 (for BV421), 640 670/30 (for APC), 561 750LP (for PE/Cy7), 640 750LP (for APC/Cy7), 405 520/35 (for BV510), 640 720/40 (for AF700), and 561 670/30 (for 7-AAD). Cells were sorted in either purity or single sort mode. Single HSCs were isolated using the single-cell deposition unit of the sorter placing 1 cell into the wells of round bottom 96-well plates, each well having been preloaded with 50 µL medium (described below). Cells were sorted at the Cambridge Institute for Medical Research Flow Cytometry Core Facility.

Table 2: Antibodies used for phenotypic mouse HSCs isolation.

Antibody	Clone	Fluorophore	Manufacturer
Biotinylated HSPC cocktail	145-2C11; M1/70; 6D5; RA3-6B2; RB6-8C5; TER-119	N/A	SCT
CD150	TC15-12F12.2	PE/Cy7	Biolegend
CD34	RAM34	Alexa Fluor 700	Biolegend
CD45	30-F11	FITC	Biolegend
CD48	HM48-1	APC	Biolegend
CD117/c-Kit	2B8	APC/Cy7	Biolegend
EPCR	RMEPCR1560	PE	SCT
SCA-1	D7	BV421	Biolegend
SCA-1	D7	BV605	Biolegend
Streptavidin	NA	BV510	Biolegend

2.3 Phenotypic human haematopoietic stem cell isolation

2.3.1 Isolation of mononuclear cells

Cord blood was obtained from Cambridge Blood and Stem Cell Biobank with informed consent from healthy donors and collected in accordance with regulated procedures approved by the relevant Research and Ethics Committees. Mononuclear cells (MNC) were isolated by using Pancoll lymphocyte separating medium (Pancoll, PAN Biotech, Aidenbach, Germany). Cord blood was mixed with equal parts of PBS and layered on Pancoll. Layered blood was then centrifuged at 500 g for 25 min. at RT with the brake turned off. The MNC layer was subsequently carefully aspirated and transferred to a new tube.

2.3.2 Erythrocyte depletion

The MNC cell fraction was subsequently treated with red blood cell lysis buffer (Biolegend, San Diego, CA, USA (Biolegend)) for 15 min. at 4°C to deplete erythrocytes.

2.3.3 CD34 enrichment

Erythrocyte depleted cord blood MNCs were enriched for CD34 using the human CD34 microbead kit (Miltenyi Biotec, Bergisch Gladbach, Germany) with the following quantity modifications: 30 µL/10^8 cells CD34 Microbeads, FcR Blocking Reagent 30 µL/10^8 cells, PBS+3% FCS 90 µL/10^8 cells. Cells were separated using the AutoMACS cell separation technology (Miltenyi Biotec).

2.3.4 Fluorescent-activated cell isolation

$CD34^+$ enriched cells were subsequently stained for the antibodies listed in Table 3 for 20 min. at RT. Cells were then washed and taken up in PBS + 2% FCS. Zombie Aqua (Biolegend) was used as a cell viability marker. HSCs ($CD34^+CD38^{neg}CD45RA^{neg}CD19^{neg}CD49f^+CD90^+$) were then sorted on a BD FACS Aria III or on BD FACS Aria Fusion sorters available at the NIHR Cambridge BRC Cell Phenotyping Hub facility.

Table 3: Antibodies used for phenotypic human HSCs isolation.

Antibody	Clone	Fluorophore	Manufacturer
CD19	SJ25-C1	Alexa Fluor 700	Biolegend
CD34	561	APC/Cy7	Biolegend
CD38	HIT2	Pe/Cy7	Biolegend
CD45RA	HI100	FITC	Biolegend
CD45RA	HI100	PE	Biolegend
CD90	5E10	APC	Biolegend
CD90	5E10	PE	Biosciences
CD49f	GoH3	Pe/Cy5	Biosciences

Cells were sorted in single sort mode. Single HSCs were isolated using the single-cell deposition unit of the sorter placing 1 cell into the wells of round bottom 96-well plates, each well having been preloaded with 50 µL StemSpan medium (described below).

2.4 *In vitro* culture of HSCs

2.4.1 Liquid cell culture

Phenotypic HSCs were sorted and culture into StemSpan serum-free expansion medium (StemSpan SFEM, SCT) supplemented with 20 ng/mL human Interleukin-11 (IL-11, R&D Systems, Bio-Techne, Minneapolis, MI, USA, (R&D)), 300 ng/mL Stem Cell Factor (SCF, R&D or SCT), 2 mM L-Glutamine (Sigma), 1000 U/mL-100 µg/mL Penicillin-Streptomycin (Sigma), 100 µM 2-Mercaptoethanol (Life Technologies).

Standard SCF concentration was 300 ng/mL unless stated otherwise. In SCF-free conditions, StemSpan SFEM was additionally supplemented with 10% FCS.

As FCS is of undefined nature and has been shown to differ significantly from batch to batch, a serum-free alternative medium was implemented: Cells were sorted into Ham's F12 nutrient mixture (Gibco, ThermoFisher, Waltham, MA, USA (Gibco)) supplemented with 20 ng/mL human IL-11 (R&D), 300 ng/mL SCF (SCT or R&D), 2 mM L-Glutamine (Sigma), 1000 U/mL-100 µg/mL Penicillin-Streptomycin (Sigma), 1% ITS-X (Insulin-Transferrin-Selenium-

Ethanolamine, Gibco), 100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, Sigma), 100 mg/mL human serum albumin (HSA, Albumin Bioscience, Huntsville, AL, USA).

2.4.2 Single cell kinetics and clone size determination

Single phenotypic HSCs were sorted and cultured as described above. Cells were counted by visual inspection every 22–24 h to assess kinetics of cell division. On day 10 of culture, clone size was scored as follows: Very small (VS, <50 cells), small (S, 50–500 cells), medium (M, 501–5,000 cells), large (L, 5,001–10,000), and extra-large (XL, >10,001 cells), and the clone size estimation was previously validated using fluorescent counting beads as described in Kent et al. 2013[252].

2.4.3 Short-term liquid culture for RNA sequencing

To minimise cell transferring steps, which would lead to loss of cells, phenotypic HSCs were sorted into a 5 mL polypropylene tube containing 500 µL of StemSpan supplemented with either 30 ng/mL or 300 ng/mL SCF in addition to 20 ng/mL human IL-11, 2 mM L-Glutamine, 1000 U/mL-100 µg/mL Penicillin-Streptomycin, and 100 µM 2-mercaptoethanol. The tube was closed with a blue filter cap allowing air exchange. The 5 mL tube was subsequently placed into a 50 mL tube containing 5 mL of prewarmed PBS.

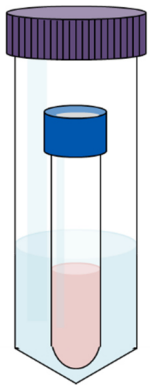


Figure 8: Schematic of 16h culture method prior to RNA sequencing.

A lid was loosely placed on the larger tube and it was subsequently placed into a humidified incubator at 37°C for 16h (Figure 8). 15 min. prior to the end of incubation, CD45-FITC antibody was added to the cells. After the incubation time, the cells were washed with 2 mL PBS. The remaining cells were resuspended in PBSFE containing DAPI (4′,6-diamidino-2-phenylindole, Cambridge Bioscience, Cambridge, UK) and resorted for viable $CD45^+$ cells into a 96 well PCR plate containing lysis buffer (see below).

Table 4: Antibodies used to analyse colony forming units.

Antibody	Clone	Fluorophore	Detected lineage
CD41	MWReg30	FITC	Megakaryocytes/Platelets
CD61	2C9.G2 (HMβ3-1)	PE	Megakaryocytes/Platelets
Ter119	TER-119	PE/Cy7	Erythrocytes
CD45.2	104	APC/Cy7	Haematopoietic cells
Ly6G/Gr1	1A8	BV421	Granulocytes
CD11b/Mac1	M1/70	APC	Monocytes/Granulocytes

2.4.4 Colony-forming assays

To assess viability and multi-lineage differentiation capacity of single cells, cells were transferred from liquid culture into 600 µL of semi-solid MethoCult GF M3434 (SCT). Cells were cultured for 14 days and colony type scored either manually by morphology or by antibody staining. Antibodies used are listed in Table 4. All antibodies were purchased from Biolegend). Samples were acquired on BD LSRFortessa cell analysers. Laser and filter configurations for analysers used are listed in Table 5. Flow cytometry data was analysed using FlowJo v10 (FLOWJO LLC, Ashland, OR, USA).

Table 5: Laser and filter configurations of LSRFortessa cell analysers.

Fluorophore	LSRFortessa 1	LSRFortessa 2
7-AAD	561 670/30	532 710/50
AF700	640 730/45	640 730/45
APC	640 670/14	640 670/14
APC/Cy7	640 780/60	640 780/60
BV421	405 450/50	405 450/50
BV605	405 610/20	405 610/20
FITC	488 530/30	488 515/20
PE	561 585/15	532 585/15
PE/Cy7	561 780/60	532 780/60

2.4.5 Culture in agarose-based gels.

Hydrogels were made by heating SeaPrep agarose (Lonza, Basel, CH) in IMDM (Iscove's Modified Dulbecco's Medium, Gibco) until dissolved and adding thrombin from human plasma (Sigma) to the agarose solution once cooled down to 40ºC. The mixture is then kept at 40ºC. Fibrinogen from human plasma (Sigma) dissolved in IMDM was added to IMDM and mixed with either Laminin (Sigma) or human plasma Fibronectin (Merck Millipore, Burlington, MA, USA). All mixtures were made at 2X concentration. The thrombin containing solution was then quickly added to the fibrinogen containing solution in a 96 well plate and left to polymerise on ice. Figure 9 shows the layout of the 96 well plate to produce 96 individual gels. The gels were subsequently topped up with 50 µL of culture medium. Single or multiple cells were sorted

as described onto the gels and cultured for up to 21 days. Cells were isolated from the gels by digestion with trypsin (Gibco) at 37°C for 30 min. The trypsin reaction was stopped with PBSFE and subsequently stained as described in 2.4.4.

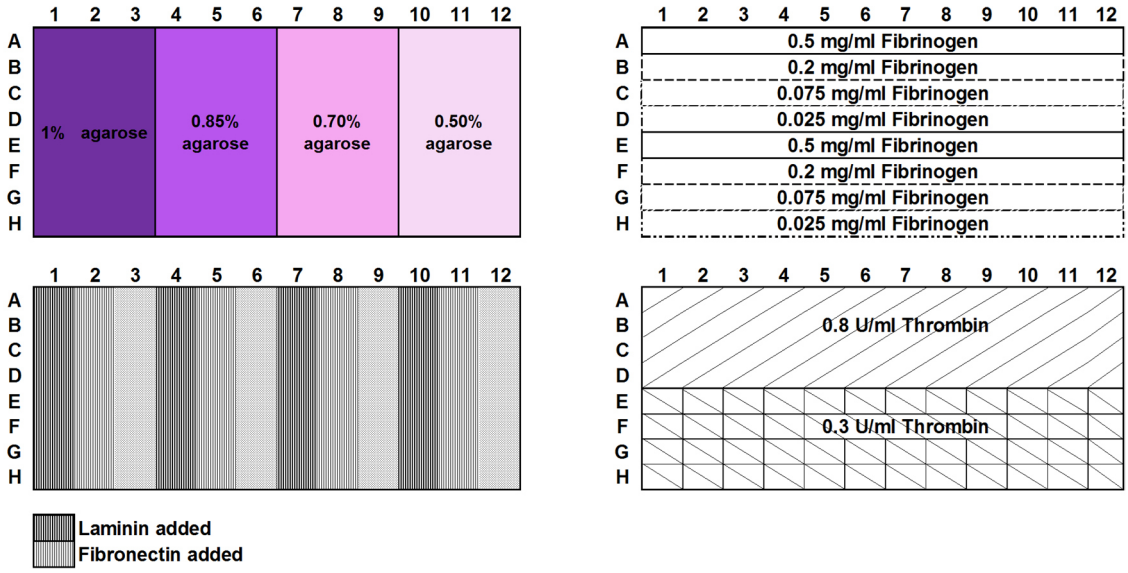


Figure 9: Plate layout for the production of agarose based hydrogels.
Combination of agarose, Fibrinogen, Thrombin and ECM proteins are used to generate 96 different hydrogels.

2.5 Atomic force microscopy

Bone marrow plugs were obtained by flushing the bone marrow of Nestin-GFP^{+} mice (gifted by Mendez-Ferrer lab[36]) with a 21 gauge hypodermic needle and 3 mL IMDM. Bone marrow plugs were mounted in a well in a 10% agarose plate and covered with IMDM.

Stiffness measurements of bone marrow plugs were carried out using an atomic force microscope (AFM, JPK Instruments, Berlin, Germany). Samples of exposed bone marrow were transferred onto an x/y motorised stage (AxioObserver A1, Zeiss, Jena, Germany) under an inverted microscope onto which the AFM was mounted. Tipless silicon cantilevers (0.1 to 0.3 N/m, Sicon-tl, Nanosensors, Neuchâtel, Switzerland) with a polystyrene bead glued at their tip (radius r = 18.64 µm ± 0.17 µm, microParticles GmbH, Berlin, Germany) were used to perform indentations on the bone marrow, with an approach speed of 10 µm/s and a set force of 10 nN. Cantilever position relative to the tissue was monitored using a CCD camera (The Imaging Source, Bremen, Germany) mounted on top of the setup. Indentations were converted into stiffness values (K) by applying the Hertz model to the recorded force-distance curves: F = 4/3 K r1/2 δ3/2, for an indentation depth of δ = 2 µm; using a custom written automated algorithm based in Matlab (Mathworks, Natick, MA, USA).

2.6 Generation of haematopoietic chimaeras

Donor cells were obtained from either WT or WT-CD45.1 mice. All donor mice were between 8 and 16 weeks of age. Recipient mice were either WT or W41. All singe cell transplants were carried out using W41 as recipient mice. The mice carry a mutation in the SCF receptor c-Kit, giving WT donor cells a competitive advantage[218].

Recipient W41 mice were sublethally irradiated with a single dose 400cGy using a caesium source. Recipient C57BL/6-Ly5.2 were lethally irradiated with split doses of 550 cGy (total = 1100 cGy), with at least 3h between doses. All recipients were >8 weeks of age.

Transplantations were performed by intravenous tail vein injection using a 29.5G insulin syringe. Limiting dilution transplantations were carried out into WT recipients by mixing the desired amount of WT-HSCs with 200,000 CD45.1/.2 whole bone marrow helper cells and subsequently transplanting the cell suspension.

For single cell transplants, single cells were sorted into 100 µL of medium in a 96-well U-bottom plate. 100 µL of PBS is mixed with the well content, all liquid is subsequently aspirated into the insulin syringe avoiding any air bubbles and injected into the tail vein.

For secondary transplantations, bone marrow was harvest from the primary recipient by flushing tibiae and femurs with PBSFE followed by red blood cell lysis. An equivalent of one femur was transplanted per mouse. Each primary donor mouse was transplanted into at least two secondary recipients.

In case of early death or sacrifice of the primary recipient, bone marrow was obtained from tibiae and femurs by crushing or flushing and subsequently frozen in 10% DMSO (Dimethyl sulfoxide, Fisher Scientific, Hampton, NH, USA) in FCS. On the day of transplantation, frozen cells are gently thawed in a water bath set to 37°C. The freshly thawed cell suspension is then added to 20 mL prewarmed IMDM in a dropwise fashion. Cells were washed to remove any DMSO and all remaining cells were transplanted into two recipients.

2.7 Peripheral blood analysis

Recipient mice were considered successfully repopulated when overall donor chimaerism >1% in the peripheral blood at 16 weeks post transplantation or later.

To assess donor chimaerism in recipient mice, peripheral blood (PB) samples were obtained from the tail vein every 4 weeks, starting 8 weeks post transplantation up to 28 weeks, unless otherwise stated. Blood was collected in EDTA coated microvette tubes (Sarstedt AG & Co, Nuembrecht, Germany). Blood samples were treated with NH_4Cl to deplete erythrocytes and

subsequently stained for lineage markers using the antibodies listed in Table 6 p.45. All antibodies were obtained from Biolegend. Samples were acquired on BD LSRFortessa cell analysers. Filter configurations for analysers used are listed in Table 5, p. 42. Cells of the B cell lineage were defined as viable single cells, myeloid negative ($CD11b^{neg}$, $Ly6g^{neg}$), $CD3e^{neg}$, $B220^{+}$. Cells of the T cell lineage were defined as viable single cells, myeloid negative ($CD11b^{neg}$, $Ly6g^{neg}$), $B220^{neg}$, $CD3e^{+}$. Cells of the myeloid lineage (GM, Granulocyte/ Monocyte) were defined as viable single cells, lymphoid negative ($B220^{neg}$, $CD3e^{neg}$), $CD11b^{+}$, $Ly6g^{+}$ or $CD11b^{+}$, $Ly6g^{low/neg}$. All flow cytometry data was analysed using FlowJo v10. An example of the gating strategy is displayed in Figure 10, p. 46.

Table 6: Antibodies used to analyse donor chimaerism in peripheral blood.

Antibody	Clone	Fluorophore	Detected lineage
B220	RA3-6B2	APC	B cells
CD11b/Mac1	M1/70	PE/Cy7	Monocytes/Granulocytes
CD11b/Mac1	M1/70	BV605	Monocytes/Granulocytes
CD3e	17A2	PE	T cells
CD45.1	A20	AF700	Recipient/Donor
CD45.2	104	FITC	Recipient/Donor
Ly6G/Gr1	1A8	BV421	Granulocytes

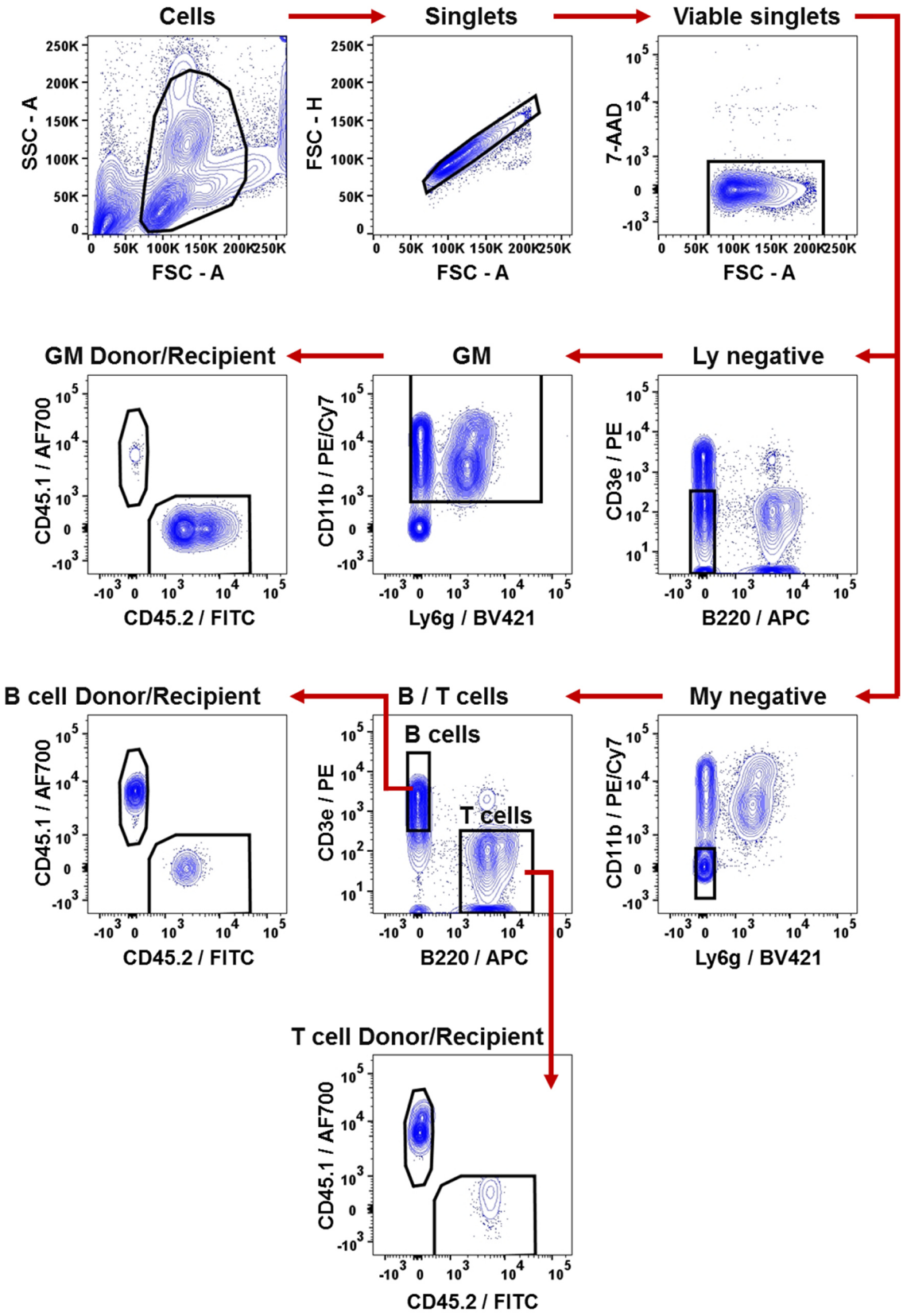


Figure 10: Gating strategy to assess donor chimaerism in peripheral blood.

2.8 RNA sequencing

2.8.1 Single cell RNA sequencing analysis

Single cell RNA sequencing (scRNA seq) analysis was carried out as previously described in Picelli et al. 2014 (Smart-seq2). Briefly, single cells were sorted by FACS directly into 96-well PCR plates containing lysis buffer. Lysis buffer contained 0.2% Triton X-100 (Sigma) and Rnase inhibitor (SUPERase, Thermofisher) in nuclease-free water (Thermo Fisher).

Libraries were prepared using the Illumina Nextera XT DNA preparation kit. Libraries were pooled and run on the Illumina Hi-Seq4000 at the CRUK Cambridge Institute Genomics Core. scRNAseq data was analysed by Dr. Fiona Hamey, Rebecca Hannah, and Evangelia Diamanti (all Goettgens' lab), and Hugo Bastos (Kent lab). Quality control settings for single cell data was as follows:

To exclude cells from which low quality libraries with insufficient sequencing depths were generated, the threshold of number of mapped reads was set to $>2*10^5$, with mapped reads encompassing nuclear genes, mitochondrial genes and ERCCs. Empty wells and dead cells were excluded by setting a minimum threshold of 20% for reads mapping to known genes. Additionally, the threshold for reads mapping to mitochondrial genes was >0.2 ensuring that a minimum of 20% of reads map to non-mitochondrial genes. As shown by in Appx-A Figure 1 and Appx-A Figure 2 in Appendix A, setting further threshold using other parameters would not eliminate any more cells than those mentioned above and thus were not applied. For example, setting the threshold ratio of "ERCC" to "mapped genes" to <1 was not necessary as to exclude more empty wells and dead cells than those excluded by above used parameters.

2.8.2 Bulk RNA sequencing analysis

Bulk RNA sequencing was carried out at the Genomics core facility of the Cambridge Stem Cell Institute upon submission of sample RNA. Libraries were prepared as described above using the Smart-seq2 protocol.

RNA was extracted from the samples using the PicoPure RNA Isolation Kit (Thermo Fisher) according to protocol. RNA sequencing data was analysed by Dr. Fiona Hamey (Goettgens lab) and Hugo Bastos (Kent lab).

2.9 Normalisation of single cell index-sorting data

Surface marker intensity of single HSCs across different experiments were normalised and batch corrected using the flowCore (version 1.42.3) and sva (version 3.24.4) R packages. Single

HSCs were sorted in 96-well format and each plate was classified as an independent batch prior to batch correction. All recorded surface markers were arranged in a flow frame and subsequently subject to logicle transformation prior to batch correction. The analysis and all resulting figures were computed in R (version 3.4.2).
Normalisation was performed by Daniel Bode (Kent lab). The original script was developed by Blanca Pijuan Sala in Elisa Laurenti's lab.

2.10 Statistical analyses

Extreme limiting dilution analysis (ELDA) was carried out using the ELDA software provided by the Walter and Elisabeth Hall Institute of Medical Research Bioinformatic resources, as described in Hu, Y, and Smyth, GK (2009)[253]. Gene ontology annotation was carried out using Enrichr[254,255]. Statistical data visualisation through violin plots was carried out using Python Seaborn. All other statistical analyses was performed using Graphpad Prism 6.07 for Windows (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 Differential SCF stimulation *in vitro* does not affect engraftment capability but alters HSCs expansion *in vivo*

Stem cell factor (SCF) is a cytokine widely used as part of various cytokine and growth factor cocktails aimed at human and mouse haematopoietic stem cell (HSCs) maintenance and expansion[10,256,257]. Research has shown that SCF independently can alter HSCs fate choice[10]. In 300 ng/mL supplemented with human Interleukin-11 (IL-11), nearly 20% of 4-day single cell derived clones are able to long-term reconstitute the haematopoietic system of recipient mice, whereas 10 day clones fail to repopulate secondary recipients[64]. Interestingly, the concentration of SCF has been shown to affect maintenance of HSCs activity, with a 30-fold reduction in SCF resulting in a 3-fold reduction of repopulation ability in 4-day clones. Importantly, this change in stem cell activity appears to occur prior to the first division between 8 and 16h of culture (Figure 11), without concurrent changes in division kinetics or survival[10]. Notably, a low concentration of SCF was shown to promote differentiation whereas a high concentration stimulated HSCs self-renewal. These experiments were performed on HSCs isolated based on the phenotype CD45^{mid}Lin^{-}Rho^{-}SP, which contain ~30% long-term repopulating cells[64].

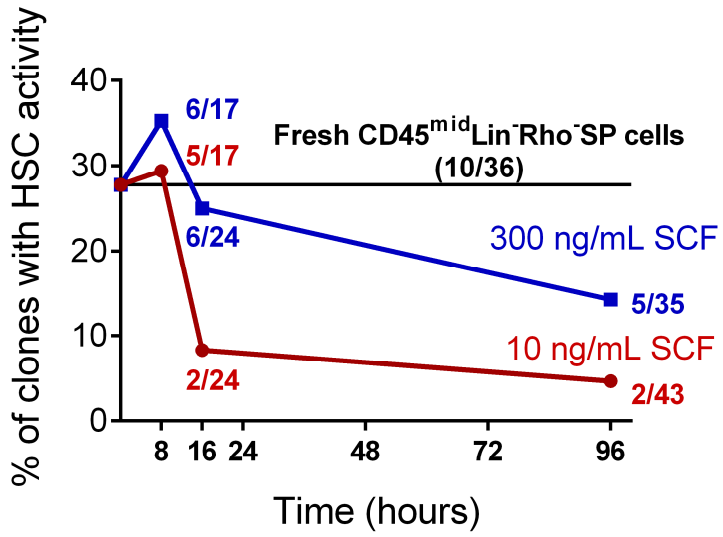


Figure 11: Differential Stem Cell factor stimulation alters HSCs activity.
Single cell HSCs (CD45^{mid}Lin^{-}Rho^{-}SP) were transplanted directly harvested from the bone marrow or following 8h, 16h or 96h in culture. A 30-fold reduction of SCF reduces HSCs activity 3-fold within 16h of culture, when compared to unstimulated, uncultured HSCs. This reduction is maintained throughout the remaining culture period terminating at 96h. Modified from Kent et al. 2008, Blood.

It is of interest to advance our understanding of the underlying molecular mechanisms driving these different fate choices. Furthering our knowledge about molecular drivers of HSCs self-renewal may be applied to human HSCs leading to the establishment of culture conditions aimed at the expansion of human HSCs. These can ultimately be used in HSCs transplantation for the treatment of malignant or non-malignant blood disorders such as leukaemia or anaemia[235,239].

In addition, understanding the involvement of key molecules instructing specific lineage differentiation may aid in the targeted generation of mature blood types such as erythrocytes and megakaryocytes from either human embryonic stem cells, induced pluripotent stem cells or primary HSCs[258].

Therefore, my aim was to assess how HSCs self-renewal and differentiation is controlled by stem cell factor signalling. For this purpose an improved HSCs isolation phenotype was used as input material: Sca-1^{high}EPCR^{high}CD45^{+}CD48$^{lo/-}$CD150^{+} (Figure 12), isolating HSCs at 67% purity in primary[68] and ~50% secondary (unpublished) transplantations. This more highly purified HSCs population did not respond in the same way, resulting in significant differences in cell cycle kinetics and lower overall survival of HSCs. SCF concentrations were therefore adjusted to accommodate only a 10-fold difference between the high and low SCF dose. To assess functional differences between HSCs, single cell transplantations of differentially stimulated HSCs were carried out. The functional data was complemented by single cell RNA sequencing to detect any alterations in the cell's transcriptome. All transplanted HSCs were index-sorted, allowing the linking of functional output to surface marker profile.

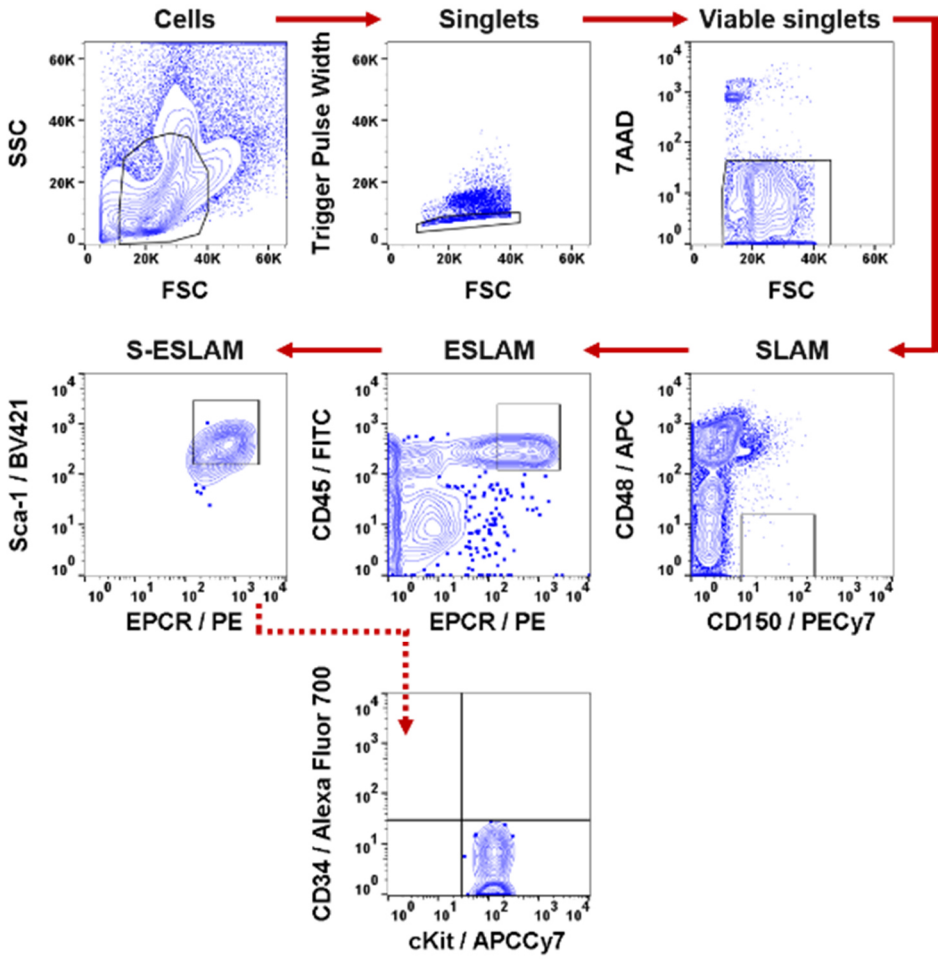


Figure 12: Representative gating layout to purify phenotypic S-ESLAM HSCs.
Sca-1^{high}EPCR^{high}CD45^{+}CD48$^{lo/-}$CD150^{+} HSCs are isolated from lineage-depleted bone marrow using FACS. The dotted line indicates that further gating is not part of the standard gating strategy for E-SLAM HSCs.

3.1.1 Low concentration of stem cell factor negatively affects cell survival and division kinetics of highly purified HSCs *in vitro*

FACS isolated single HSCs were cultured for a period of 10 days in serum-free medium (SFM) containing human IL-11 and mouse stem cell factor (SCF). SCF was used at three different concentrations: 10 ng/mL, 30 ng/mL and 300 ng/mL. To assess the effect of alteration of SCF concentration on single HSCs clone survival, cell division kinetics, and clone size were monitored daily for 10 days.

In contrast to a previous report using a less purified HSCs populations (~30% vs 67%), differences in cell survival were observed across the three SCF concentrations. Day 10 clone survival was 1.3x and 2.6x higher in medium containing 300 ng/mL mSCF (35.6% ± SD 15.83%) when compared to 30 ng/mL (25.6%, ± SD 12.8%) and 10 ng/mL (13.6%, ± SD 3.9%) respectively (Figure 13A). While these differences were not significant, they did represent a

nearly 3-fold reduction in cell survival, making future molecular experiments more challenging and potentially introducing an element of cell selection. Supporting this latter possibility, the surviving HSCs treated with 10 ng/mL SCF were significantly delayed in the time to first division and subsequent divisions showed significant delays at day 3, day 4, and day 7. In contrast, divisional kinetics between 300 ng/mL and 30 ng/mL were similar at all time points (Figure 13B, C, D). Consequently, single HSCs stimulated with 10 ng/mL almost exclusively made very small (VS) and small (S) clones, at the expense of larger clones (Figure 13E and Table 7). While HSCs treated with 30 ng/mL did give rise to a significantly higher proportion of VS clones than those stimulated with 300 ng/mL (p=0.0248, see Table 7), the percentages of small, medium, large, and very large clones did not differ between the two treatments (Figure 13E).

As there were virtually no differences in cell survival, cell cycle entry and cell cycle kinetics in HSCs stimulated with 30 ng/mL and 300 ng/mL SCF, these conditions were chosen to further investigate the molecular and functional changes in HSCs following differential SCF stimulation.

Table 7: Percentage of very small and small clones after SCF treatment.

Clone size	300 ng/mL SCF	30 ng/mL SCF	10 ng/mL SCF
VS	21.6%, ± SEM 5.0%)	45.1%, ± SEM 6.0%	65.2%, ± SEM 10.3%
S	36.6%, ± SEM 6.8%	45.0%, ± SEM 7.9%	33.3%, ± SEM10.5%

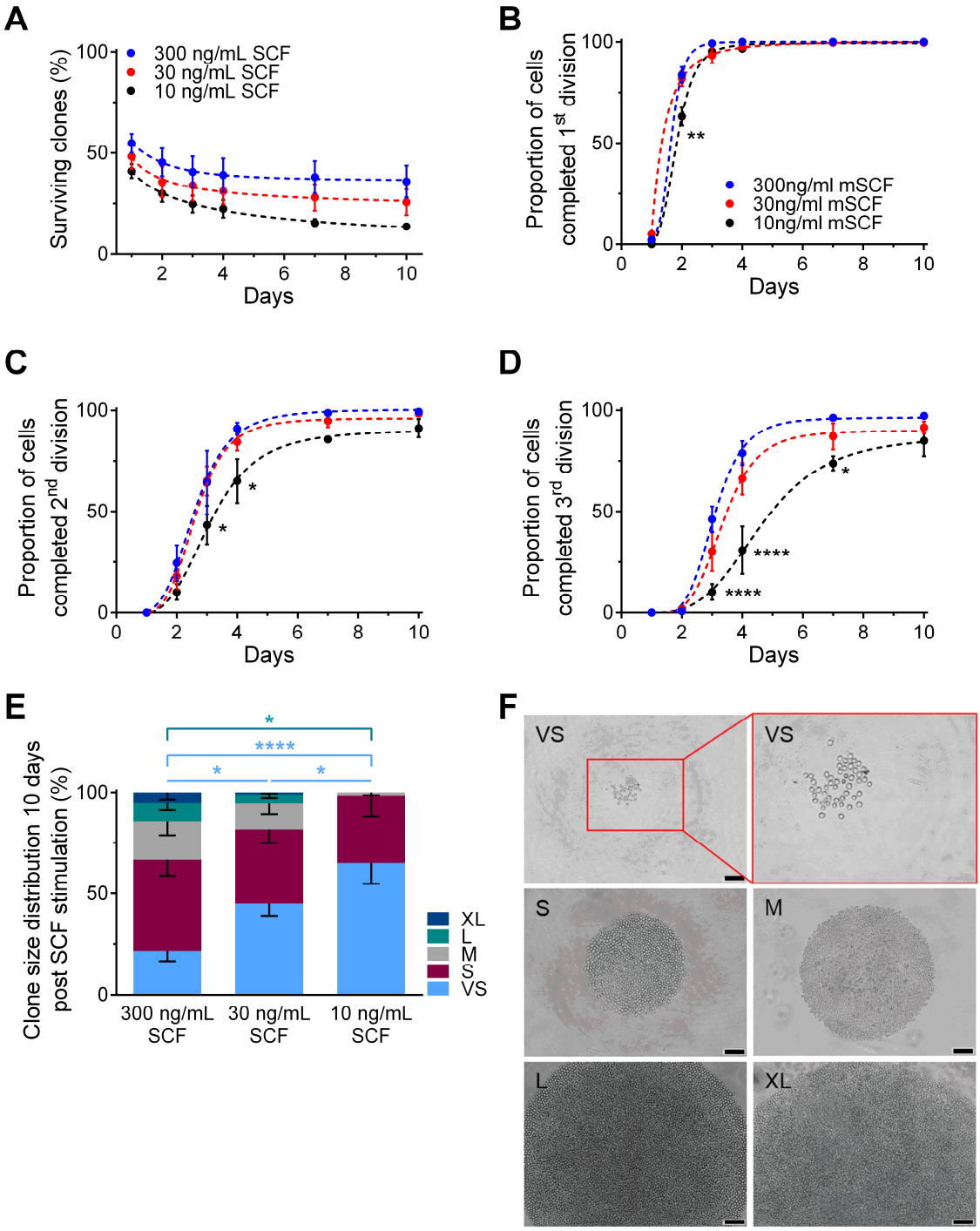


Figure 13: A 10 fold reduction in stem cell factor concentration does not alter HSCs kinetics or survival *in vitro*.

A) Survival of clones treated with different concentration of SCF. B-D) Division kinetics of cells treated with different concentration of SCF: E) Clone size distribution of 10 days after treatment with different concentration of SCF, n= 4 exp. F) Exemplary micrographs of different clone sizes. Scale bar = 100 µm. Error bars show standard error of the mean (SEM). 2way ANOVA, Tukey's multiple comparison test. Dashed line represents non-linear regression fit, [Agonist] vs. response, variable slope (four parameters). VS=very small, S=small, M=medium, L=large, XL=very large. 300ng: n=180, 4 exp; 30ng: n=128, 4 exp; 10ng: n=49, 4 exp.

3.1.2 Limiting dilution analysis does not reveal significant functional differences between short-term differentially stimulated HSCs.

To investigate the effect of different concentrations of SCF on the functional output of HSCs, 50 LT-HSCs were bulk sorted followed by a 16h treatment with high (300 ng/mL) or low (30 ng/mL) concentrations of SCF. Sublethally irradiated W41 mice were transplanted with 3 cells or 6 cells of the culture from each treatment and compared post-transplantation for their contribution to mature cell production. As seen in Figure 14A,B in both conditions 2 of 3 mice transplanted with 3 cells and 3 of 3 mice transplanted with 6 HSCs showed donor chimaerism >1% up to 20 weeks post transplantation, equivalent to an estimated stem cell frequency of 1 in 2.64 (i.e. 37.9%). At 16 weeks, all mice show multi-lineage repopulation (Figure 14C,D) with a possible tendency to have a less balanced lineage output from HSCs stimulated with a low concentration of SCF. In both mouse m1.4 and mouse m1.8 a biased lineage production towards myeloid cells can be observed, in contrast to mouse m1.9 which seems to generate exclusively lymphoid cells.

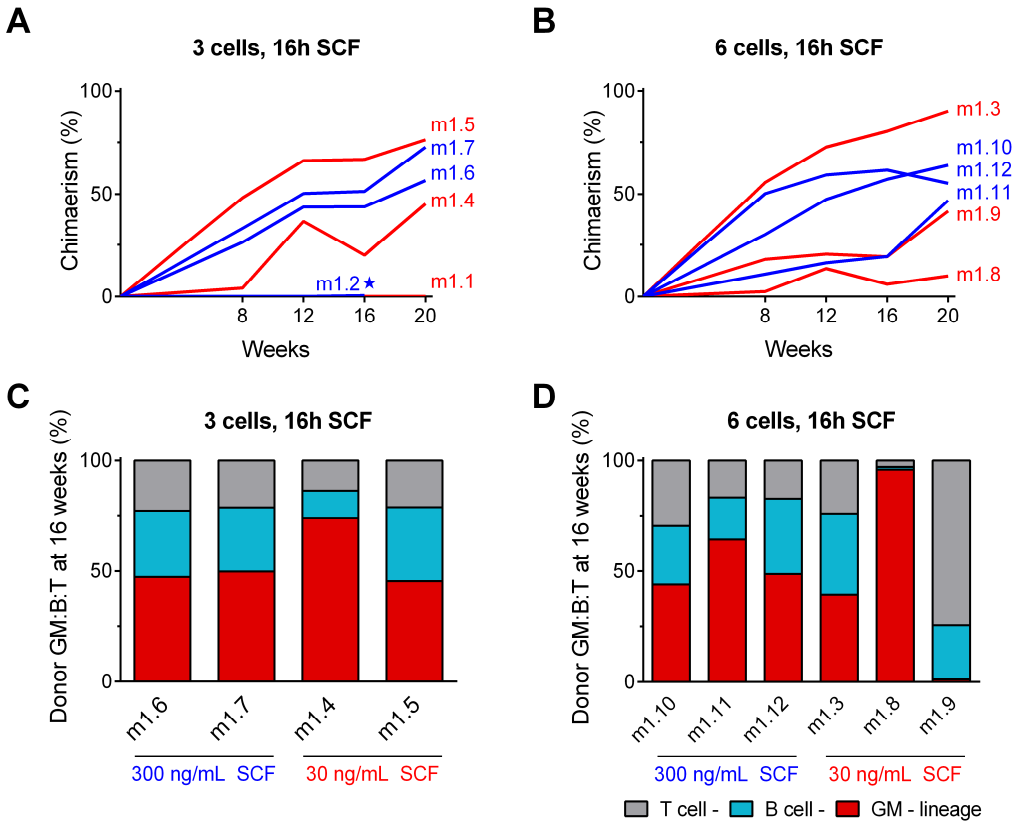


Figure 14: Primary transplantation of differentially treated HSCs.
A-B) Donor chimaerism in peripheral blood of W41 recipient mice over the course of 20 weeks post primary transplant. Blue: treatment with 300 ng/mL SCF. Red: treatment with 30 ng/mL SCF. Star indicates that this mouse was sacrificed for non-experimental reasons. C-D) Ratio of myeloid (GM) to lymphoid (B:T) lineage at 16 weeks post primary transplantation. All ratios were determined from peripheral blood samples.

As these results are not conclusive, a limiting dilution assay was performed at a 1, 3 and 10 donor cell doses.

At 20 weeks post transplantation, none of the mice transplanted (0 of 5) with 1 cell equivalent doses showed any repopulation (Figure 15A), whereas 2/6 (high SCF) and 2/7 (low SCF) mice that received 3 cells were repopulated (Figure 15B). All mice transplanted with 10 cells showed repopulation at 20 weeks (3/3, high SCF. 2/2 low SCF, Figure 15C). These results represent a non-significant (p=0.592) 1.2-fold difference in stem cell frequency between cells that were treated with 300 ng/mL SCF (1/6.69) and 30 ng/mL SCF (1/8.18, Figure 15D).

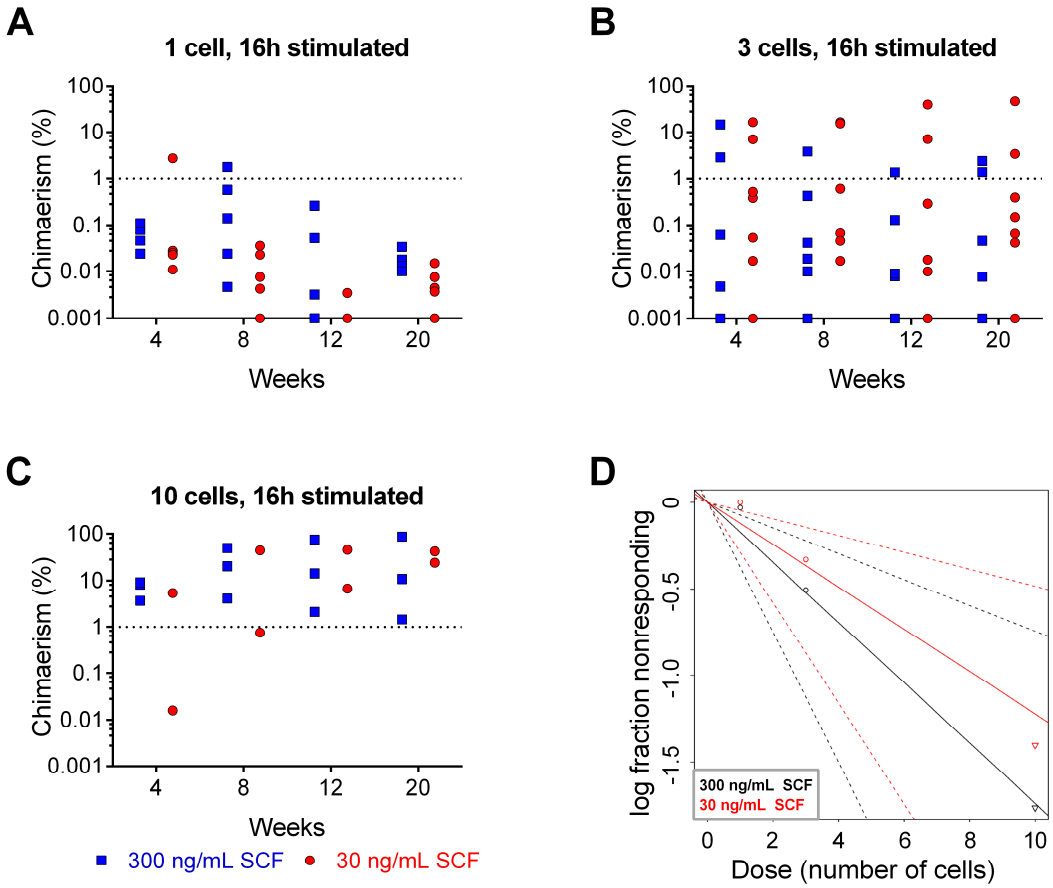


Figure 15: Limiting dilution transplantation of 16h stimulated HSCs.
A-C) Peripheral blood donor chimaerism in WT recipient mice. Recipients were transplanted with different cell doses of either 300 ng/mL or 30 ng/mL 16h treated HSCs in addition to $2*10^5$ whole bone marrow helper cells. Chimaerism at 4, 8, 12, and 20 weeks post primary transplant is displayed. D) Extreme Limiting Dilution Analysis (ELDA) plot, estimating stem cell frequency.

When comparing the lineage composition of the two differentially treated input HSCs, in both treatments unbalanced lineage contribution could be observed, independent from the cell dose (Figure 16A-D). In a bulk transplantation setting it is probable that multiple HSCs contribute to the repopulation in the recipient mouse and a balanced repopulation pattern should be

observed. Therefore, in mice receiving 10 HSCs, a balanced lineage output (like m3.3 in Figure 16C) would be expected as the pooled contribution of multiple HSCs subtypes. Yet, only 1 of 3 mice in high SCF and 1 of 2 mice in low SCF (m3.3 and m3.20, Figure 16C-D) display a balanced lineage output suggesting that one or a few HSCs dominate the lineage production in the other recipients.

From these data it can be concluded that low cell dose transplantations do not offer a high enough resolution to assess any functional changes between highly purified HSCs treated with different doses of SCF. This is likely due to the high reconstitution capabilities of Sca-1^{high}-ESLAM HSCs as well as their cell intrinsic heterogeneity[64,68]. Furthermore, transplanting 2% of the total culture as an equivalent of a single cell may result in none of the cells being physically transplanted. Therefore, it was necessary to perform single cell transplantation of HSCs to eliminate the possibility of comparing recipients that have multiple or only one dominant HSCs contributing to the lineage production.

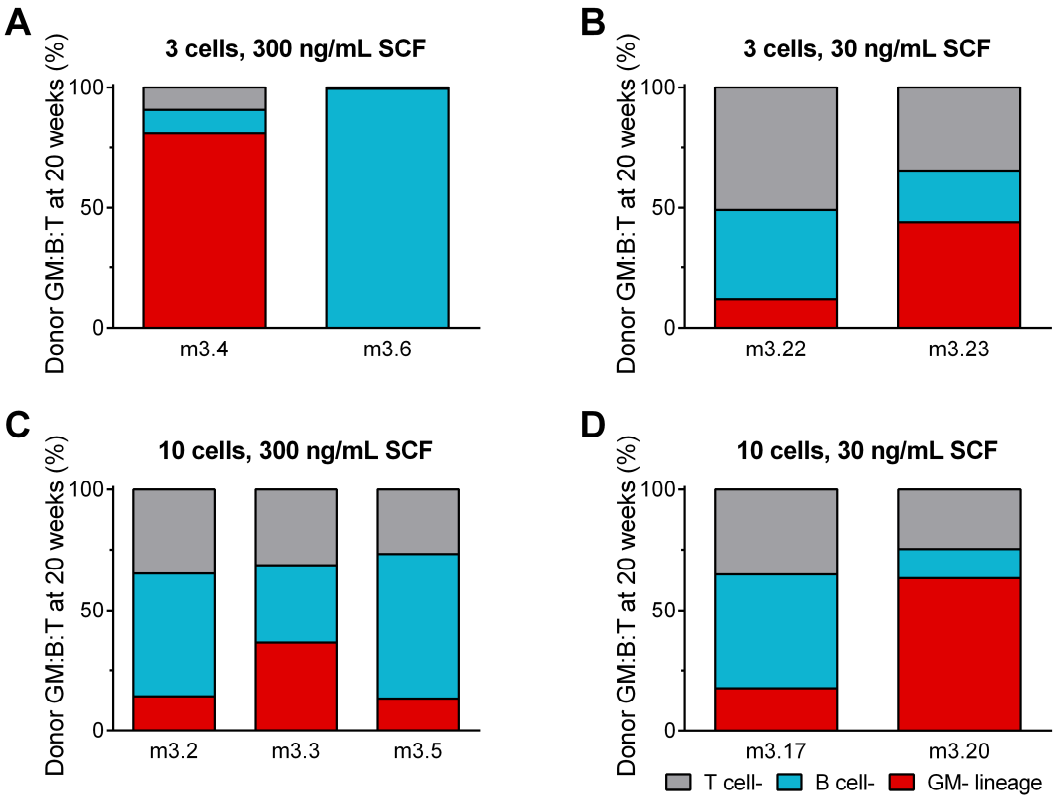


Figure 16: Ratio of lineage contribution in recipients of 16h stimulated HSCs.
A-B) Ratio of myeloid (GM) to lymphoid (B:T) lineage 20 weeks post primary transplantation in recipient of 3 treated HSCs. C-D) Ratio of myeloid (GM) to lymphoid (B:T) lineage 20 weeks post primary transplantation in recipient of 10 treated HSCs. All ratios were determined from peripheral blood samples.

3.1.3 Single cell transplantation shows that SCF does not alter HSCs frequency but may impact clonal expansion post-transplantation.

For the single cell transplants, single HSCs were sorted and stimulated for 16h with a high or low concentration of SCF in individual wells of a 96 well plate and presence of a single cell was confirmed prior to transplant. This approach removes the uncertainty associated with transplanting a proportion of total liquid and ensures that 1 single cell is delivered per recipient animal. The number of recipients successfully repopulated in primary transplantation does not differ between those from HSCs treated with 300 ng/mL SCF to those treated with 30 ng/mL with 14/40 (37.5%, Figure 17A and B) and 15/40 (35%, Figure 17C and D) respectively, from two separate experiments. In both settings, one mouse showed >0.5% and <1.0% donor chimaerism but was also serially transplantable, indicating the presence of functional HSCs. In secondary recipients 8 of 40 and 10 of 40 mice for high and low dose treated HSCs respectively, displayed multi-lineage repopulation. Notably the levels of donor contribution were significantly higher ($p=0.0274$) in secondary recipients of HSCs receiving a high dose of SCF, suggesting that more (or more potent) daughter HSCs were generated in the primary recipient compared to cells treated with a low dose of SCF (Figure 17E). In both treatments, one mouse retrospectively determined to have been transplanted with a gamma-HSCs (m7.14 and m10.23) showed repopulation in secondary recipients. However, only lymphoid lineages were detected and chimaerism steadily decreased over time (Figure 17A&D). It can therefore be assumed that the secondary engraftment is due to lymphocyte progenitors and long-lived lymphocytes.

This data does not reflect previous findings in that low SCF reduces HSCs frequency however, it does suggest that the concentration of initial SCF stimulation alters HSCs biology resulting in differential stem cell expansion post-transplantation. It is possible that this has not been observed previously due to the less pure population of HSCs. Alternatively, the lower dose of 10 ng/mL may exacerbate the reduction in HSCs activity not only resulting in lower HSCs expansion (as seen in 30 ng/mL) but also leading to loss of self-renewal activity. This may indicate that 30 ng/mL SCF is sufficient to maintain HSCs activity but not enough to stimulate HSCs expansion. To fully understand the impact of SCF signalling on HSCs fate choice, it would be necessary to perform dose response studies in combination with timed exposure.

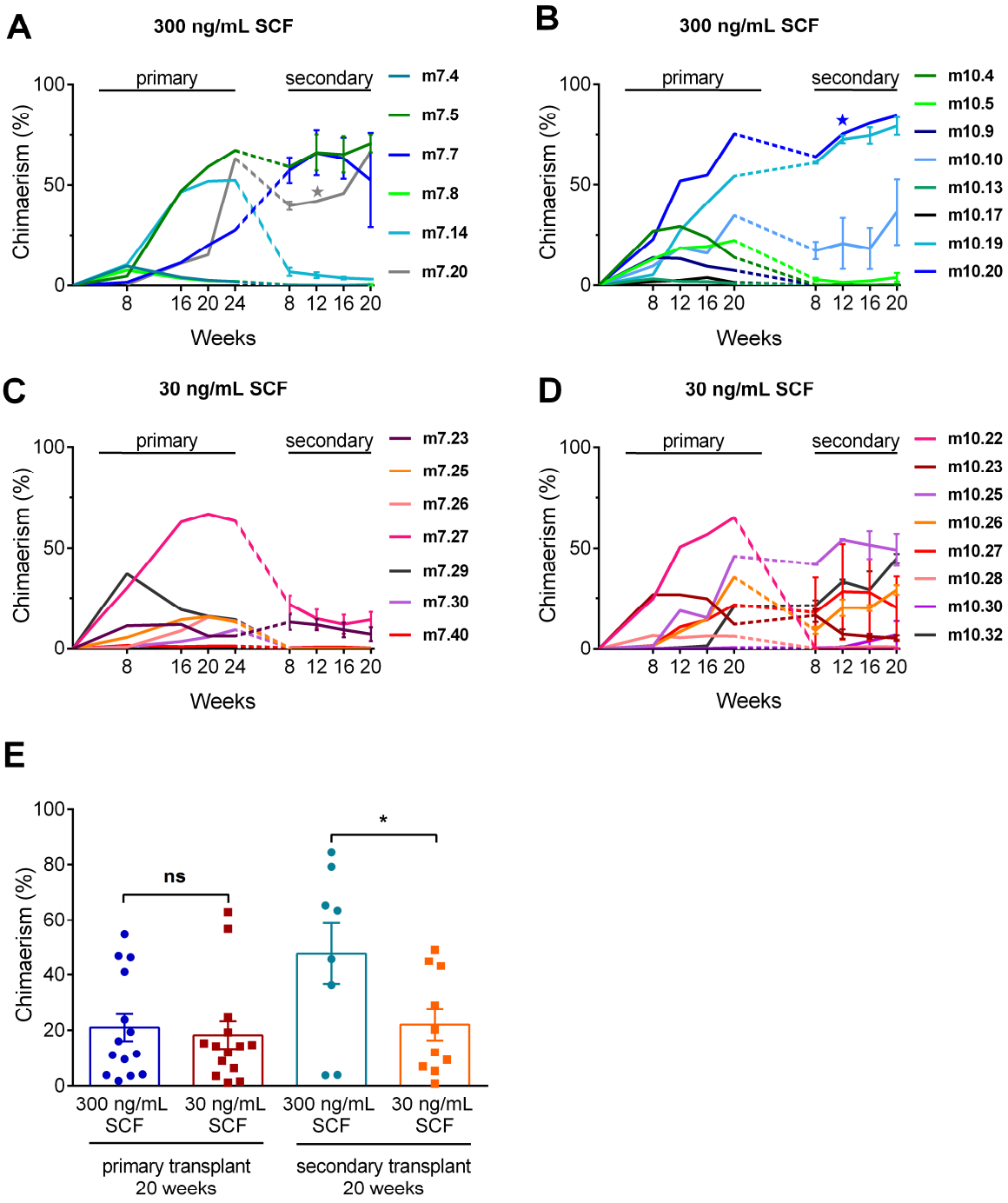


Figure 17: Stem cell activity but not secondary donor chimaerism is nearly identical between recipients of differentially treated HSCs.

A-B) Peripheral blood donor chimaerism in W41 mice transplanted with 300 ng/mL, 16h treated HSCs. 14/40 mice were positive in primary transplantation >1% (35%), 8/14 repopulated secondary recipients. C-D) Donor chimaerism in mice transplanted with 30 ng/mL, 16h treated HSCs. 15/40 mice were positive in primary transplantation >1% (37.5%), 10/15 engrafted in secondary recipients. n=80, 2 experiments. Star indicates recipient animal was sacrificed for non-experimental reasons. E) Donor chimaerism in primary and secondary recipients of differentially treated HSCs. Unpaired t-test, *=p<0.05, ns= not significant. Error bars represent data ± SEM.

3.1.4 Single cell transplantations reveal that *in vitro* SCF stimulation does not alter HSCs subtype

Single cell transplantation allows the classification of donor HSCs based on their lineage outcome at 16 weeks. According to findings from the Eaves lab, HSCs can be categorised into alpha, beta, gamma and delta, as determined by the ratio of GM to B/T-cells. Alpha-HSCs are lymphoid deficient, whereas beta-HSCs give rise to an equal amount of myeloid and lymphoid cells. Both alpha and beta-HSCs subtypes are capable of long-term lineage reconstitution in secondary recipients. In contrast, gamma and delta-HSCs primarily produce lymphoid cells with delta-HSCs completely lacking myeloid lineage contribution past 16 weeks in primary transplantation. These two HSCs subtypes typically fail to reconstitute the haematopoietic system in secondary recipients[64].

As can be seen from Table 8 and Figure 18 both treatments result in a similar distribution of HSCs subtypes with a tendency of a more balanced lineage production from those HSCs treated with a low concentration of SCF. More single cell transplants will be necessary to confirm this trend. Interestingly, in the low SCF treated mice 3 out of 6 retrospectively assigned beta-HSCs failed to reconstitute secondary recipients which would be expected. This is again supports the hypothesis that a high SCF concentration stimulates more self-renewal divisions.

Table 8: HSCs subtype distribution in primary recipients of differentially stimulated single HSCs

HSCs subtype	16h stimulation of single HSCs	
	300 ng/mL SCF	30 ng/mL SCF
Alpha	5	5
Beta	2	6
Gamma	2	2
Delta	5	2

At present, from the data it can be concluded that there are no significant differences in primary lineage reconstitution ability and lineage production between HSCs stimulated with a high or low concentration of SCF. This shows that the amount of 30 ng/mL is sufficient to retain HSCs stemness within the first 16h of culture. Longer time-courses will be necessary to determine if changes arise after HSCs have undergone several cell divisions.

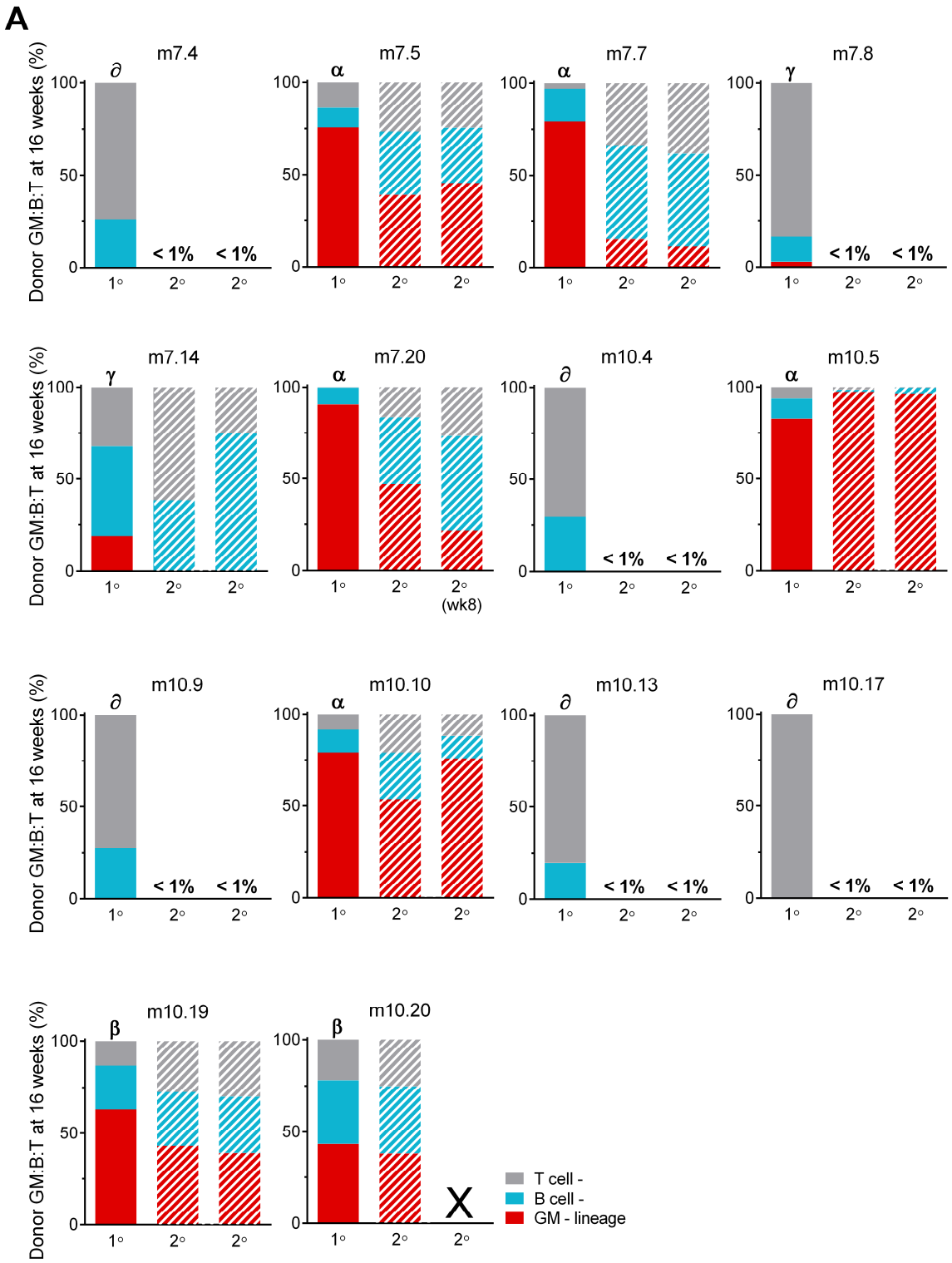


Figure 18: Peripheral blood donor cell ratio of myeloid to lymphoid progeny from single cell primary and secondary recipients at 16 weeks post transplantation.

A) Recipients of single 300 ng/mL, 16h treated HSCs. B) Recipients of single 30 ng/mL, 16h treated HSCs. <1% indicates unsuccessful secondary transplants. X indicates sacrifice of the recipient for non-experimental reasons before any data could be obtained. *Legend continued on next page*.

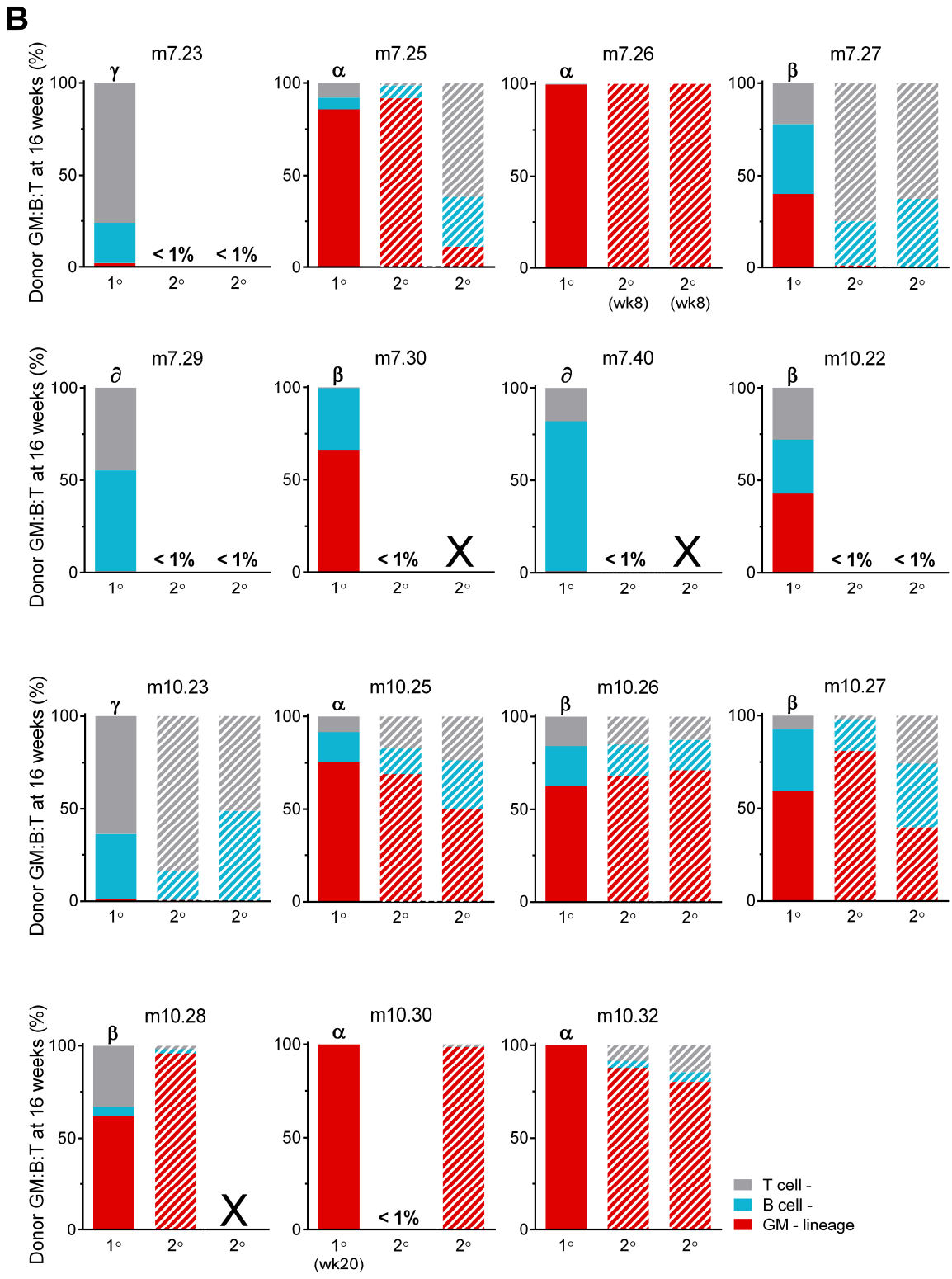


Figure 18: Peripheral blood donor cell ratio of myeloid to lymphoid progeny from single cell primary and secondary recipients at 16 weeks post transplantation.

α (alpha), β (beta), γ (gamma), δ (delta) indicate the HSCs subtype determined at 16 weeks based on peripheral blood myeloid (M) to lymphoid (L) ratio. M/L>2 = alpha-HSCs. M/L>0.25,<2 = beta-HSCs. M/L<0.25 = gamma/delta-HSCs. Gamma HSCs that fail to contribute to the myeloid lineage at 20 weeks are categorised as delta-HSCs.

3.1.5 Single cell RNA sequencing of cultured and stimulated HSCs shows that cell cycle activation signature drives main differences between cell populations

To assess transcriptional changes between freshly isolated HSCs and those that were stimulated with a high or low dose of SCF, single cell RNA sequencing was carried out. 191 freshly isolated cells were sequenced of which passed 127 quality control. For both 300 ng/mL and 30 ng/mL SCF treated conditions 96 cells were processed of which 63 and 80 passed the thresholds set for quality control (Figure 19, Appx-A Figure 1 and Appx-A Figure 2).

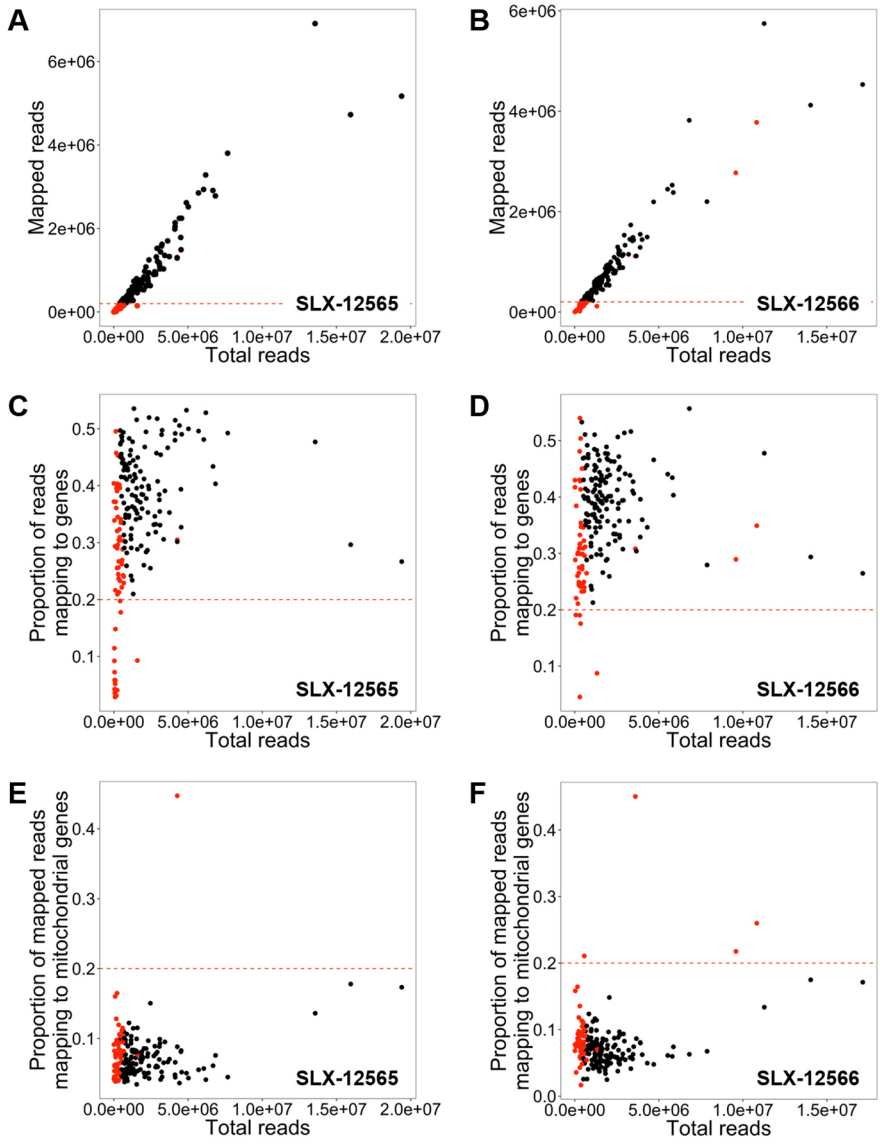


Figure 19: Quality control of raw read data in single cells.
RNA sequencing ran in two lanes SLX-12565 and SLX-12566 with libraries prepared from single freshly isolated HSCs divided up into both lanes at molarity equal to that of the libraries from treated single HSCs (SLX-12565= 300 ng/mL treatment, SLX-12566= 30 ng/mL treatment). Red depicts cells that did not pass thresholds set for the respective QC criteria listed on the y-axis of each graph. A-B) Mapped reads including nuclear genes, mitochondrial genes and ERCC (>2*10^5). C-D) Reads mapping to genes (>0.2). E-F) Reads mapping to mitochondrial genes (<0.2). Cells that did not pass quality control were excluded from analysis. The black dots depict cells that passed quality control.

A total of 21,616 gene transcripts were analysed, with 2480 genes being differentially expressed ($p<0.01$) between freshly isolated and 300 ng/mL SCF stimulated cells (1780 upregulated, 700 downregulated). Gene expression of 2103 transcripts was altered between naïve HSCs and those stimulated with 30 ng/mL SCF (951 upregulated, 1152 downregulated).
Of note, 824 upregulated and 554 downregulated genes were found significantly differentially expressed from naïve HSCs in both treated cell populations as depicted in the venn diagram of Figure 20.

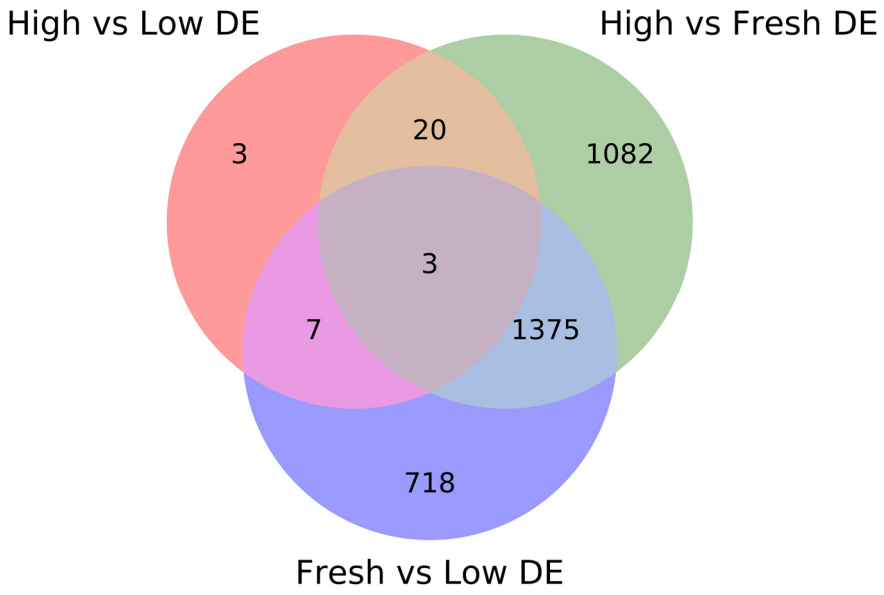


Figure 20: Venn diagram of differentially expressed genes between freshly isolated HSCs and SCF stimulated HSCs.
Freshly isolated BM HSCs were sorted directly into lysis buffer. Cultured HSCs were stimulated 16h with high (300 ng/mL) or low (30 ng/mL) concentration of SCF. The top left cluster shows that 33 genes were differentially expressed (DEX) between HSCs treated with 300 ng/mL and 30 ng/mL SCF. Of these 33 DEX genes, 30 were upregulated and 3 downregulated. 20 of those upregulated genes were also upregulated when compared to freshly isolated HSCs in the top right cluster. The top right cluster shows 2480 DEX genes between freshly isolated and 300 ng/mL SCF treated HSCs. Of those genes 1780 transcripts were upregulated in high SCF treated HSCs and 700 were downregulated. The bottom cluster displays the differential gene expression between freshly isolated HSCs and 30 ng/mL SCF treated HSCs. Of the 1378 DEX genes, 824 are found upregulated and 554 downregulated in treated HSCs.

Not surprisingly, gene ontology (GO) terms observed in SCF stimulated cells upregulated genes functionally clustered were mostly involved in initiation of proliferation, such as DNA replication initiation, DNA synthesis and repair, G1/S transition and DNA repair (Figure 21, Appx-B Table 1, and Appx-B Table 2). In contrast, none of the downregulated genes in SCF stimulated genes were significantly enriched in any GO terms. This indicates that SCF treated

cells are stimulated to divide, preparing the necessary machinery to do so, while shutting down other cellular processes that might be related to preserving the quiescence programme.

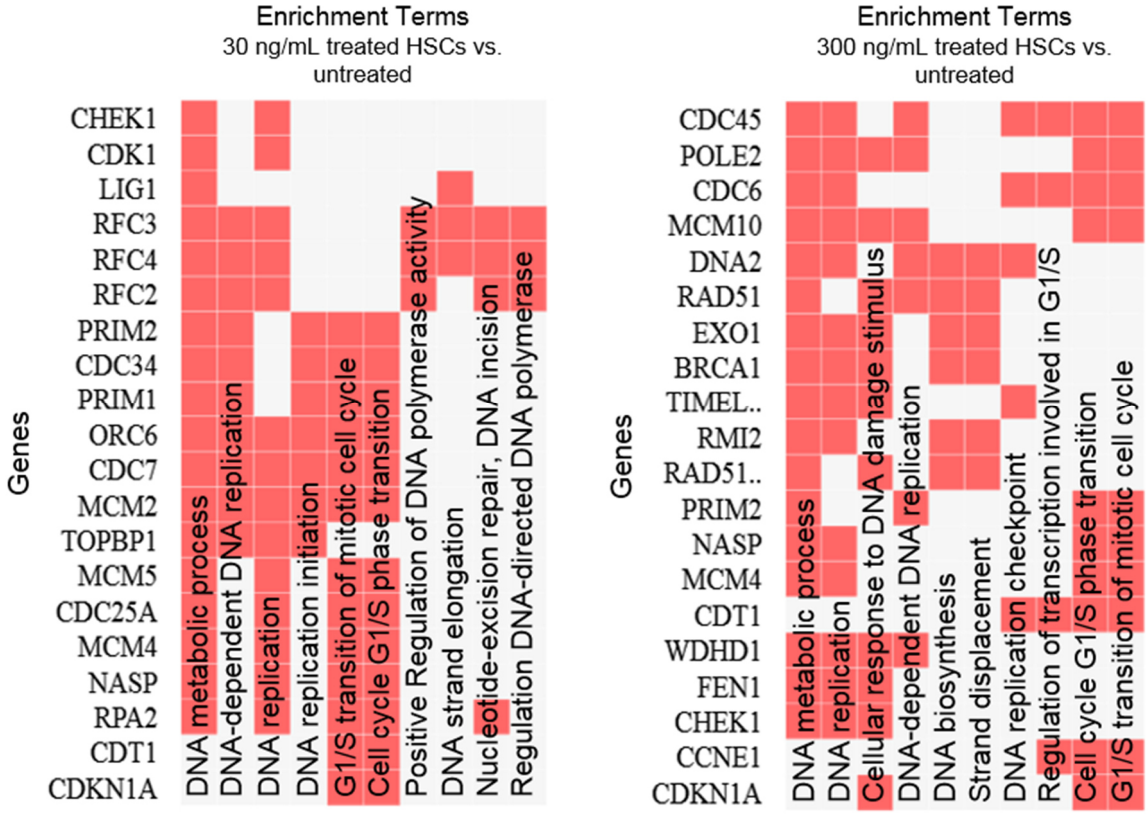


Figure 21: Clustergram of gene ontology displaying biological processes upregulated in treated HSCs.

The first 10 significant enriched terms are displayed in the columns, and the first 20 enriched genes in the rows. Red matrix indicate the association of the input gene with the respective ontology term.

When comparing the transcriptome of high and low SCF treated HSCs 33 genes were differentially expressed (Figure 20). 30 if these 33 genes being significantly upregulated and 3 downregulated (Table 9 and Table 10) in HSCs treated with a high dose of SCF compared to a low dose. As shown in Table 10, all significantly upregulated genes in HSCs treated with 30 ng/mL SCF encompassed predicted genes. Therefore, the analysis was restricted to protein coding annotated genes leaving 12,831 transcript to be analysed. However, following exclusion of pseudogenes, no transcripts were found to be significantly overexpressed in low SCF treated HSCs. This suggests that the activation from quiescence is a much more dominant set of molecular changes than differential SCF signalling.

Table 9: Upregulated genes in HSCs stimulated with 300 ng/mL SCF for 16h compared to HSCs stimulated with 30 ng/mL SCF for 16h.

Gene name	ENSEMBLE gene ID	Adjusted P-value	Log_2 fold change
Zfp385a	ENSMUSG00000000552	0.00596	3.08476
Nabp1	ENSMUSG00000026107	0.00581	2.56111
Gm12420	ENSMUSG00000081775	0.00001	2.04650
Fahd2a	ENSMUSG00000027371	0.00002	1.90934
Cav2	ENSMUSG00000000058	0.00752	1.65520
Tmem120a	ENSMUSG00000039886	0.00012	1.47429
Tubb6	ENSMUSG00000001473	0.00415	1.46703
Plek	ENSMUSG00000020120	0.00752	1.30837
Gem	ENSMUSG00000028214	0.00021	1.28827
Pidd1	ENSMUSG00000025507	0.00626	1.26505
Ash2l	ENSMUSG00000031575	0.00596	1.10319
Zdhhc13	ENSMUSG00000030471	0.00862	1.04585
Lyn	ENSMUSG00000042228	0.00720	1.03073
Tap1	ENSMUSG00000037321	0.00720	1.02628
Myadm	ENSMUSG00000068566	0.00596	0.97459
Hmgcr	ENSMUSG00000021670	0.00596	0.97231
Naa40	ENSMUSG00000024764	0.00752	0.96858
Galk1	ENSMUSG00000020766	0.00752	0.96756
Ndrg3	ENSMUSG00000027634	0.00539	0.90353
2810474O19Rik	ENSMUSG00000032712	0.00596	0.86782
Lypla1	ENSMUSG00000025903	0.00720	0.84400
Ctnna1	ENSMUSG00000037815	0.00563	0.81197
Serinc1	ENSMUSG00000019877	0.00720	0.79858
Pafah1b2	ENSMUSG00000003131	0.00720	0.74582
Vps35	ENSMUSG00000031696	0.00539	0.68410
Kdelr2	ENSMUSG00000079111	0.00752	0.65759
Rnf187	ENSMUSG00000020496	0.00596	0.49646
Msn	ENSMUSG00000031207	0.00019	0.45594
Bzw1	ENSMUSG00000051223	0.00626	0.41193
Serinc3	ENSMUSG00000017707	2.75E-07	0.37956

Table 10: Differential gene expression in cells treated 16h with 30 ng/mL compared to 300 ng/mL SCF

Gene name	ENSEMBLE gene ID	Adjusted P-value	Log_2 fold change
Gm3511	ENSMUSG00000105031	0.002582	0.163444
Gm28437	ENSMUSG00000101111	0.00744	0.335708
Gm12967	ENSMUSG00000080944	0.008061	0.547697

The close relationship between these two treated cell populations is visualised in Figure 22A by principle component analysis (PCA). Here, differentially treated single cells clearly cluster together and away from freshly isolated cells. To test if the short culture period drives this separation, a total of 1378 genes with shared differential expression in treated and freshly isolated HSCs were excluded from the PCA. However, cultured cells still clustered together

(Figure 22B). Therefore, genes involved in cell cycle activation are the most likely to drive this clustering.

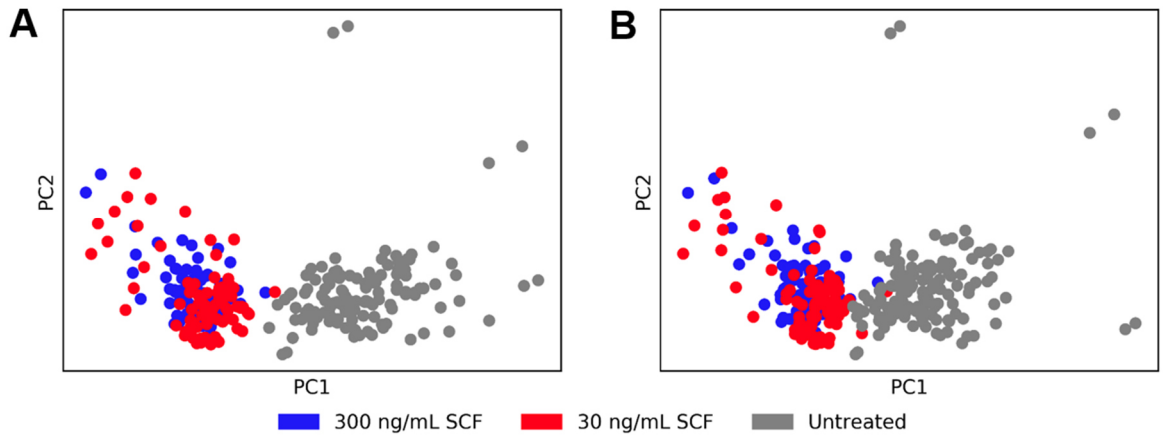


Figure 22: PCA clustering of freshly isolated BM HSCs and cultured, SCF treated HSCs. Freshly isolated, untreated HSCs are depicted in grey and cluster away from HSCs treated for 16h with 300 ng/mL SCF (blue) and 30 ng/mL SCF (red). A) Highly variable genes are plotted without additional filtering. B) Clustering of cells following exclusion of genes differentially expressed in treated cells compared to untreated.

Of the 30 genes upregulated in high vs. low SCF treated HSCs, 20 were also upregulated in high vs freshly isolated HSCs (Appx-B Table 3). Analysis of transcription factor occurrence shows that *Lyn*, *Tubb6*, *Cav2*, *Plek*, and *Msn* are significantly associated with the transcription factor *Elf4* (Table 11 and Figure 23).

Table 11: Transcription factor-gene occurrence of genes upregulated in high SCF treated HSCs

Term	P-value	Adjusted P-value	Input genes
ELF4	9.31E-06	0.00421	LYN;TUBB6;CAV2;PLEK;MSN
ETS1	0.000196	0.01775	LYN;TUBB6;CAV2;MSN
ELK3	0.000196	0.01775	TUBB6;CAV2;MSN;GEM
NFKB2	0.000196	0.01775	LYN;TUBB6;PLEK;GEM
STAT3	0.000196	0.01775	LYN;TUBB6;CTNNA1;MSN
KLF6	0.003123	0.042776	TUBB6;MSN;GEM
PRDM1	0.003123	0.042776	LYN;PLEK;GEM
REL	0.003123	0.042776	LYN;PLEK;GEM
CBFB	0.003123	0.042776	TUBB6;MSN;BZW1
LARP6	0.003123	0.042776	TUBB6;CAV2;GEM

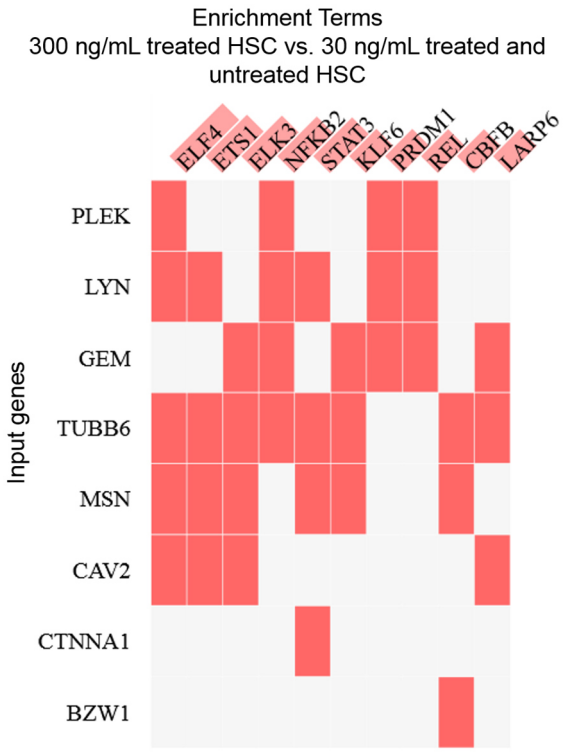


Figure 23: Clustergram of transcription factor-gene occurrence of genes upregulated in high SCF treated HSCs.

The transcription factor Elf4 has the strongest association with the input genes that were upregulated in high SCF treated HSCs compared to low SCF treated and unstimulated HSCs.

As seen in Figure 24 *Plek*, *Tubb6*, and *Cav2* but not *Msn* and *Lyn* are indeed higher expressed in the majority of cells treated with a high dose of SCF.

Plek encodes for the phosphoprotein Pleckstrin, which is the main substrate for phosphorylation by protein kinase C in thrombocytes, following platelet activation. Over-expression of Pleckstrin in cell lines has been shown to alter their actin cytoskeleton and cell spreading[259-261]. Little is known about its role in haematopoiesis although its expression seems to be enriched in myeloid progenitor cells[262].

The class V β-tubulin subtype 6 is encoded by *Tubb6*. It is a vital component of the cell's cytoskeleton and required for orchestrating DNA segregation during cell division. β6 Tubulin is thought to be restricted to megakaryocytes and platelets, however not much is known about its expression in HSCs[263,264].

Caveolin-2 (Cav2) is a component of the caveolae, which are situated within lipid rafts and mediate signal transduction to intracellular space and in doing so facilitate signal activation of a variety of pathways[265].

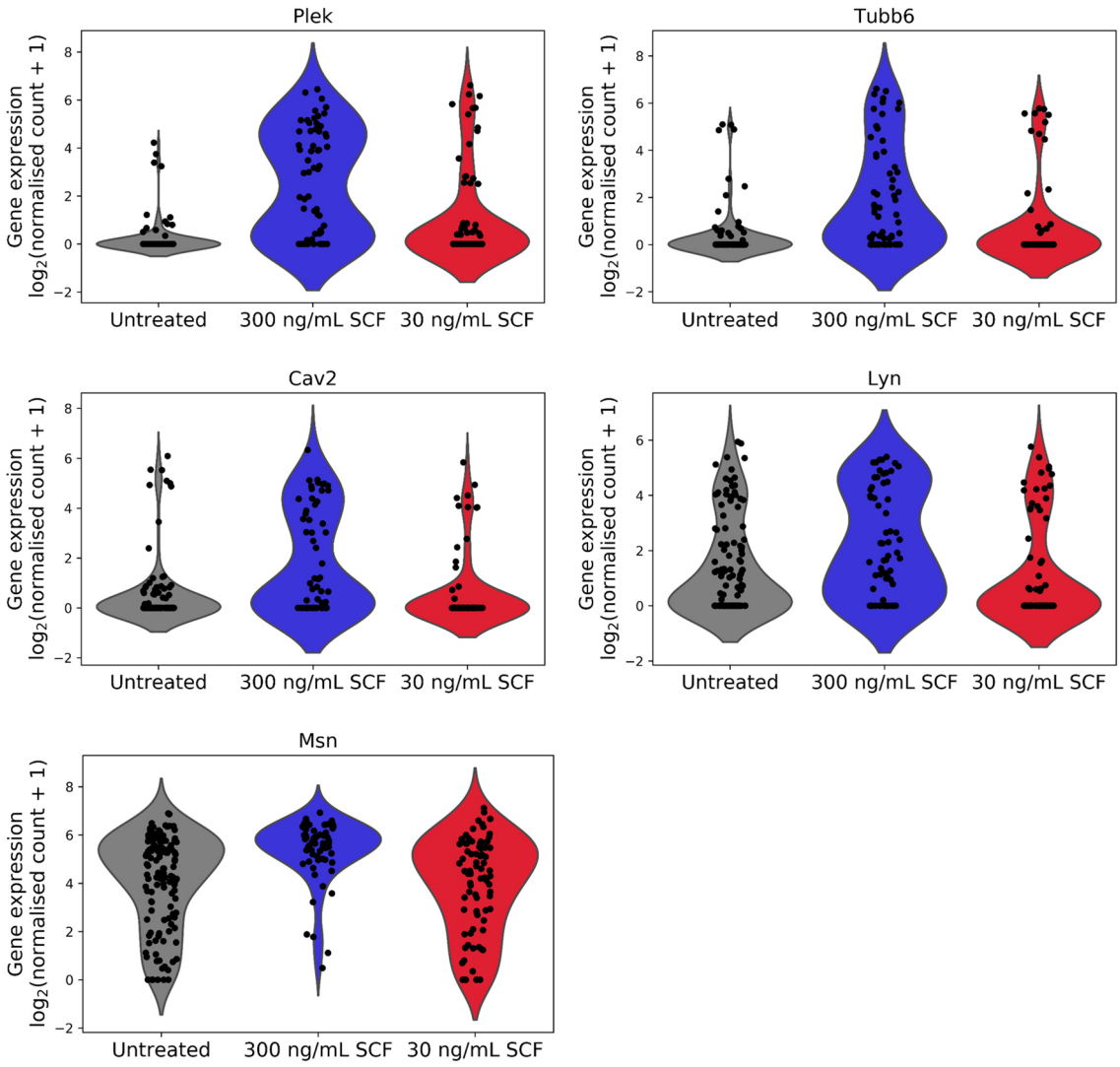


Figure 24: Violin plots displaying gene expression distribution among differentially treated HSCs.

The ETS transcription factor Elf-4 (E74-like factor, also known as Myeloid Elf-1-like factor (MEF) is known to play a crucial role in HSCs cell cycle entry and proliferation show increased quiescence and radioprotection[108,266,267]. The common co-occurrence of *Plek*, *Tubb6*, and *Cav2* with Mef suggests that these genes are activated upon entry into cell cycle. This is in congruent with the finding that HSCs are stimulated to proliferate which may occur in a dose dependent fashion (Figure 24).

Overall, in this chapter my aim was to investigate how differential SCF stimulation alters HSCs fate choice on a functional and molecular level. Functionally, I was able to show through single cell transplantation of differentially stimulated HSCs that a higher concentration of SCF results in an increased level of donor cell contribution in secondary but not primary recipients. This is

suggests that HSCs are programmed to undergo more self-renewal divisions when stimulated with a high concentration of SCF *in vitro*. To assess if a high concentration indeed stimulates more self-renewal divisions *in vivo*, the transcriptome of freshly isolated single HSCs was compared to that obtained from single differentially treated HSCs. The results clearly show that SCF stimulates HSCs to enter cell cycle regardless of the concentration tested. However, treated HSCs were transcriptionally very similar thus only few genes were differentially expressed. Genes that were upregulated in HSCs treated with a high concentration of SCF associated with the transcription factor Mef, known to positively regulate exit from quiescence and cell proliferation and differentiation[108]. Therefore, it is necessary to investigate if Mef regulates self-renewal and differentiation in a dose dependent manner or if a negative regulator of Mef is overrepresented in high SCF treated HSCs.

Additionally, further self-renewal regulators need to be identified and their putative differential expression analysed. Furthermore, downstream activation of SCF/KIT signalling needs to be investigated to evaluate if differential SCF treatment modulates the response, for instance by activation of different members of the STAT family. It would also be interesting to assess how cell cycle regulators are altered in response to differential SCF stimulation. In conclusion, for statistically relevant analysis, more biological replicates need to be processed to provide insight into the optimal conditions promoting HSCs self-renewal and expansion.

3.2 HSCs remain in state of hibernation *in vitro* in the absence of stem cell factor

The results above have shown that stimulation of HSCs with different doses of SCF prior to first division does not impact HSCs retention, but instead alters their expansion capacity post-transplantation. A previous study has also shown that single phenotypic HSCs can be maintained as single cells for 5–7 days in the presence of SCF when lipid-raft clustering is inhibited resulting in abrogated SCF signalling. In this same study and supported by additional studies, it has been demonstrated that complete absence of cytokines resulted in HSCs death within 24h[111,121]. Collectively, these findings suggest that SCF signalling is necessary for HSCs self-renewal and proliferation but it is potentially dispensable for maintenance of stemness. Understanding the molecular mechanisms underlying HSCs dormancy and exit from quiescence may aid in the optimisation of HSCs maintenance and expansion *ex vivo*. Additionally, these findings may be applied to therapy resistant leukaemic cells termed minimal residual disease, which is often cause for relapse after a patient went into remission[268].

Therefore, I set out to investigate whether SCF was essential for retaining HSCs function *in vitro*. To this end, I established a minimal cytokine culture medium without SCF in which HSCs function and survival can be maintained for an extended period of time. As it has previously been shown that gp130 signalling is indispensable for effective haematopoiesis and that activation of the gp130 receptor by IL-6 or IL-11 augments HSCs expansion *in vitro*, IL-11 was chosen as the sole stimulatory cytokine in this minimal culture set up[231,269]. In this chapter, I was able to show that fully functional serially transplantable HSCs can be maintained for up to 7 days *in vitro* in the absence of SCF signalling. These HSCs can serially reconstitute recipient mice but display a bias towards myeloid differentiation. Gene expression analysis indicates that the majority of known HSCs regulators are expressed in these cultured HSCs, validating their stem cell identity on the molecular level. Yet, differences can be observed making this culture condition an intriguing system to investigate several biological questions.

3.2.1 *In vitro* maintenance of single HSCs in the absence of SCF

To assess the importance of SCF for HSCs self-renewal and survival, single HSCs were cultured in the absence of SCF but in presence of human IL-11. In standard culture conditions, none of the cells survived past 3 days in culture. The medium was therefore supplemented with 10% FCS (serum-supplemented medium, SSM) resulting in 25.5% (± SD 18.9%) survival despite continuous cell death (Figure 25A-B). Modified serum-free medium (SFM) conditions yielded 32.6% (± SD 3.6%) survival (Figure 25B), compared to 94.8% (± SD 5.2%) in SCF, IL-11 and serum containing culture. Survival in SCF-deprived conditions was exclusive to

HSCs, as single sorted progenitor fractions (LSK or SLAM ($CD48^{-}CD150^{+}$)) cells did not survive past 2 days in culture (data not shown). Together, this shows that a proportion of phenotypic HSCs are more resilient in culture, demonstrating that the HSCs population is heterogeneous in cell survival in stressed conditions.

The average clone size in standard SCF containing conditions would amount to ~400 cells after 7 days in culture[252]. Interestingly, 99.3% of surviving HSCs did so without undergoing cell division. Any cell that did divide would either remain as 2 cells or subsequently die. Altogether this minimal culture condition allows the culture of single HSCs for an extended time in the absence of any notable proliferation. Thus, these HSCs from here on referred to as hibernating HSCs.

3.2.2 Heterogeneity in HSCs cell cycle kinetics is maintained in minimal cytokine culture

At the time of bone marrow isolation, the vast majority of HSCs are in G_0 of the cell cycle and are subsequently activated by cytokine stimulation *in vitro*[95]. Time to first division typically comprises 24–48h[252].

To investigate the viability of these hibernating HSCs and to assess if SCF deprivation leads to synchronisation of the cell cycle state, cell division was stimulated by reintroduction of SCF on day 7 of culture. Surprisingly, heterogeneity in time to first division, cell division kinetics, and clone size distribution is maintained and close to identical to that of freshly stimulated HSCs (Figure 25C-D). Thus, these hibernating HSCs are not synchronised and do not selectively survive based on cell cycle state.

To test if these cells maintain multipotency, single cells were transferred into cytokine-rich semi-solid medium supporting colony formation. 67.9% (± SD 13.9%) and 81.4% (± SD 17.2%) of single cells from SSM and SFM culture respectively, generate clones. This is comparable to HSCs cultured for 20h (prior to first division) in standard conditions 85.4%9 (± SD 2.9%) (Figure 25E). The vast majority (79.4% ± SD 6.6% in SSM and in 83.5% ± SD 5.8% SFM) of cells retain multipotency and generate clones of at least three different lineages as seen in Figure 25F. These results provide the first evidence that phenotypic HSCs can be cultured long-term without undergoing differentiation.

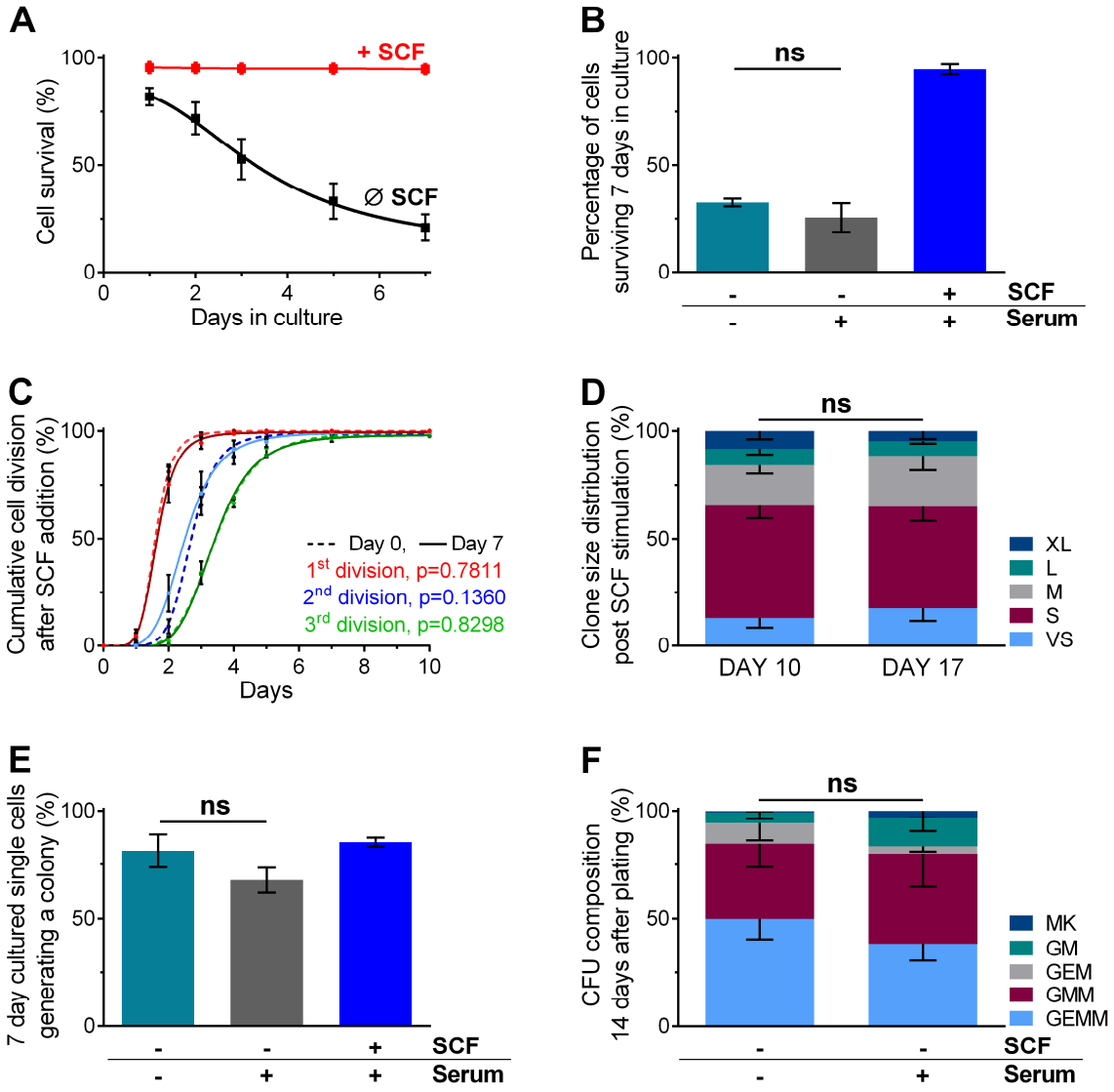


Figure 25: HSCs remain viable single cells and retain their multipotency in minimal cytokine culture.

A-B) SCF deprivation decreased HSCs survival in culture but ~25-35% remain viable single cells up to 7 days in culture. C) Cell division kinetics following cell division directly after sort (day 0, n=418, 5 exp.) or following 7 days of culture (day7, n=180, 5 exp.). Lines show non-linear regression fit, [Agonist] vs. response, variable slope (four parameters). D) Heterogeneity in clone size on day 10 following SCF addition is maintained throughout 7 day of SCF deprivation. E) ~65-80% of single cells can generate a clone in CFU assays after 7 days of SCF deprivation (-/- n=121, 5 exp., -/+ n=230, 6 repeats, +/+ n=48, 2 exp.). F) The majority of single cells (~80%) make colonies of at least three lineages in CFU assays showing their retention of multipotency (-/-, n=70, 4 exp., -/+ 166, 5 exp.). Paired t-test. Error bars represent SEM.

3.2.3 Hibernating single HSCs retain full *in vivo* functionality

The CFU assay does not measure the cell's capacity to differentiate into the lymphoid lineage and has limited capacity to assess self-renewal. Therefore, hibernating HSCs were transplanted

into sublethally irradiated mice either as single cells or at a 3-cell dose (3 pooled single cells). Figure 26 shows the experimental set up for the transplantation.

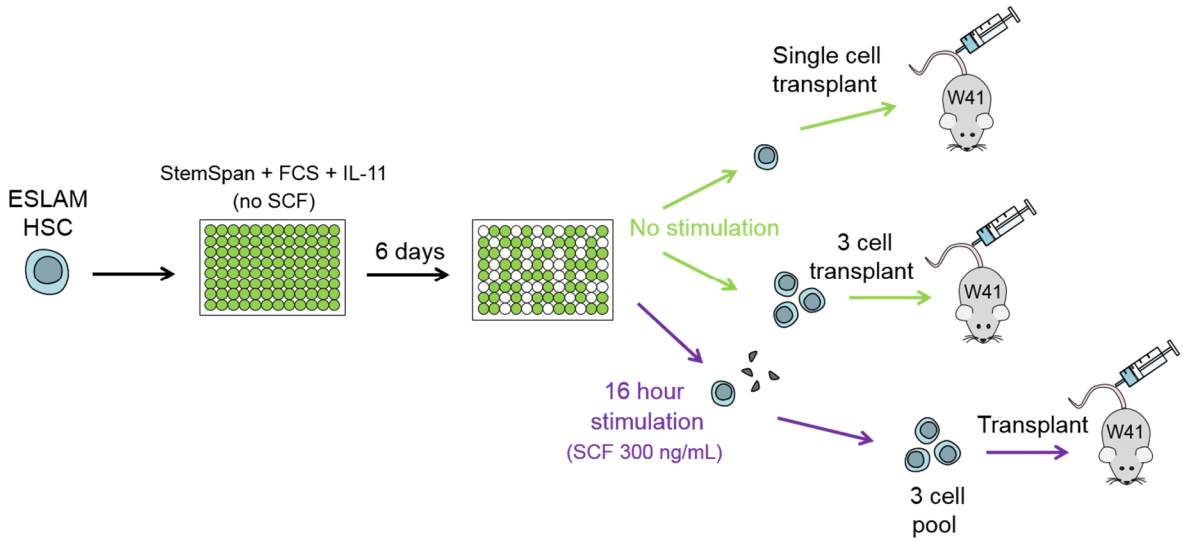


Figure 26: Experimental design for the transplantation of one or three hibernating HSCs. HSCs were cultured 7 days in minimal cytokine conditions and subsequently transplanted into W41 recipients as either single cells or three pooled unstimulated single cells or three pooled single cells that were stimulated 16h with SCF. 10 mice per condition.

4 of 10 mice transplanted with 3 cells showed donor chimaerism of >1.0% in peripheral blood. One of the mice transplanted with 3 cells (m6.6) presented exclusively lymphoid lineage reconstitution at 16 weeks and failed to engraft a secondary recipient, as would be expected. Overall, 2/10 mice successfully engrafted secondary recipients. Similarly, 3/10 mice were successfully engrafted from primary recipients of single HSCs. All of these (3 of 3) were serially transplantable. This demonstrates that at least 30% of cells surviving 7 days in culture without SCF stimulation are indeed stem cells capable of multi-lineage long-term reconstitution and durable self-renewal.

Interestingly, all three secondary repopulating single HSCs predominantly exhibited myeloid lineage output 16–28 weeks post primary transplant and would be classified as alpha-HSCs based on the definition published by Dykstra et al. Importantly, this potential lineage bias was retained in 2/3 secondary transplants, in accordance with the 50% rate of alpha programme retention observed in Dykstra et al. Possible bias towards the myeloid lineage is partially obscured in the 3 cell transplants since multiple HSCs would contribute but even so, the myeloid cell bias is observed in 2/5 recipients, suggesting an over-representation of alpha-HSCs.

Of note, m6.25 (Figure 27E-F) did not show any donor chimaerism >0.5% at 16 weeks post transplantation and therefore would have been excluded from further analysis and serial transplantation by many standard definitions of LT-HSCs. By week 28 however, 13.6% donor chimaerism was observed in the primary recipient and subsequent secondary animals were successfully reconstituted. This emphasises the need for longer term assessment of donor chimaerism in single cell transplantation studies in particular.

To investigate if short-term SCF stimulation after 7 days of minimal culture alters HSCs chimaerism, mice were also transplanted at a 3-cell dose with HSCs that were stimulated 16h with SCF. As seen in Figure 27C, 4/10 mice showed donor chimaerism >1.0% in peripheral blood, comparable to the reconstitution efficiency seen from non-stimulated cells. However, the overall chimaerism was higher in mice reconstituted with 3x16h stimulated HSCs compared those transplanted with 3 non-stimulated HSCs in both primary (32.2% ± SD 32.7% vs. 12.8% ± SD 9.1%) and secondary recipients (41.2% ± SD 46.0% vs. 15.0% ± SD 13.0%). The possibility cannot be excluded, that any of the recipients have not received all 3 cells however. Therefore, single cell transplants of stimulated compared to non-stimulated HSCs would need to be carried out to verify that short-term SCF stimulation indeed improves donor chimaerism.

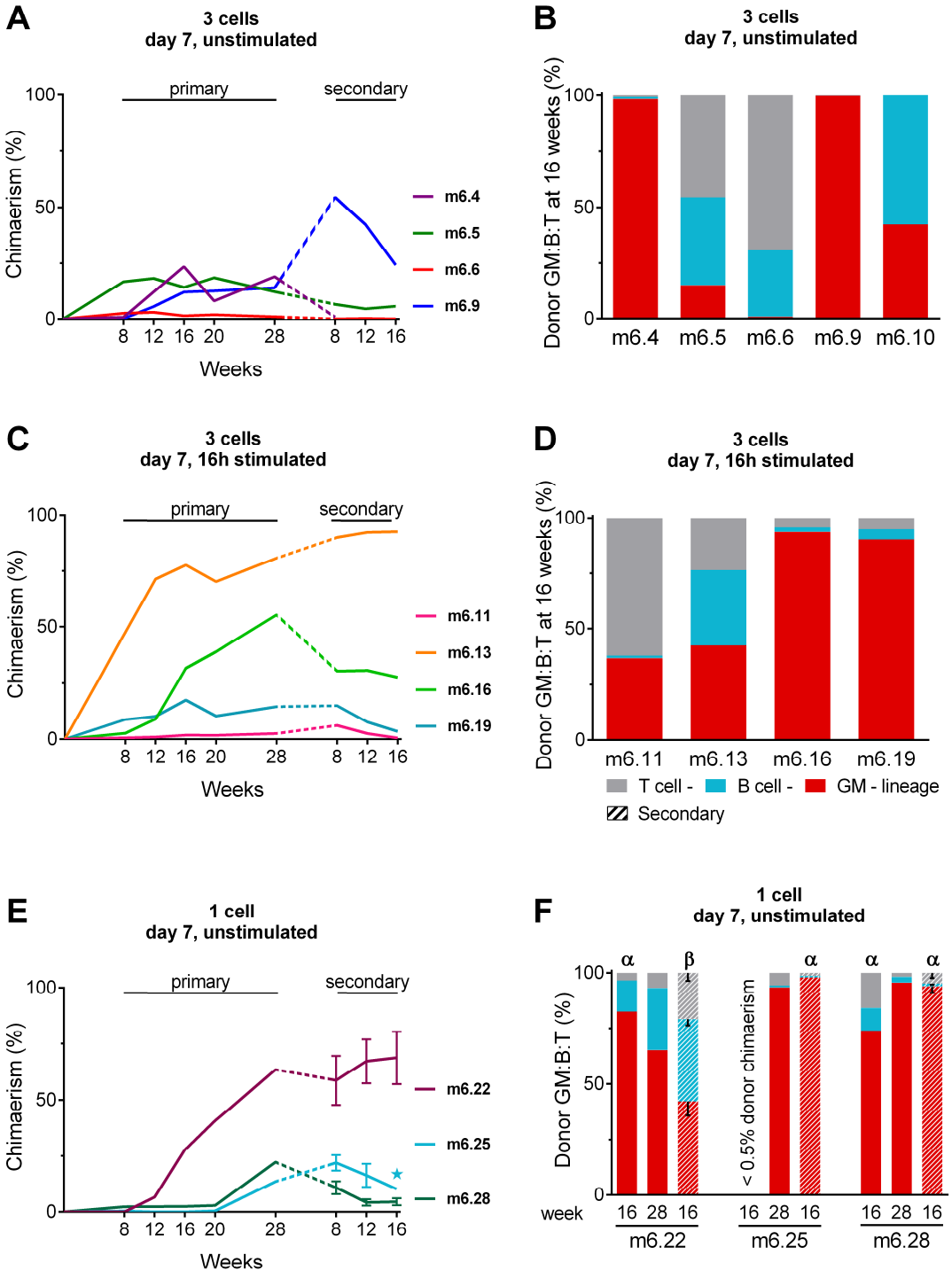


Figure 27: Donor chimaerism in W41 recipients of 7 day cultured HSCs.
Donor chimaerism in peripheral blood of primary and secondary recipients of 3 pooled HSCs cultured for 7 days in the absence of SCF. B) Donor myeloid to lymphoid ratio at 16 weeks in primary recipients of A. C) Donor chimaerism in primary and secondary recipients of 3 pooled HSCs cultured for 7 days in absence of SCF followed by 16h stimulation of SCF. D) Donor myeloid to lymphoid ratio at 16 weeks in primary recipients of C E) Donor chimaerism in primary and secondary recipients of single HSCs cultured for 7 days in the absence of SCF. F) Donor myeloid to lymphoid ratio at 16 and 28 weeks in primary recipients and at 16 weeks in secondary recipients of E. Error bars represent SEM. n=30 mice, 10 primary recipients per condition. Star indicates mouse was culled for non-experimental reasons.

3.2.4 HSCs that tolerate SCF deprivation are predominantly alpha subtype HSCs

Despite low numbers, these results suggest that SCF deprivation selects for HSCs with a robust myeloid lineage output in both primary and secondary transplants, which may need longer than average to contribute significantly to the production of all lineages. This would be in accordance with previous data reporting a delayed onset of lineage production by alpha-HSCs[64,65]. To test this hypothesis, further single cell transplants were carried out of HSCs cultured for 7 days in both serum containing (SSM) and serum free (SFM) conditions as depicted in Figure 28.

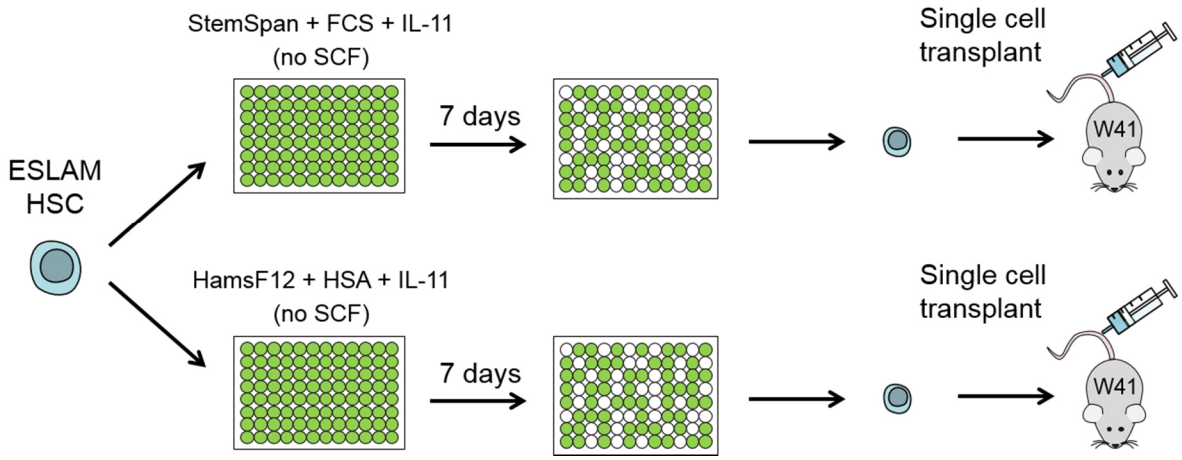


Figure 28: Experimental design for the transplantation of single serum-free or serum-supplemented, cultured HSCs.

HSCs were cultured 7 days in minimal cytokine conditions in either serum-supplemented StemSpan (21 recipients) or serum-free Ham's F12 (22 recipients) base medium.

12/21 Mice (57.1%) transplanted with SSM cultured HSCs, successfully repopulated primary recipients (Figure 29A) with >1% overall donor contribution at some point post transplantation. One mouse (m12.10) was culled for non-experimental reasons before 16 week data could be obtained but serial transplantation of its bone marrow showed secondary reconstitution. At 8 weeks in the primary transplantation, this mouse displayed an exclusively myeloid phenotype. 5/11 (45.4%) transplanted HSCs were classified as alpha-HSCs, with a predominant myeloid lineage output, which is a significantly higher ratio of alpha-HSCs compared to freshly isolated HSCs. 4/11 were characterised as beta-HSCs, displaying a balanced lineage production of both myeloid and lymphoid cells (Figure 29B). Data from secondary transplants (16 weeks) show that 10/21 (47.6%) mice are successfully engrafted. As expected, gamma and delta-HSCs did not serially transplant. Again, one recipient (m12.22) did not exhibit donor chimaerism >1% at 16 weeks (0.52%) in the primary recipient and very low contribution (4.4%) at 20 weeks. Yet, this mouse successfully serially engrafted (16 weeks: 29.2% ± SD 6.9%, 3 secondary

recipients). Out of 22 mice transplanted with SFM cultured HSCs, 14 (63.6%) showed >1% donor contribution at 16 weeks (Figure 29C). Again, the majority (9/14, 64.2%) were classified as alpha-HSCs (Figure 29D). In secondary recipients, 11/22 (50%) successfully engrafted at 12 weeks. Overall, half of single cultured HSCs (50.2%, ± SD 14.6%) repopulated primary recipients. Since one of the caveats of single cell transplantation is that the cell may remain in the syringe at the time of transplantation, this percentage may even be slightly higher. These results show that the established minimal cytokine culture is able to maintain fully functional HSCs for an extended period of time, allowing the manipulation at the single cell level as well as providing a platform to study exit from quiescence.

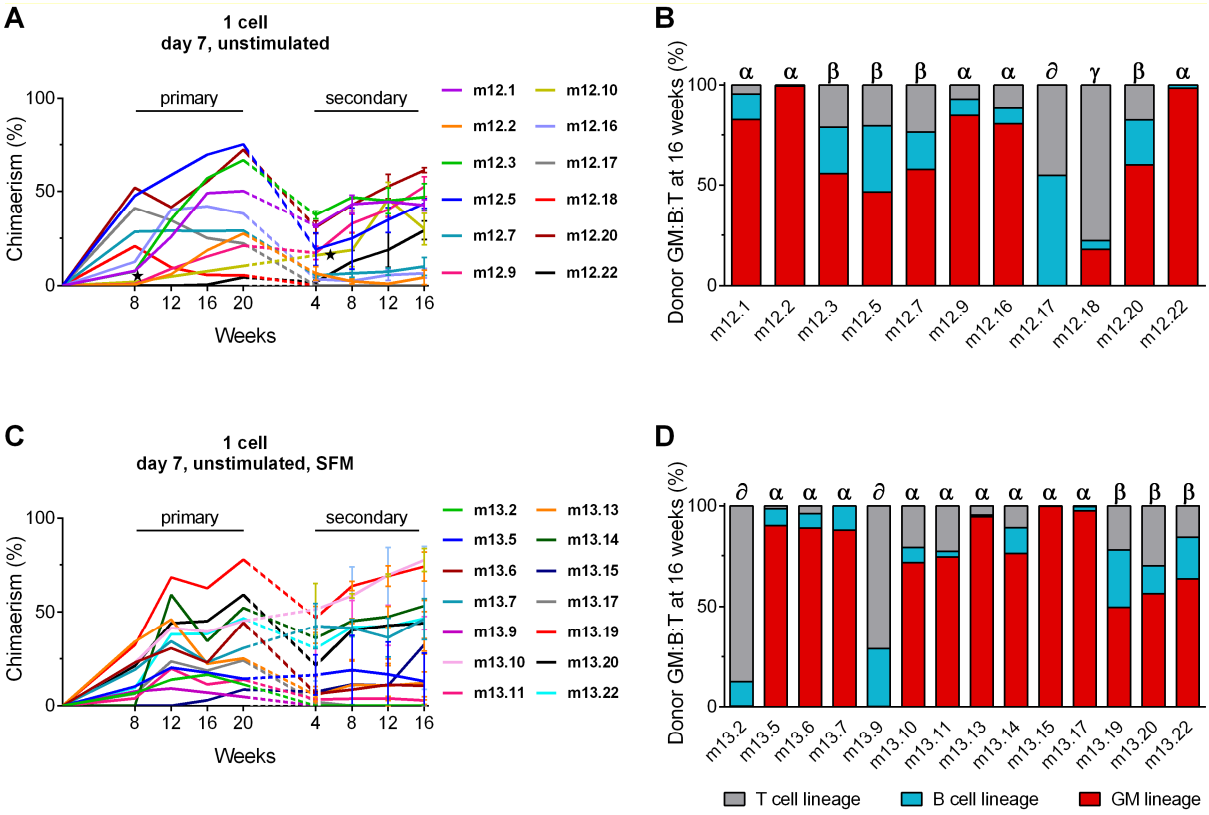


Figure 29: Donor chimaerism in W41 recipients of single 7 day cultured HSCs.
A) Overall donor contribution in peripheral blood of primary and secondary recipients of single HSCs cultured for 7 days in SSM without SCF. The star indicates that this mouse had to be sacrificed for non-experimental reasons. Recipient bone marrow was then secondary transplanted. n=22 primary recipient mice. B) Donor myeloid to lymphoid ratio at 16 weeks in primary recipients, subdividing the input HSCs into α (alpha), β (beta), γ (gamma), δ (delta) based on myeloid to lymphoid ratio. C) Overall donor contribution in primary and secondary recipients of single HSCs cultured for 7 days in SFM deprived of SCF. n=21 primary recipient mice D) Donor myeloid to lymphoid ratio at 16 weeks in primary recipients, subdividing the input HSCs into the classes discussed in B. Error bars represent SEM. Star indicates that this mouse was culled for non-experimental reasons and bone marrow was successfully serially transplanted.

Interestingly, repopulated recipients predominantly displayed an alpha-HSCs lineage output (54.9%, ± SD 13.3%, Figure 30B-C). All mice received HSCs that were obtained from mice between 8 and 16 weeks of age. Strikingly, when comparing the frequency of alpha-HSCs present in our recipients, it resembles the lineage output of HSCs derived from aged mice (38–46 weeks) as seen in Figure 30B-E. Cultured HSCs consists of significantly more alpha-HSCs at the expense of beta-HSCs (p=0.031), whereas the ratio to gamma/delta-HSCs remains unchanged (p=1.0). This indicates that the alpha-HSCs subtype is more resilient both *in vitro* and *in vivo*. Possibly, alpha-HSCs represent the most primitive stem cell that only convert to beta-HSCs when lymphoid cell production is required. This hypothesis can only be investigated by comparing purified alpha and beta-HSCs, which to date is not possible based on their surface marker profile.

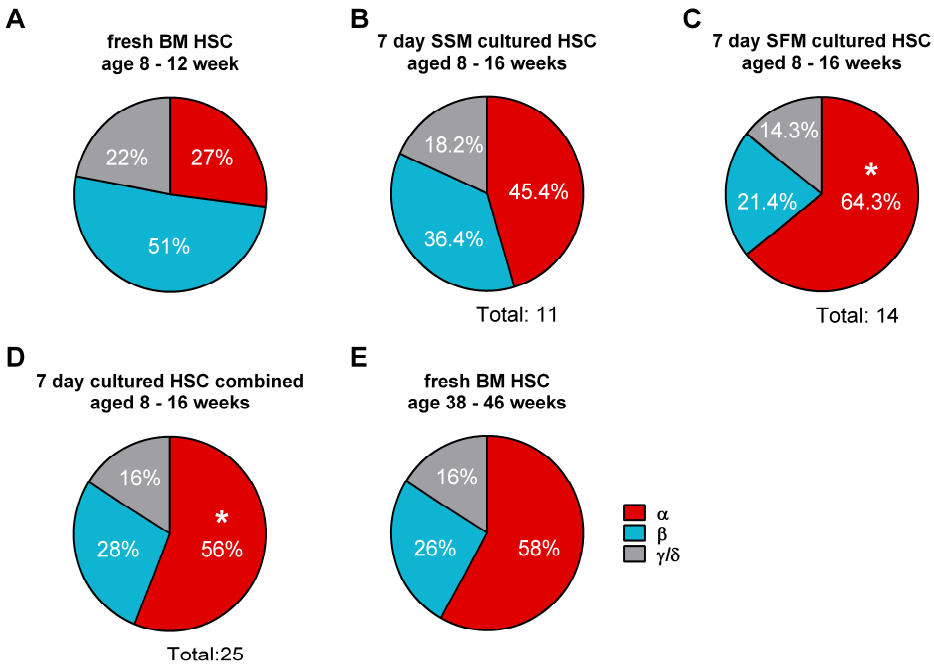


Figure 30: Lineage differentiation of 7 day cultured HSCs is more similar to those derived from aged mouse HSCs.

A) Distribution of HSCs subtypes in mice transplanted with freshly isolated HSCs obtained from mice aged 8–12 weeks. B) Distribution of HSCs subtypes in mice transplanted with 7 day HSCs cultured in serum-supplemented minimal medium and obtained from mice aged 8–16 weeks. C) Distribution of HSCs subtypes in mice transplanted with 7 day HSCs cultured in serum-free medium and obtained from mice aged 8–16 weeks. D) Combined data from B and C. E) Distribution of HSCs subtypes in mice transplanted with freshly isolated HSCs obtained from mice aged 38–46 weeks. A/E) modified from Benz et al. 2012. α (alpha), β (beta), γ (gamma), δ (delta) HSCs. *=P<0.05, Fisher's exact test.

3.2.5 High CD150 expression enriches for HSCs able to tolerate SCF deprivation

Since all HSCs were index-sorted, it was possible to analyse their surface marker profile in order to try and prospectively isolate alpha-HSCs with robust *in vitro* survival. Most strikingly, higher levels of CD150 expression correlated with surviving HSCs. Other markers that were modestly but significantly different on hibernating HSCs include higher CD45 and EPCR expression, as well as lower CD48 expression. Interestingly, expression levels of the SCF receptor c-Kit did not select for surviving HSCs (Figure 32).

To verify the use of CD150 as a selective marker for hibernating HSCs, Sca-1^{high}-ESLAM HSCs were sorted from CD150^{mid} or CD150^{high} population (Figure 31A) and cultured in absence of SCF. As seen in Figure 31B, day 7 survival of CD150^{high} HSCs is significantly higher (44.2% ± SD 14.6%) than that of CD150^{mid} HSCs (18.2% ± SD 17.7%), confirming that adjustment of the sorting gate selects for HSCs with a higher propensity to survive in minimal culture conditions. These results and the correlating enrichment in alpha-subtype HSCs after 7 day culture suggest that alpha-HSCs may prospectively be so isolated based on their higher than average expression of CD150. This would also be agreement with previous findings by two other groups[65,270] who demonstrated an enrichment of alpha-HSCs in CD150^{high} cell fractions. To assess if these cultured alpha-HSCs are different from those freshly isolated, more single cell bone marrow transplantations will need to be performed.

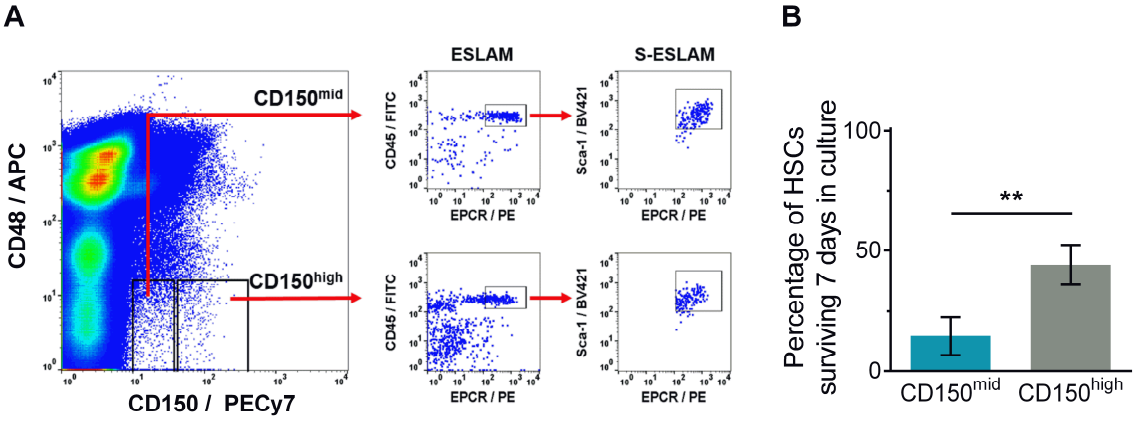


Figure 31: CD150^{high} expression correlates with higher survival of single HSCs in minimal culture.

A) Gating strategy for the isolation of CD150^{mid} or CD150^{high} ESLAM Sca-1^{high} HSCs. First gate shows viable single lineage depleted bone marrow cells. B) Survival of prospectively sorted CD150^{high} HSCs is 5-fold higher than that of CD150^{mid} HSCs. n=480, 5 exp. ***=P<0.01, Paired two-tailed t-test.

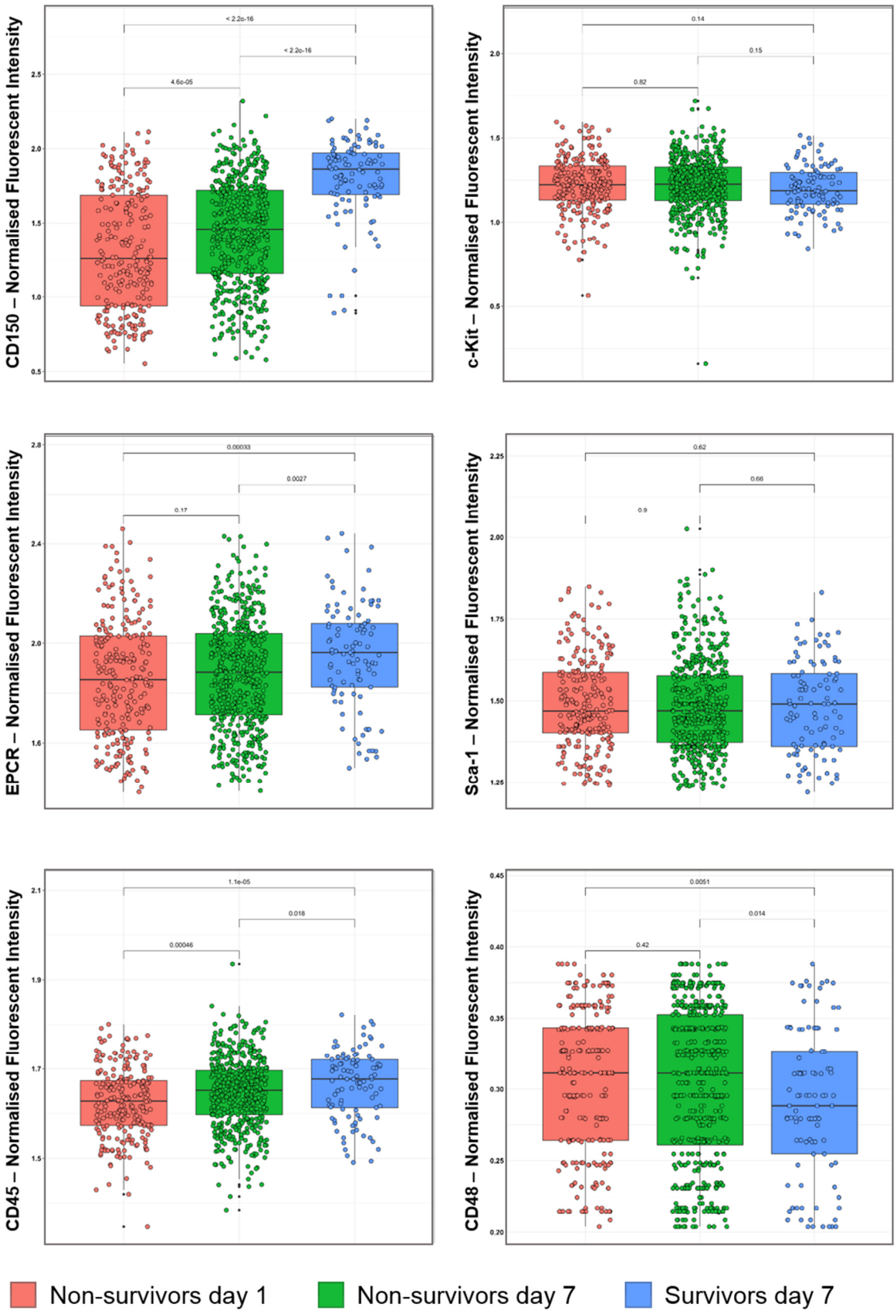


Figure 32: Surface marker expression on single HSCs at time of sort.
Normalised fluorescent intensity for the respective cell surface markers at the time of sort. Cells surviving 7 days in culture have elevated expression of CD150, EPCR and CD45 and lower expression of CD48.

Higher expression of CD150 is also observed in cells that survive 7 days in culture and are ultimately candidates for transplantation, as shown in Figure 33A-B. When investigating CD150 expression on transplanted HSCs, no bias can be observed between those that successfully repopulate the recipient and those that do not. Equally, no evident clustering in the CD150 high fraction can be observed of those HSCs that are retrospectively determined to be an alpha-HSCs (blue diamonds). In both conditions, mice repopulated with short-term gamma/delta-HSCs have a CD150 expression below average (squares), potentially indicating their lower self-renewal potential.

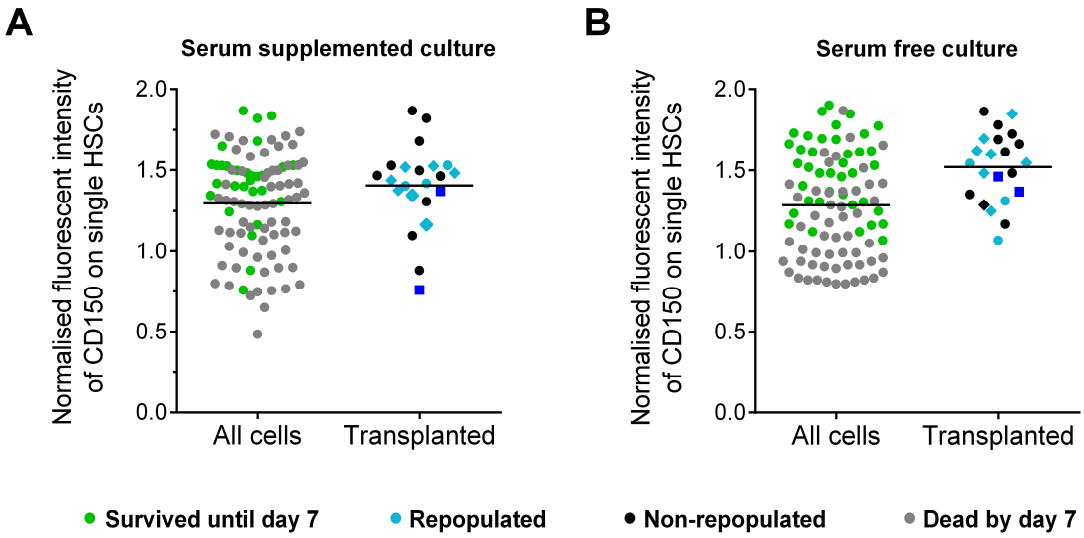


Figure 33: Normalised CD150 expression on single HSCs at the time of sort.
Surviving HSCs (green) generally have a higher than average expression of CD150 in both serum supplemented (A) and serum free culture (B). Repopulating (blue) and non-repopulating cells (black) do not cluster to a specific region of CD150 intensity. Blue diamonds represent repopulated alpha-HSCs. Dark blue squares represent HSCs that were retrospectively assigned a gamma or delta subtype.

3.2.6 Human HSCs can be maintained as single cells in minimal culture conditions

Altogether, these findings demonstrate that the cytokine stem cell factor is not essential for the maintenance of HSCs in culture but enhances survival *in vitro* and promotes proliferation. In the absence of SCF HSCs remain in a hibernating state retaining full *in vivo* functionality as shown by single cell transplant. These hibernating HSCs can be induced to proliferate and self-renew and may be isolated based on a higher than average expression of CD150. Transcriptional analysis of these hibernating HSCs may provide insight into the underlying mechanisms of exit from quiescence as well as self-renewal.

These results may have major clinical implications if they can be applied to human HSCs, with regard to HSCs isolation and retention, and study of exit from quiescence. A better understanding of what drives human HSC proliferation and differentiation may aid in the development of culture conditions aimed at the expansion of HSCs for bone marrow transplantation as well as contribute to establishing protocols for the *ex vivo* production of specialised mature blood cells.

Therefore, human HSCs ($CD34^{+}CD38^{neg}CD45RA^{neg}CD19^{neg}CD49f^{+}CD90^{+}$, at 10% purity[81]) were single-cell sorted and cultured in StemSpan with or without serum supplementation. Preliminary results depicted in Figure 34A-B show that 79.1% survived in FCS supplemented minimal culture conditions, with 97.3% remaining single cells. As expected, survival was lower in serum-free conditions (33.3%) but 100% of surviving cells remained undivided. Notably, even in SCF stimulated culture a small proportion of cells did not enter the cell cycle (Figure 34C-D). Next, I assessed cell cycle kinetics following SCF stimulation after 7 days of deprivation in HSCs cultured in serum-supplemented minimal conditions. HSCs did enter cell cycle within the first 24h of stimulation, albeit at a slower rate than the control which received SCF from day 0. This indicates that human HSCs need a longer stimulation period to become activated than mouse HSCs. However, minimal culture conditions will need to be optimised and more repeats carried out to confirm and validate this observation. These results may provide an exciting opportunity to study exit from quiescence in human HSCs, without the need for induction of quiescence through chemical inhibitors such as the specific CDK4-CDK6 inhibitor PD033299 (PD). PD blocks G_1 to S phase transition, however these non-dividing HSCs are not truly reflective of quiescent HSCs[87].

In conclusion, I was able to show that single mouse HSCs can be cultured for a period of up to 7 days without undergoing cell division while maintaining the capacity to self-renew and serially repopulate recipients with multi-lineage reconstitution. The majority of these hibernating HSCs exhibit an alpha phenotype and may be prospectively identified using high CD150 expression. These findings may allow the study of how extrinsic stimulants effect cell survival and exit from quiescence on the single cell level. In addition, molecular changes within freshly isolated and cultured HSCs can be assessed including transcriptional changes using RNA sequencing. The latter may allow the identification of genes that are dispensable for HSCs stemness and possibly detect genes that are overrepresented in the alpha-HSCs subtype. Finally, a proof-of-principle experiment in human HSCs demonstrates that this phenomenon may not

be restricted to mouse HSCs, making it particularly important to validate whether single human HSCs maintained in culture still retain the functional properties of input HSCs.

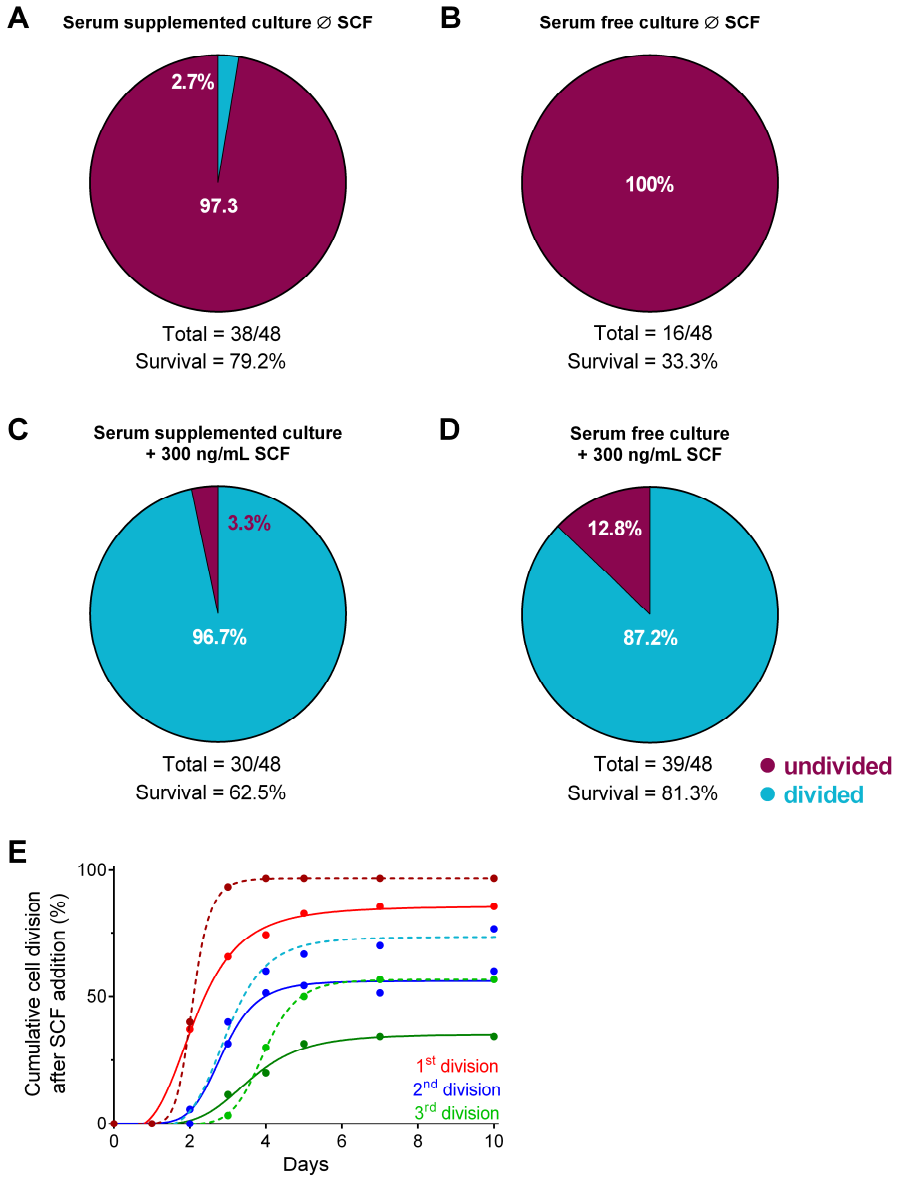


Figure 34: Distribution of quiescent and proliferating human HSCs in minimal culture conditions.

A) Percentage of cells that remain undivided in serum-supplemented minimal culture conditions. B) Percentage of cells that remain undivided in serum-free minimal culture conditions. C) Percentage of cells that remain undivided in serum-supplemented standard conditions. D) Percentage of cells that remain undivided in serum-free standard conditions. E) Cell division kinetics following cell division directly after sort (dashed curve, n=30, 1 exp.) or following 7 days of culture (solid line, n=35, 1 exp.). Lines show non-linear regression fit, [Agonist] vs. response, variable slope (four parameters).

3.2.7 RNA sequencing of cultured HSCs reveals genes potentially driving myeloid lineage choice and maintenance of HSCs activity

Functionally, the results have conclusively shown that the majority of hibernating HSCs retain their stem cell activity and thus are able to reconstitute the haematopoietic system of primary and secondary recipients. Investigation of the transcriptome of these cells may aid in the identification of genes that are indispensable for HSC function, under the premise that those genes shared between freshly isolated HSCs and hibernating HSCs are essential for the maintenance of stemness.

To this end, RNA sequencing was performed on a small bulk population (200–300 cells) of hibernating HSCs and compared to freshly isolated cells. In total 17,860 gene transcripts were analysed of which 2,067 were significantly differentially expressed ($p<0.01$). 1107 genes were upregulated in the cultured cell population, which clustered mainly with GO terms usually associated with synaptic signalling activity such as excitatory extracellular ligand-gated ion channel activity, acetylcholine receptor activity, neurotransmitter:sodium symporter activity, and acetylcholine-gated cation-selective channel activity (Table 12). GO analysis of the 960 downregulated genes indicates that the majority are involved in protein and small molecule binding (Table 13).

Table 12: Gene ontology associated terms based on upregulated genes in hibernating HSCs compared to naïve BM HSCs.

Annotation cluster	Gene count	P-value elimFisher
calcium ion binding	695	9.10E-06
actin binding	370	0.00168
heme binding	170	0.01830
motor activity	136	0.00918
excitatory extracellular ligand-gated ion channel activity	52	0.00128
calcium channel activity	98	0.00803
peptide hormone binding	38	0.00204
acetylcholine receptor activity	24	0.00134
neurotransmitter:sodium symporter activity	21	0.00070
acetylcholine-gated cation-selective channel activity	17	0.00024

Table 13: Gene ontology associated terms based on downregulated genes in hibernating HSCs compared to naïve BM HSCs.

Annotation cluster	Gene count	P-value elimFisher
protein binding	504	0.00028
organic cyclic compound binding	261	0.00630
heterocyclic compound binding	260	0.00415
metal ion binding	189	8.50E-05
transferase activity	117	0.00085
GTP binding	29	0.00017
carboxylic acid binding	13	0.00192
protein kinase inhibitor activity	8	0.00830
transferase activity, transferring pentosyl groups	6	0.01341
peroxidase activity	6	0.00257

As GO terms are inconclusive and meant to guide research questions rather than answer them directly, normalised tag counts were also compared between freshly isolated and hibernating HSC samples in two biological replicates. Most significant differences were due to very low or undetectable gene transcripts in one of the replicates, which may be true absence, but could also be due to RNA degradation prior to library preparation. Upon exclusion of these genes, a list was generated with the most reliably differentially expressed genes listed in Table 14.

Table 14: Manually curated list of genes that are downregulated in hibernating HSCs

Gene ID	BaseMean	log_2FoldChange	P-value	Adjusted P-value
Fos	21784.53	-14.24	-5.16044	2.46E-07
Jun	11497.15	-16.76	-5.01921	5.19E-07
Ier2	10827.07	-11.72	-4.27136	1.94E-05
Ifi44	2804.25	-14.72	-4.40949	1.04E-05
Adck5	2794.90	-14.72	-4.40575	1.05E-05
Fes	2609.28	-14.62	-4.37729	1.20E-05
Hn1	2006.60	-14.24	-4.26249	2.02E-05
Cx3cl1	1984.74	-14.22	-4.25883	2.06E-05
Dfna5	1802.99	-14.08	-4.21785	2.47E-05
Prdx4	1735.20	-14.03	-4.197	2.70E-05
Anxa1	1672.70	-13.98	-4.17868	2.93E-05
Retnlg	1488.00	-13.81	-4.13012	3.63E-05
Atl3	1438.01	-13.76	-4.11912	3.80E-05
Ptger3	1432.09	-13.75	-4.11715	3.84E-05
Slc25a13	1356.67	-13.67	-4.08802	4.35E-05
Sestd1	1350.56	-13.67	-4.08552	4.40E-05
Slc25a24	1298.69	-13.61	-4.07234	4.65E-05

The two most downregulated genes in this list are *Fos* and *Jun* whose gene products form the transcription factor complex Activator Protein-1 (AP-1). Little is known about the role of

AP-1 in the regulation of HSCs fate choice. *c-Fos*$^{-/-}$ mice have severe defects in bone formation, and by proxy aberrant deficient haematopoiesis. *Jun-B* has more intensively been studied and has been shown to be involved the regulation of myelopoiesis and HSCs expansion. Overexpression of *JunB* in mouse HSCs leads to loss of HSCs and vice versa, loss of *JunB* results in the expansion of HSCs and myeloid progenitors ultimately leading to the development of myeloproliferative disease. However, *JunB* deficiency does not affect HSCs self-renewal activity *in vivo*[271,272]. These findings indicate that AP-1 activity may be essential to drive HSCs proliferation *in vitro*. However, more extensive gene analysis need be carried out to verify these findings and potentially uncover the network in which AP-1 plays a regulatory role. Potential targets need to be verified by qPCR and single cell RNA seq could be employed to provide a greater resolution into the transcriptional networks that drive myeloid differentiation.

As hibernating HSCs retain their long-term, multipotent lineage engraftment abilities, investigating genes that are not expressed in this population may provide insight into the genes that are not essential for HSCs self-renewal. Since α-catulin[47], Hoxb5[48], and Fgd5[49] are used as *in vivo* HSCs reporters, their differential expression was assessed in freshly isolated BM HSCs and HSCs cultured for 7 days in minimal cytokine conditions. Both *Ctnnal1* and *Fgd5* are not differentially expressed, while Hoxb5 is significantly downregulated in cultured HSCs (log_2fold change 12.1506, p-adj. 0.005024) suggesting that Hoxb5 may not be suitable for the identification for HSCs *in vitro* or at least not the alpha-HSCs subtype.

Wilson et al. recently identified a gene signature shared between phenotypic HSCs isolated based on different isolation strategies. These HSCs were termed MolO (Molecular overlapping) cells. The MolO signature was proposed to identify LT-HSCs on the molecular level and comprises transcription factors involved in differentiation and self-renewal, epigenetic regulators, as well as surface markers. Therefore, the expression data of cultured HSCs was probed for the presence or differential expression of MolO genes.

As can be seen in Table 15, normalised tag counts widely differ between biological replicates in some samples. This may be due to RNA integrity or read depth during sequencing. However, this data is indicative of significant transcriptional changes occurring in cultured HSCs without a change in their functional output, thereby allowing us to better resolve the key molecules involved in the self-renewal machinery.

Table 15: Tag counts and log$_2$fold change of MolO genes in hibernating HSCs

Gene ID	Freshly isolated tag counts		Cultured tag counts		Log$_2$Fold change in cultured	Adjusted P-value
	Repeat 1	Repeat 2	Repeat 1	Repeat 2		
Ets1	1.456	0.000	653.527	0.000	8.411	0.1060
Etv6	6320.094	2890.007	31397.328	665.283	1.666	0.9941
Vwf	3387.897	6059.953	38414.486	150.164	1.588	0.9998
Tal1	1131.240	5796.241	15291.860	818.774	1.319	0.9998
Pbx1	4519.137	6656.346	16027.903	1725.935	1.229	0.9998
Mecom	1485.026	7309.539	12763.571	1393.293	1.209	0.9998
Cbfa2t3	11022.675	12137.487	24395.022	2754.273	0.838	0.9998
Kit	34406.005	76182.844	184013.921	7165.575	0.779	0.9998
Lyl1	28206.751	10809.463	47360.868	2850.263	0.643	0.9998
Nfe2	35352.345	27032.448	65367.504	4256.862	0.446	0.9998
Erg	8610.237	3030.653	13108.488	643.899	0.422	0.9998
Fli1	22879.586	5715.100	18716.273	1681.741	0.041	0.9998
Gata2	10917.850	19067.011	20133.897	1646.576	-0.085	0.9998
Mpl	125234.247	76769.771	132221.945	9628.549	-0.148	0.9998
Lmo2	78483.603	89618.603	158273.888	3730.338	-0.205	0.9998
Meis1	22141.441	20063.705	29184.249	1490.234	-0.307	0.9998
Tet2	2330.908	2357.174	1113.966	117.850	-1.359	0.9998
Procr	26705.710	15603.601	16966.934	159.668	-1.607	0.9998
Prdm16	2153.287	2270.623	670.030	97.892	-1.732	0.9775
Ets2	5372.299	490.909	0.000	139.234	-2.185	0.9846
Gata3	1595.675	2489.706	1.650	19.483	-4.611	0.3110
Hoxb4	69.884	0.000	0.000	0.000	-6.310	NA
Gfi1b	5183.031	5539.292	0.000	2.851	-8.799	0.0230

The transcription factor *Ets1* is upregulated in HSCs cultured in minimal culture condition, although it was only detected in one replicate in each condition making it difficult to make robust claims about its involvement. Conversely, the Ets transcription factor family members *Fli-1* and *Erg*[273] were not differentially expressed. *Ets1* plays a known role in megakaryocyte differentiation and is highly expressed in embryonic endothelial cells where it potentially is involved in co-regulation the endothelial to haematopoietic transition via *Runx-1*. Little is known about *Ets1* function in HSCs[274].

On the other hand, the negative transcriptional regulator *Gfi1b* is significantly downregulated in cultured HSCs, suggesting that this gene is not necessary for the maintenance of stem cell identity. This is in accordance to published data that shows Gfi1b is predominantly expressed in MEP and not the HSCs population, which suggests that *Gfi1b* is involved in driving differentiation in HSCs[70,275]. This is in agreement with reports suggesting that *Gfi1b* is involved in megakaryocyte/erythrocyte lineage specification[68]. It cannot be excluded however, that bulk

RNA sequencing does not provide a high enough resolution to detect changes in certain cells, despite the low amount of input cells.

These first data from HSCs cultured in minimal conditions demonstrate that transcriptional differences can be observed between naïve and hibernating HSCs with fully retained functional capacity. Targets identified through RNA sequencing would need to be verified using RT-qPCR and potentially modulated to demonstrate their role in HSCs biology. Single cell RNA sequencing could further provide intercellular differences in cultured HSCs, where only 20-40% retain a beta-HSCs programme. scRNA seq would also provide a better understanding of the molecular drivers of HSCs activity and exit from quiescence if performed in a defined time course post 7 day culture. Most intriguingly, it will enable the investigation of the transcriptional program of myeloid biased HSCs, potentially identifying novel markers for the specific isolation of alpha-HSCs. This may also be of clinical interest as myeloid cancers are suspect to arise from HSCs which are deregulated in their ability to generate lymphoid cells.

3.2.8 Optimisation of serum-free culture to ensure single cell survival in absence of SCF

As virtually no cells survived in StemSpan based medium without SCF stimulation in absence of foetal calf serum (FCS), I sought to improve survival in serum-free culture conditions to exclude the possibility that the described phenomenon is due to an unidentified molecule present in FCS. Literature searches showed that the addition of Collagen I (Col I) and nerve growth factor (NGF) to the standard conditions commonly used in our lab resulted in 97% viability of clones over a period of 7 days, with 74% or 9% of clones retaining short-term or long-term repopulating activity after transplantation respectively[164]. However, I was unable to replicate these findings in multiple experiments. HSCs were cultured as single cells in StemSpan supplemented with either 10% FCS (SSM) or NGF plus 10% Collagen I (NCM).
By day 4, only 21.4%, ± SEM 6.8% of cells had generated viable clones in NCM compared nearly 80% in SSM (79.1%, ± SEM 10.3%) as seen in Figure 35A. From day 1 in culture, cell cycle entry of HSCs in NCM was delayed compared to SSM, with entry into second and third division being significantly later on day 2 and 3 respectively (Figure 35B-D). To assess if NGF is in fact cytotoxic, cells were cultured in SSM with the addition of NGF but not Collagen I. Only 16.5% of clones survived in these conditions, compared to 80-90% in SSM (data not shown). Thus, it seems that NGF adversely affects HSCs survival and proliferation *in vitro*. These findings do not exclude the possibility that Col I still has beneficial effect on cultured

HSCs, concealed by the overpowering negative effects of NGF. I therefore opted to test conditions suggested by Adam Wilkinson (Nakauchi lab, unpublished) which are described in 3.2.1, p. 70.

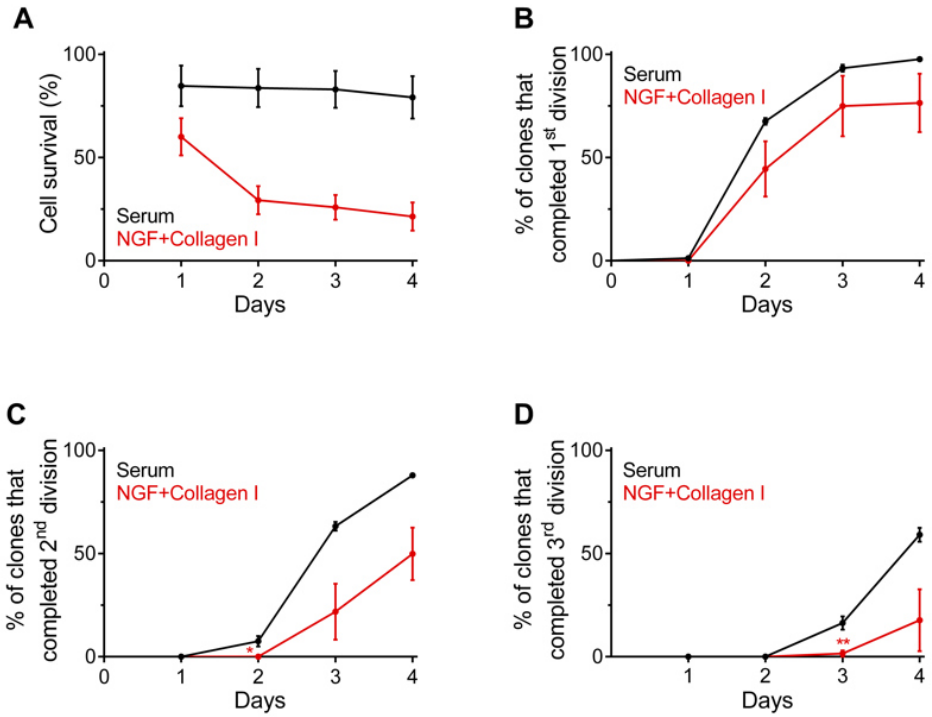


Figure 35: NGF and Collagen supplementation does not improve HSCs survival or proliferation *in vitro*.

A) 4 Day clonal survival in medium either supplemented with serum or NGF+Collagen I. B) Percentage of cells that have undergone a1st division. C) Percentage of cells that have undergone a 2nd division. D) Percentage of cells that have undergone a 3rd division. Serum: 10% FCS in standard medium supplemented with SCF and IL-11. n=274, 3 exp. NGF+Collagen I: 10% Collagen I + 250 ng/mL NGF in standard medium + SCF + IL11. n=282, 3 exp. *=p<0.05, **=p<0.001. Error bars represent data ± SEM.

3.3 Investigating physical forces supporting HSCs survival and expansion *ex vivo*

Adult HSCs reside in the bone marrow niche, which is a complex microenvironment comprising soluble factors, transmembrane receptor ligands and extracellular matrix molecules, as well as differentiated cells[18,242]. Besides collagen type II and III, collagen type I is part of the of the extracellular matrix in the bone marrow[276], suggesting that mimicking the mechanical properties of the HSCs microenvironment is likely to influence HSCs survival and proliferation *in vitro*. Additionally, defined conditions that mimic the 3D architecture of the bone marrow niche *in vitro* would allow the teasing apart of mechanical effects from biochemical cues and enable us to study how HSCs biology is affected by matrices of different elasticity or stiffness[242,244]. Therefore, I set out to first estimate the stiffness in the stem cell niche followed by the construction of agarose/ECM composite gels that would mimic a range of stiffness close to this value.

Material or tissue stiffness can be measured using micro-indentation atomic force microscopy (AFM)[277]. In a preliminary experiment, AFM was used to narrow down the range of elasticity within the bone marrow using bone marrow plugs obtained from Nestin-GFP$^+$ mice. Areas close to GFP positive cells were measured, as Nestin-GFP expressing mesenchymal stromal cells (MSCs) are considered to be important HSCs niche cells[278] (described in 1.1.2, p. 3). These measurements indicated that the BM elastic stiffness ranged from 300 to 450 Pa (data not shown), with areas of Nes-GFP$^+$ being slightly stiffer than GFP-negative areas. This range is consistent with previous findings reporting a stiffness of 0.3 kPa[245] and several orders of magnitude softer than tissue culture plastics (2–4 GPa)[246]. I therefore moved forward with this range in mind for the first screen of supportive substrates.

3.3.1 HSCs can be cultured on agarose-based matrix gels

To screen for optimal substrate stiffness conditions retaining and expanding HSCs *in vitro,* agarose based hydrogels were made covering a stiffness range of approximately 100 Pa–1 kPa. ECM proteins laminin and fibronectin were added to some of the gels to test whether extracellular matrix (ECM) components influence HSCs retention *in vitro*. Agarose was chosen for its neutral, biocompatible and scalable properties. It is non-cytotoxic in its single component prior to polymerisation in contrast to synthetic polymers such as poly(vinyl alcohol or polyacrylamide. Additionally, it is stable for extended periods of time *in vitro*, and importantly,

it is tuneable in its mechanical properties. Agarose can be functionalised with peptides and other small molecules and allows their diffusion-controlled release. Therefore, agarose can be used to form functionalised droplets encapsulating single cells to perform high throughput screenings of various factors[279,280].

In a pilot experiment 3 cells were sorted onto the agarose hydrogel. However, as seen in Figure 36, in some wells all three sorted cells were viable and generate clones with different cell cycle kinetics and diverse cell compositions, as can be determined by the different granularity and clone size. Furthermore, in this setting it cannot be excluded that cells of one clone do not produce factors that affect another clone. Additionally, it was not possible to physically separate these clones and analyse them individually. To avoid these caveats, single cells were sorted onto the hydrogels for all future experiments. Our usual liquid culture follows single cells for up to 10 days, from which time on cells begin to undergo apoptosis. By culturing cells for 14 days, I aimed to assess if softer gel mechanics extends viability compared to stiff tissue culture plastic.

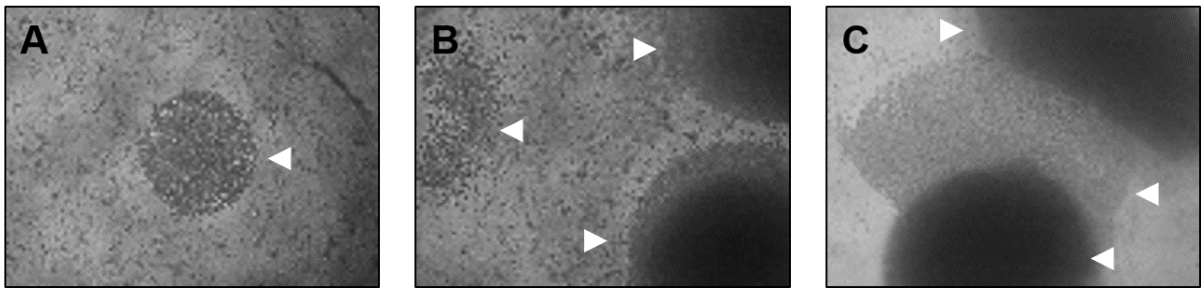


Figure 36: Representative well that supported the development of three separate clones. One well at different time points shows the development of three separate clones that heterogeneous in cell composition as well as cell cycle kinetics. A) Day 7. B) Day 10. C) Day 14 in culture. Arrow heads point out the clones

The proportion of HSPCs (as measured by the LSK markers), relative to the amount of viable cells was used as the readout for HSCs stemness retention (i.e., cultures that retain HSCs should also retain more primitive progenitors relative to the more mature progeny).

Single HSCs were sorted and clonal growth was followed over time by visual inspection. Due to the opacity of the gel, cell division kinetics could not be carried out. As seen Figure 37 broad changes in clone size could be observed across the 96 different conditions on day 14.

In liquid culture, small clone size generally correlates with retention of HSPCs. However, in this setting small clone size does not equate to HSPC retention as seen from clone E1 when compared to clone F1, with the former containing only very few LSK cells. Another factor to

consider, is that gel digestion conditions (30 min. trypsin at 37°C) may result in cell death of some cells before flow cytometry is carried out, although analysed cell number broadly correlated with size of colonies.

The screening also gave an indication whether any of the conditions are detrimental to HSCs survival as no clone would grow from the single cell (e.g. well F2 in Figure 37). However, none of the tested conditions consistently had empty wells, suggesting that survival was not negatively affected as seen from Figure 38. When comparing the average survival of each well, 60 out of 96 wells had consistently a cell survival rate >50%. A consistent >70% survival rate was only observed in 18 of 96 wells. Survival was enriched in lane 10 and 12, which contained the lowest concentration of agarose (0.5%). In both cases 3 of 18 wells had the highest survival. In addition both row B and F contained each 4/18 wells with >70% survival. Both these rows contained 0.2 mg/mL fibrinogen but differed in the amount of thrombin. Thus, softer substrate seems to support cell survival better than stiffer gels, while extracellular matrix components do not greatly affect survival.

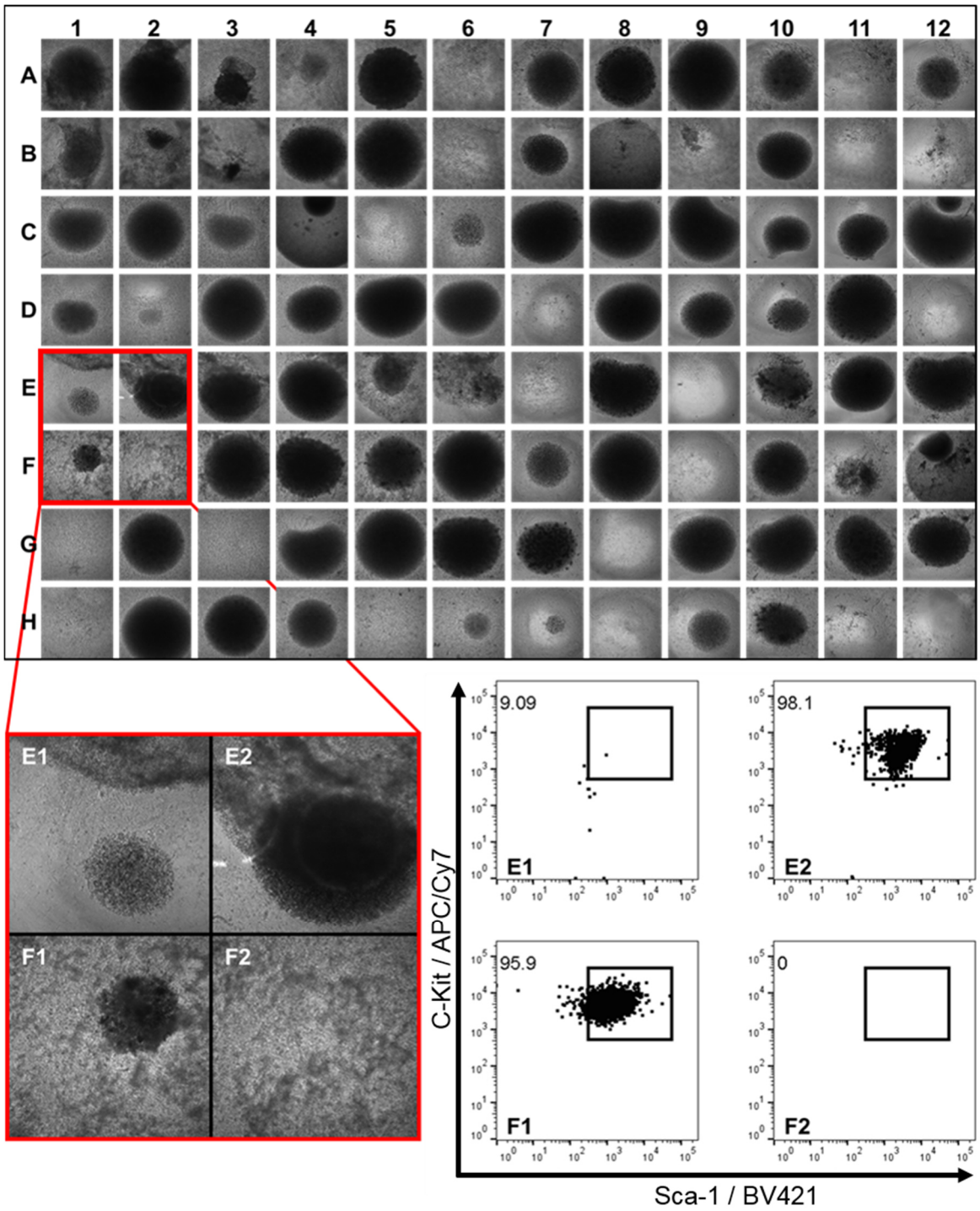


Figure 37: Day 14 visualisation of clones grown on 96 different matrix gels in Plate D.
Broad changes in clonal growth and survival can be observed. Clone size is not necessarily proportional to HSPC content. The depicted LSK percentage is frequency of parent. LSK are gated from lineage-negative viable cells.

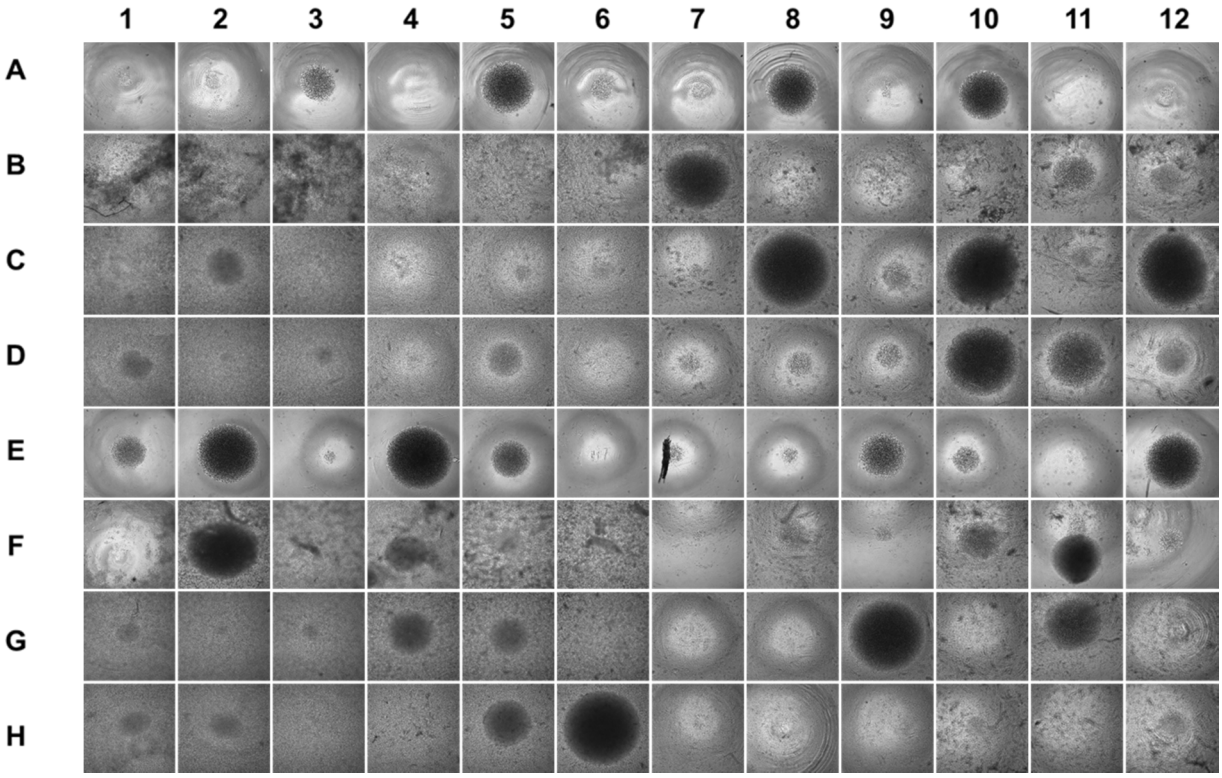


Figure 38: Day 10 visualisation of clones grown on 96 different matrix gels in Plate A.

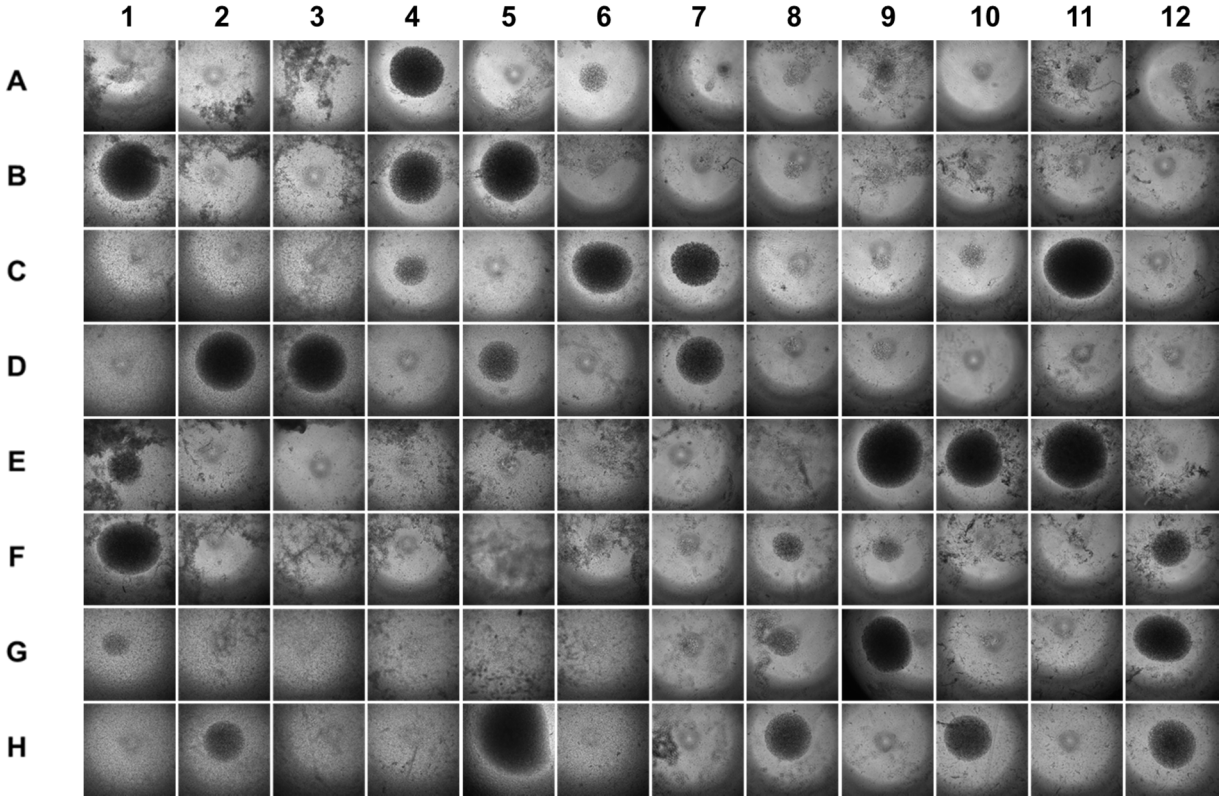


Figure 39: Day 10 visualisation of clones grown on 96 different matrix gels in Plate B.

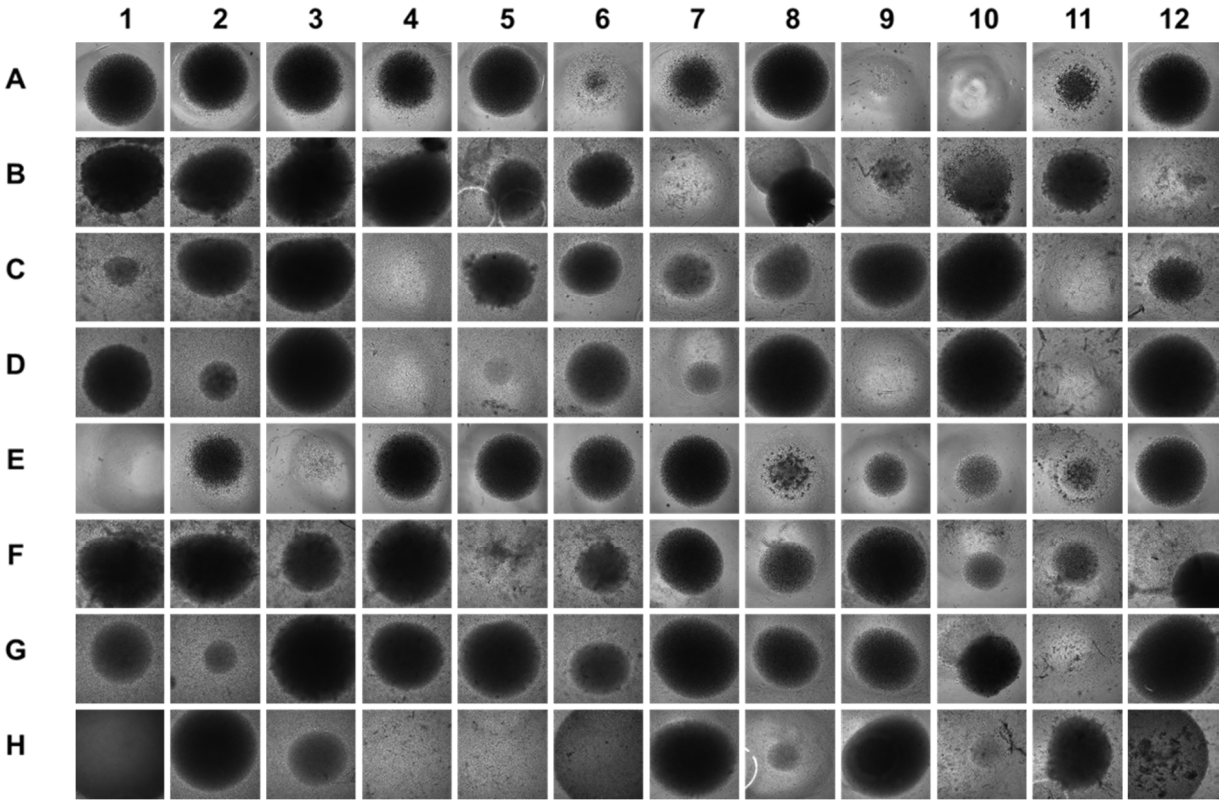


Figure 40: Day 14 visualisation of clones grown on 96 different matrix gels in Plate C.

On day 10 of culture, 28 of 96 wells contained clones with >70% LSK in at least one of the two repeats, whereas on day 21 none of the wells was found to comprise >50% LSK. Row A and F contained most wells (4 of 16) with an LSK content >70% on day 10. Both rows contain higher amounts of fibrinogen, which also enriched for improved cell survival. Lane 10 (4/16) and lane 11 (3/16) contain most wells with LSK retention >70%, both wells are manufactured with the lowest concentration of agarose. These results suggest that low concentration of agarose supports cell survival and HSPC maintenance retention.

To compare HSPC retention in 96 different conditions, LSK frequencies were displayed in a heat map like fashion. When focussing on the influence of agarose concentration on HSPC retention on day 10 it becomes evident that lower concentrations of agarose, thus gels of lower mechanic stiffness, tended to favour the production of HSPCs. The addition of the ECM proteins laminin and fibronectin seem to have a positive effect on HSPC retention but not consistently, this may be due to HSCs heterogeneity (Figure 41B). When the protease thrombin is mixed with the plasma protein fibrinogen the mixture polymerises forming a fibrin gel[281]. As seen in Figure 41C-D, it appears that gels containing a higher concentration of both thrombin and fibrinogen have an overall slightly higher proportion of clones retaining LSK. Altogether,

the main driver of differences between clonal LSK proportions seems to be the agarose concentration.

These results indicate that matrix composition and stiffness affect HSCs fate choice and that soft matrices containing ECM proteins may benefit HSCs expansion. To date, very little research has investigated the effect of mechanical property changes on HSCs. This screen provides the first evidence on the single cell level, that substrate stiffness can indeed effect HSCs fate choice. Heterogeneity within the HSCs compartment hamper the analysis of different conditions on single cells as many replicates are needed to compensate for this. This concern may be resolved through the development of high throughput screening devices, for instance microfluidic chips. Nevertheless, these experiments set the stage for future experiments, which could test individual factor combinations to narrow down specific conditions that are optimal for HSCs expansion and testing of these best candidates by *in vivo* transplantation assays. It might also be possible to use the minimal medium conditions described in Chapter 3.2 to help identify conditions that would support *in vitro* survival. In the long term, beyond the scope of my PhD work, such HSCs expansion conditions (physical and molecular) could be applied to the culture of human HSCs with the goal of expanding human HSCs for clinical applications.

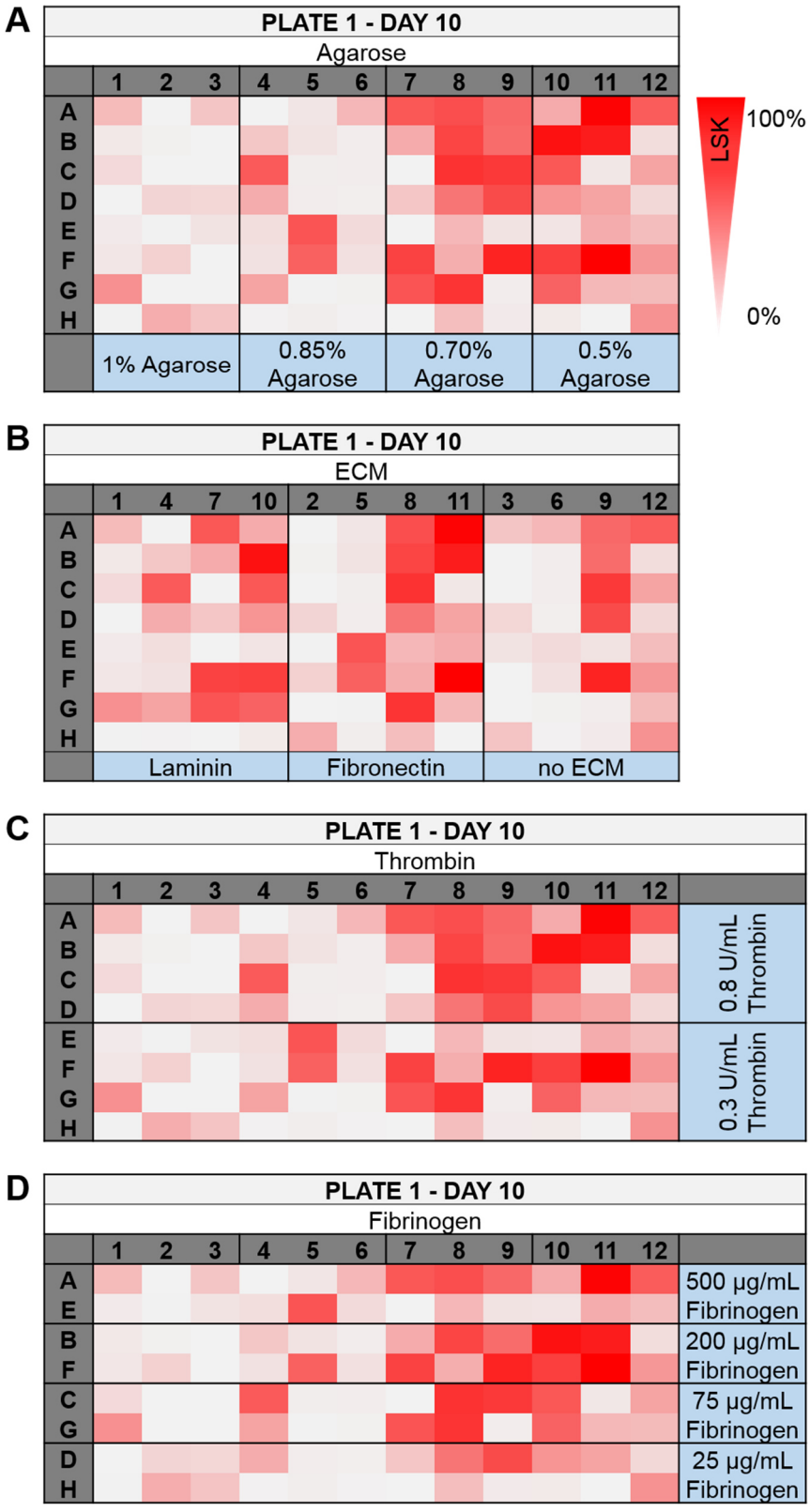


Figure 41: Day 10 LSK retention in clones grown from single cells in 96 different matrix conditions.

A-D all show the same plate but ordered according to the matrix component in focus. A) Agarose concentration. B) ECM additives. C) Thrombin concentration. D) Fibrinogen concentration. LSK percentage equals the LSK frequency within the viable singlets pool.

4 Discussion

Haematopoietic stem cells (HSCs) have been studied for decades. In this time a great number of genes have been implicated as intrinsic regulators of HSCs self-renewal and differentiation, yet little is known about their effects on HSCs self-renewal[99-102,282]. Overexpression screening has demonstrated that some of these genes are able to expand HSCs *ex vivo*, including *Hoxb4*, *Fos*, *Prdm16* and *Smarcc1*[283].

Additionally, numerous extrinsic regulators, including cytokines, growth factors, and chemokines have been studied for their effects on HSCs[123-125,128,130]. Yet, there is little evidence on how extrinsic regulators enhance HSCs self-renewal and the molecular regulators driving HSCs fate choice are still poorly understood. A better understanding of the machinery that drives lineage priming, self-renewal, and differentiation is of medical importance as this could aid in the development of protocols for *ex vivo* HSCs expansion, production of specific mature cell types such as platelets, and gene therapy[258]. Moreover, improving our knowledge of genes involved in lineage differentiation may elucidate which pathways are perturbed in the development of blood cancers.

Even though the study of HSCs has made vast progress in recent years, especially through the development of single cell tools, there are still many challenges to overcome to uncover what drives HSCs fate choice.

For one, it is virtually impossible to study HSCs over extended periods *in vitro*, due to their propensity to differentiate. Freshly isolated HSCs only offer a snap-shot of native haematopoiesis and rely on the purity of the isolation strategy and the stability of the cellular state being assessed. Any phenotypically isolated HSCs population contains contaminating non-HSCs cells and vice versa, true LT-HSCs may be lost through rigid gating strategies and may skew interpretation if this is non-random[75]. In addition, even a highly purified population of phenotypic HSCs displays extensive heterogeneity in lineage choice and as of yet it is not possible to isolate specific HSCs subtypes specifically based on their surface marker expression alone. Moreover, any sort of gene perturbation *ex vivo* relies on HSCs culture, which inevitably alters HSCs biology.

In vivo, the lack of reliable markers that exclusively label HSCs as well as the lack of clarity on the actual location and cell types comprising the HSCs niche challenge all available imaging technologies. *In vivo* gene perturbation studies using gene specific promoters are at present the closest we can get to understand the roles of individual genes in haematopoiesis *in situ* but these

strategies rely on targeting all HSCs and excluding any off-target effects, which is not necessarily possible.

When studying HSCs fate choice at the single cell level, it is therefore advantageous to control and minimise as many variables as possible. In this thesis, I show in Chapter 3.1 that *in vitro* activation of SCF/KIT signalling in HSCs prior to first division alters clonal stem cell expansion without affecting initial HSCs engraftment ability. Furthermore, in Chapter 3.2 I could show that absence of SCF/KIT signalling can maintain HSCs function *in vitro* for extended periods of time. Finally, in Chapter 3.3, I explore the role of physical forces on HSCs retention, demonstrating that changes in the composition and stiffness of various matrices can also alter HSCs fate.

Differential SCF stimulation prior to first division alters HSCs clonal expansion *in vivo* without affecting initial HSCs repopulation potential

It has long been known that the SCF/KIT signalling pathway is crucial for functional haematopoiesis[195] and as one of the key players in this, SCF has been one of the primary standard cytokines used in various growth factor combinations to maintain and expand stem and progenitor cells *in vitro*[124,125,130,284]. Furthermore, it has been shown that changes in SCF concentration *in vitro* can alter HSCs fate choice (self-renewal versus differentiation) *in vivo* prior to first division, with altered self-renewal frequency[10].

In this thesis, I was able to show that a concentration as low as 10 ng/mL has a negative effect on HSCs survival and proliferation *in vitro*, whereas there is virtually no difference when HSCs are treated with 30 ng/mL or 300 ng/mL. While this is different from previously published data, the input HSCs fraction I used in this thesis are a more highly purified population of HSCs with durable self-renewal, suggesting that the effects previously described were primarily on HSCs with finite self-renewal.

In both limiting dilution and single cell transplants, no significant differences in repopulation ability were detected in cells treated with a high (300 ng/mL) or low (30 ng/mL) dose of SCF prior to first division. In both settings, ~35% of single transplanted HSCs were able to reconstitute the recipient mouse with similar proportions of HSCs subtypes as defined by their lineage output at 16 weeks. Thus 30 ng/mL SCF is sufficient to maintain HSCs function in culture prior to a first division. Interestingly, donor chimaerism was significantly higher in secondary recipients of HSCs that were stimulated with a high dose of SCF. This indicates that

prior to a first division, SCF stimulation alters the self-renewal activity in single cells, with HSCs producing more daughter HSCs following treatment with a higher dose of SCF, presumably in the early stages post-transplantation.

To thoroughly assess if the increase in self-renewal activity indeed occurs in a dose-dependent manner, more single cell transplantations need to be carried out at different doses of SCF and within different time windows. HSCs expansion and number in recipients of differentially treated bone marrow can be quantitatively assessed by limiting dilution transplantation of the primary recipients bone marrow at different weekly time points within the first month of transplantation[256]. These experiments will narrow down which functional changes occur and when this is most evident. Concurrent RNA sequencing of these differentially treated HSCs may pinpoint which transcriptional changes occur driving these self-renewal divisions. SCF stimulation leads to the clustering of c-Kit in membrane lipid rafts[121]. Potentially, this occurs in a dose-dependent fashion and high concentrations of SCF therefore augment SCF/KIT signalling more than lower doses. However, it has been shown that lipid raft clustering in HSCs occurs as early as 30 minutes post *in vitro* SCF stimulation[121], suggesting that after 16h lipid raft clustering should have reached its saturation point, even at low doses. Transplantation of single cells beforehand stimulated at a high dose of SCF in the presence of an inhibitor of lipid raft clustering may be useful to investigate if this mechanism is indeed essential for the improved self-renewal outcome.

Another possibility could be that HSCs homing ability is differentially affected by SCF stimulation, with cells stimulated with a higher amount of SCF homing to the bone marrow at earlier time points thus having an earlier opportunity to undergo self-renewal divisions. This may happen under the assumption that SCF induces c-Kit expression on the cell surface making them more receptive for signals from the bone marrow, such as more SCF produced by BM MSCs. However, a role of SCF in bone marrow homing has not been described, in contrast to the CXCL12/CXCR4 axis[285]. Thus it would be interesting to investigate if these cells preferably home to putative niche cells that secrete SCF. One possible way to examine this would be the use of recipient transgenic mice that express fluorescent reporters in their bone marrow niche cells such as the Nestin-GFP$^+$ mouse. The main caveat of this approach is the necessity of a high number of labelled, purified donor stem cells as only the calvarium can be imaged in a live animal, which represents only a small proportion of the total BM[36,286].

Another potentially more feasible approach to answer questions surrounding alterations in c-Kit level post SCF stimulation is flow cytometric analysis or immunocytochemistry as shown by Yamazaki et al.[121], with flow cytometry having a higher and more quantitative throughput

but with the caveat of requiring significantly more input material of an already rare population of cells.

Measuring the levels of c-Kit on the HSCs post SCF stimulation may answer the question whether differentially treated cells have distinct levels of c-Kit expression that positively correlate with stimulation dose. Contradictory to this hypothesis would be the finding that W41 mice, which carry a c-Kit mutation, are not reported to display any homing defects but this was not assessed in LT-HSCs shortly after transplantation[218]. A simpler way to assess the relative homing capacities would be to investigate this putative increased bone marrow translocation by transplanting differentially stimulated and labelled HSCs and assessing their homing to the bone marrow tracked in first days post transplantation.

SCF has been shown to be radioprotective with the drawback that patients display severe allergic reactions mediated by the activation of mast cells[223,225]. The discussed findings show that on the single cell level, SCF may indeed exert some radioprotective measure onto transplanted HSCs in a dose dependent manner. This may be of clinical relevance, firstly in the pre-treatment of patients prior to stem cell transplant and secondly in stimulation of HSCs prior to the transplant. In patients, allergic responses may be mitigated through the use of engineered recombined SCF featuring an altered dimerisation interface[213], which selectively activates HSPCs but not mast cells.

Encouraging results from an ongoing clinical trial using stabilized Prostaglandin E2 (PGE2) demonstrates that treatment of HSCs prior to allogeneic transplant can indeed positively influence the engraftment capability of these cells. In this phase I trial it was shown that HSCs derived from umbilical cord blood (CB) that were pre-treated with PGE2 have higher multi-lineage engraftment than HSCs obtained from a co-transplanted untreated umbilical cord[241]. Prior to this study, it had been shown that PGE2 enhances mouse HSCs self-renewal following 2h *ex vivo* pre-treatment, but its effect on adult human HSCs has not been assessed[240]. Therefore, it is of interest to assess if pre-treatment of cord blood as well as human adult HSCs with SCF would yield a similar increase in self-renewal division *in vivo* as seen in mouse HSCs and PGE2 treated CB HSCs.

LT-HSCs can be maintained as single cells *in vitro* in the absence of SCF stimulation and independent of serum-supplementation

The results discussed above suggest that the concentration of SCF does not affect the ability of HSCs to repopulate recipients. It was therefore of interest to investigate how SCF deprivation would influence HSCs survival, proliferation, and maintenance.

It has previously been shown that activation of the gp130 receptor by IL-6 or IL-11 enhances HSCs expansion *in vivo* and *in vitro*[231,269]. The importance of gp130 signalling in stem cells has also been demonstrated in embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC), where the IL-6 family member LIF (Leukaemia Inhibitory Factor) facilitates the maintenance of stem cell pluripotency and morphology as well as the inhibition of differentiation[287,288]. Here we could show that activation of gp130 signalling alone is sufficient to maintain LT-HSCs as single cells in culture for a period of 7 days. Interestingly, serum supplementation does not drive differentiation as is commonly believed. While 67–75% of sorted phenotypic HSCs die in these conditions, those that are resilient show full retention of multipotent long-term repopulation ability and do not show upregulation of genes associated with apoptosis. These results are comparable to findings showing that inhibition of lipid raft clustering prevents HSCs from dividing *in vitro,* despite activation of the c-Kit receptor[111,121].

While more differentiated progenitor fractions (LSK and SLAM) do not survive in absence of SCF, it has not been assessed if multipotent progenitors would survive in these conditions. Culture of MPP1, MPP2, MPP3 and MPP4 will determine if resiliency in these conditions is exclusive to LT-HSCs. Yamazaki et al. have shown that $CD34^{+}$LSK do not survive for more than 48h *in vitro* upon inhibition of lipid raft clustering[121], suggesting that this resilient phenotype may only apply to the most primitive cells.

Two of 31 mice showed delayed onset of donor contribution, predominantly generating cells of the myeloid lineage but capable of producing lymphoid cells in secondary recipients. These mice would have been classified as not repopulated at 16 weeks as donor chimaerism was not detectable or equalled less than 1%. This was not due to the minimal culture conditions and is more likely a cell intrinsic mechanism as it has been observed by several research groups either through barcoding experiments or single cell transplantation[64,65,289]. This emphasises the need for new standards in the detection of successfully engrafted mice, in particular in a single cell transplantation setting.

Comparison of the transcriptome of freshly isolated HSCs and those cultured for 7 days in absence of SCF may allow the identification of genes that are truly indispensable for the HSCs

state, under the premise that genes that are not expressed in the cultured population are dispensable for HSCs maintenance. A recent study comparing the transcriptome of several different phenotypically defined HSCs populations identified an overlapping molecular signature (MolO) in a subpopulation of HSCs suggesting that this signature can identify HSCs with long-term repopulation potential[68]. Most genes in this MolO signature were not differentially expressed between naïve BM HSCs and cultured HSCs, suggesting that these genes among others are indeed likely to be vital for HSCs biology and confirming the HSCs identity of cultured HSCs on the transcriptional level. The Ets1 transcription factor was found to be upregulated in cultured HSCs. Possibly, Ets1 is involved in stress response to minimal cytokine conditions. Alternatively, Ets1 may play a role in the transcriptional regulation of myeloid differentiation. In contrast, Gfi1b was downregulated in HSCs suggesting that this transcriptional regulator does not play a role in the maintenance of stemness but does not exclude its participation in the negative regulation of HSCs self-renewal or differentiation. To determine its role, transcriptional changes need to be assessed in HSCs that received SCF stimulation after 7 days of culture. Further transcriptional analysis and qPCR will be necessary to confirm these findings. Interestingly, *Jun* and *Fos* which encode for protein subunits of AP-1 were both downregulated in cultured HSCs. This suggests that AP-1 may play a role in cell proliferation although contradictory it has been shown that mice lacking *JunB* expression have an increase in cell cycle activity in addition to expansion of HSCs and myeloid cells and eventually develop myeloproliferative disease[271]. Thus, AP-1 regulation of cell proliferation may be dependent on the "dose" of Jun and Fos present. To assess how closely 7 day cultured HSCs are related to freshly isolated bone marrow HSCs and to investigate what changes occur post SCF stimulation, it would be interesting to sequence and compare the transcriptome of single cultured HSCs that either were never treated with SCF or received a pulse of SCF following 7 day culture. This may allow the identification of genes important for exit from quiescence as well as proliferation where self-renewal expansion or maintenance divisions occur. The described culture method allows the study of any molecule's effect on single HSCs. For example, due to the discrepancy in the field about the efficacy of SCF in combination with TPO it would it may be of interest to study the effect TPO has on HSCs self-renewal and proliferation.

Importantly, it will be essential to test if these culture conditions can be applied to human HSCs. As the preliminary results have shown, some phenotypic HSCs can be maintained as single cells for 7 days and be subsequently stimulated to proliferate, although they display a delay in time to entry into cell cycle. Human HSCs are isolated based on a specific set of surface

markers, however to date just 10% of the phenotypic cord blood HSCs population has repopulation potential, with even lower capacities in peripheral blood and bone marrow HSCs. Thus, if the minimal culture condition indeed enriches for resilient HSCs, which will need to be confirmed by xenotransplantation, the transcriptome of surviving HSCs may be compared to freshly isolated phenotypic HSCs to assess differentially expressed genes. The gene set overexpressed in cultured HSCs may then be probed for molecules such as surface markers that can aid further phenotypic enrichment of long-term repopulating human HSCs. Furthermore this condition could be used to extend the time window in which cells can be transduced for gene editing, while ensuring that exclusively HSCs are targeted.

Cultured single HSCs serially engraft recipient mice and generate predominantly myeloid progeny

The "hibernating" HSCs described by Yamasaki et al. were able to engraft primary recipients however, the study did not report if these donor HSCs exhibited any particular lineage bias[121]. In the transplantation data reported in this thesis, a striking tendency of HSCs to produce more myeloid cells than lymphoid cells was observed. The ratio of GM:(B+T) was >2, which are defined as alpha-HSCs.[64]. Resilient cells were enriched in the $CD150^{high}$ fraction of the phenotypic HSCs pool and prospective gating on $CD150^{high}$ did indeed improve 7 day HSCs survival. This suggests that these resilient alpha-HSCs may be phenotypically distinguishable from HSCs that have a more balanced lineage ratio (beta-HSCs). This is in agreement with previous findings that suggest $CD150^{high}$ enriches for cells with a myeloid lineage bias, whereas $CD150^{mid}$ HSCs produce both myeloid and lymphoid cells at similar ratios[65,270], and $CD150^{neg}$ HSCs have predominantly lymphoid offspring with very little potential to engraft secondary recipients[65,69,72,290]. Additionally, MolO HSCs have been shown to have a higher than average expression of CD150[68], which again is in line with my findings that minimal culture conditions select for true HSCs.

To verify that $CD150^{high}$ expression indeed enriches for alpha-HSCs, more single cell transplantations need to be carried out. However, based on our data, CD150 alone would not be sufficient to prospectively isolate highly purified alpha-HSCs. Therefore, it would be useful to investigate if any other surface marker molecules or intracellular molecules are enriched in the resilient cultured HSCs fraction. This could first be performed by screening for survival in 7 day cultures and then validated by HSCs transplantation.

Previous findings suggest that HSCs with a lymphoid bias proliferate faster than those with a predominantly myeloid output[69], which may result in their premature death in minimal cytokine conditions as the environment is not supportive of proliferation.

The enrichment in alpha-HSCs after 7 day minimal culture suggests that cells of this subtype are more resilient in culture and putatively also *in vivo* since myeloid-biased HSCs accumulate with age[151]. Interestingly, the distribution between HSCs generating balanced or "myeloid-biased" HSCs resembles that of HSCs derived from aged donor mice (38–46 weeks) rather than young adult mice (8–16 wks) which were used as donors. It would be interesting to assess if aged HSCs have generally a better survival outcome in minimal culture conditions as they have already proven to be more resilient *in vivo*. Furthermore, comparison of the transcriptional programs in young naïve, aged, and young cultured HSCs could putatively identify pathways that are involved in the extended survival of the latter two cell population.

In agreement with Morita et al. I hypothesise that alpha-HSCs represent the apex of the haematopoietic hierarchy, predominantly generating myeloid cells (most cells of the innate immune system), and transforming to beta-HSCs when cells of the adaptive immune system are required. This hypothesis is formed under the premise that any organism possessing both an innate and adaptive immune system should encounter the vast majority of pathogens in the early years of life. Therefore, activation of the adaptive immune response with age would be mediated through already existing B and T cell progenitors and would not stem from HSCs. In contrast, cells of the innate immune system have a limited life span and therefore require regular replenishing. Therefore, HSCs capable of producing a vast amount of myeloid cells are required until death explaining why these cells accumulate in aged individuals. Tools to test this hypothesis in mouse are currently not available, as a system is required in which both lymphoid and myeloid progenitors carry different labels, which are also distinct from a label present in the common ancestral HSCs and which in turn is altered with ageing. If the hypothesis holds true, an aged HSCs specific label should be undetectable in lymphoid cells.

However, a recent study in an aged, healthy human individual made use of randomly occurring somatic mutations in both primitive and differentiated cells to track the population dynamics during homeostasis. While this cannot formerly prove the hypothesis on the single cell level, the study did show that not all HSCs contribute equally to haematopoiesis and that T cells and granulocytes separate earlier in life[291].

Understanding the molecular mechanisms that drive HSCs fate choice, in particular the decision to branch into the myeloid or lymphoid lineage may aid in the identification of molecules and

pathways aberrantly regulated in the development of leukaemia and present druggable targets for treatment.

Softer hydrogels favour HSPC maintenance and survival *in vitro*

HSCs reside in the bone marrow niche which is greatly different from the tissue culture plastics they are commonly cultured in. These differences comprise extracellular signalling molecules, lack of cell-to-cell interaction, and importantly a lack or change in matrix elasticity. Defining conditions that mimic the 3D architecture of the bone marrow niche *in vitro* would allow the teasing apart of mechanical effects from biochemical cues and enable us to study how HSCs biology is affected by matrices of different stiffness[242,244].

To investigate the role physical forces play in HSCs retention, I cultured single HSCs on agarose based hydrogels covering a range of matrix elasticity similar to that of the bone marrow. HSCs thrived better on matrices that were composed of lower concentrations of agarose, making them less stiff. Better retention of the HSPC population was also observed on softer hydrogels. This is in agreement with previous reports showing that HSCs are better maintained on soft tropoelastin coated plates when compared to non-coated controls[247].

However, other gel components such as extracellular matrix proteins and fibrin did have no apparent effect on HSCs survival and HPSC retention. Therefore, it may be needed to individually assess the impact these factors have on HSCs fate choice. Particularly, the hydrogel set up may not be ideal to study the effect of fibrin on HSCs, as this protein may degrade in culture. The addition of tranexamic acid or Aprotinin, both anti-fybrinolitic molecules, may extend the half-life of the fibrin network[292]. To assess HSCs survival in a more stringent manner, it may be useful to implement the minimal cytokine conditions in the culture of HSCs on hydrogels. Due to the great decline of HSCs on tissue culture plastic in minimal culture, any conditions significantly improving cell survival would certainly become apparent.

Further screens are necessary to narrow down specific conditions that are optimal for HSCs expansion. Ideally, once the optimal conditions have been identified, these then can be employed to generate agarose droplets, which can be functionalised for high-throughput screening of factors affecting HSCs fate choice. Ultimately, HSCs expansion conditions can be

applied to the culture of human HSCs with the target of human HSCs expansion for clinical applications.

Overall, in this PhD thesis I was able to show that short term *ex vivo* stimulation of SCF/KIT signalling modulates the clonal expansion of HSCs *in vivo* without altering HSCs engraftment capabilities. This shows that the SCF/KIT signalling axis is vital for HSCs self-renewal but not maintenance of stem cell function. The latter is also reflected in the finding that HSCs can be maintained as single cells *in vitro* for extended periods of time in the absence of SCF/KIT signalling while maintaining HSCs function. Additionally, I could show that HSCs are better retained on hydrogels with a soft matrix, similarly to the elasticity found in the bone marrow and thus formally prove that physical forces can alter HSCs fate choice.

These findings may have great implications for the culture of human HSCs aiming at the expansion for autologous stem cell transplantation as well as possible pre-treatment applications prior to allogenic stem cell transplantations.

5 References

1 Ratcliffe, M. J. H. Encyclopedia of immunobiology. (2016).

2 Bryder, D., Rossi, D. J. & Weissman, I. L. Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. Am J Pathol 169, 338-346, doi:10.2353/ajpath.2006.060312 (2006).

3 Doulatov, S., Notta, F., Laurenti, E. & Dick, J. E. Hematopoiesis: a human perspective. Cell stem cell 10, 120-136, doi:10.1016/j.stem.2012.01.006 (2012).

4 Calvi, L. M. & Link, D. C. The hematopoietic stem cell niche in homeostasis and disease. Blood 126, 2443-2451, doi:10.1182/blood-2015-07-533588 (2015).

5 Silbernagl, S. & Despopoulos, A. Taschenatlas Physiologie. (2007).

6 Kersaudy-Kerhoas, M. & Sollier, E. Micro-scale blood plasma separation: from acoustophoresis to egg-beaters. Lab on a Chip 13, 3323-3346, doi:10.1039/C3LC50432H (2013).

7 Eaves, C. J. Hematopoietic stem cells: concepts, definitions, and the new reality. Blood 125, 2605-2613, doi:10.1182/blood-2014-12-570200 (2015).

8 Wang, J. C., Doedens, M. & Dick, J. E. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood 89, 3919-3924 (1997).

9 Kent, D., Dykstra, B. & Eaves, C. Isolation and assessment of long-term reconstituting hematopoietic stem cells from adult mouse bone marrow. Curr Protoc Stem Cell Biol Chapter 2, Unit 2A 4, doi:10.1002/9780470151808.sc02a04s3 (2007).

10 Kent, D. G., Dykstra, B. J., Cheyne, J., Ma, E. & Eaves, C. J. Steel factor coordinately regulates the molecular signature and biologic function of hematopoietic stem cells. Blood 112, 560-567, doi:10.1182/blood-2007-10-117820 (2008).

11 Jagannathan-Bogdan, M. & Zon, L. I. Hematopoiesis. Development (Cambridge, England) 140, 2463-2467, doi:10.1242/dev.083147 (2013).

12 Tavian, M., Biasch, K., Sinka, L., Vallet, J. & Peault, B. Embryonic origin of human hematopoiesis. The International journal of developmental biology 54, 1061-1065, doi:10.1387/ijdb.103097mt (2010).

13 Dzierzak, E. & Speck, N. A. Of lineage and legacy: the development of mammalian hematopoietic stem cells. Nat Immunol 9, 129-136, doi:10.1038/ni1560 (2008).

14 Inlay, M. A. et al. Identification of multipotent progenitors that emerge prior to hematopoietic stem cells in embryonic development. Stem cell reports 2, 457-472, doi:10.1016/j.stemcr.2014.02.001 (2014).

15 Baron, M. H., Isern, J. & Fraser, S. T. The embryonic origins of erythropoiesis in mammals. Blood 119, 4828-4837, doi:10.1182/blood-2012-01-153486 (2012).

16 Hirschi, K. K. Hemogenic endothelium during development and beyond. Blood 119, 4823-4827, doi:10.1182/blood-2011-12-353466 (2012).

17 Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood cells 4, 7-25 (1978).

18 Morrison, S. J. & Scadden, D. T. The bone marrow niche for haematopoietic stem cells. Nature 505, 327-334, doi:10.1038/nature12984 (2014).

19 Calvi, L. M. et al. Activated parathyroid hormone/parathyroid hormone-related protein receptor in osteoblastic cells differentially affects cortical and trabecular bone. The Journal of clinical investigation 107, 277-286, doi:10.1172/jci11296 (2001).

20 Calvi, L. M. et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425, 841-846, doi:10.1038/nature02040 (2003).

21 Anthony, B. A. & Link, D. C. Regulation of hematopoietic stem cells by bone marrow stromal cells. Trends Immunol 35, 32-37, doi:10.1016/j.it.2013.10.002 (2014).

22 Zhao, M. & Li, L. Dissecting the bone marrow HSC niches. Cell research 26, 975-976, doi:10.1038/cr.2016.71 (2016).

23 Lambertsen, R. H. & Weiss, L. A model of intramedullary hematopoietic microenvironments based on stereologic study of the distribution of endocloned marrow colonies. Blood 63, 287-297 (1984).

24 Lord, B. I., Testa, N. G. & Hendry, J. H. The relative spatial distributions of CFUs and CFUc in the normal mouse femur. Blood 46, 65-72 (1975).

25 Arai, F. et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118, 149-161, doi:10.1016/j.cell.2004.07.004 (2004).

26 Lo Celso, C. et al. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature 457, 92-96, doi:10.1038/nature07434 (2009).

27 Stier, S. et al. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. The Journal of experimental medicine 201, 1781-1791, doi:10.1084/jem.20041992 (2005).

28 Zhang, J. et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 425, 836-841, doi:10.1038/nature02041 (2003).

29 Nilsson, S. K. et al. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood 106, 1232-1239, doi:10.1182/blood-2004-11-4422 (2005).

30 Greenbaum, A. et al. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. Nature 495, 227-230, doi:10.1038/nature11926 (2013).

31 Ding, L., Saunders, T. L., Enikolopov, G. & Morrison, S. J. Endothelial and perivascular cells maintain haematopoietic stem cells. Nature 481, 457-462, doi:10.1038/nature10783 (2012).

32 Kunisaki, Y. et al. Arteriolar niches maintain haematopoietic stem cell quiescence. Nature 502, 637-643, doi:10.1038/nature12612 (2013).

33 Xu, C. et al. Stem cell factor is selectively secreted by arterial endothelial cells in bone marrow. Nature Communications 9, 2449, doi:10.1038/s41467-018-04726-3 (2018).

34 Omatsu, Y. et al. The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. Immunity 33, 387-399, doi:10.1016/j.immuni.2010.08.017 (2010).

35 Ding, L. & Morrison, S. J. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. Nature 495, 231-235, doi:10.1038/nature11885 (2013).

36 Mendez-Ferrer, S. et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466, 829-834, doi:10.1038/nature09262 (2010).

37 Asada, N. et al. Differential cytokine contributions of perivascular haematopoietic stem cell niches. Nature cell biology 19, 214-223, doi:10.1038/ncb3475 (2017).

38 Taichman, R. S., Reilly, M. J. & Emerson, S. G. The Hematopoietic Microenvironment: Osteoblasts and The Hematopoietic Microenvironment. Hematology 4, 421-426 (2000).

39 Ishimi, Y. et al. IL-6 is produced by osteoblasts and induces bone resorption. Journal of immunology (Baltimore, Md. : 1950) 145, 3297-3303 (1990).

40 Sipkins, D. A. et al. In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature 435, 969-973, doi:10.1038/nature03703 (2005).

41 Kiel, M. J. et al. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121, 1109-1121, doi:10.1016/j.cell.2005.05.026 (2005).

42 Boulais, P. E. & Frenette, P. S. Making sense of hematopoietic stem cell niches. Blood 125, 2621-2629, doi:10.1182/blood-2014-09-570192 (2015).

43 Wilson, A. & Trumpp, A. Bone-marrow haematopoietic-stem-cell niches. Nature Reviews Immunology 6, 93, doi:10.1038/nri1779 (2006).

44 Haug, J. S. et al. N-cadherin expression level distinguishes reserved versus primed states of hematopoietic stem cells. Cell stem cell 2, 367-379, doi:10.1016/j.stem.2008.01.017 (2008).

45 Ehninger, A. & Trumpp, A. The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. The Journal of experimental medicine 208, 421-428, doi:10.1084/jem.20110132 (2011).

46 Itkin, T. et al. Distinct bone marrow blood vessels differentially regulate haematopoiesis. Nature 532, 323-328, doi:10.1038/nature17624 (2016).

47 Acar, M. et al. Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. Nature 526, 126-130, doi:10.1038/nature15250 (2015).

48 Chen, J. Y. et al. Hoxb5 marks long-term haematopoietic stem cells and reveals a homogenous perivascular niche. Nature 530, 223-227, doi:10.1038/nature16943 (2016).

49 Gazit, R. et al. Fgd5 identifies hematopoietic stem cells in the murine bone marrow. The Journal of experimental medicine 211, 1315-1331, doi:10.1084/jem.20130428 (2014).

50 Thomas, E. D., Lochte, H. L., Lu, W. C. & Ferrebee, J. W. Intravenous Infusion of Bone Marrow in Patients Receiving Radiation and Chemotherapy. New England Journal of Medicine 257, 491-496, doi:doi:10.1056/NEJM195709122571102 (1957).

51 Thomas, E. D. Bone marrow transplantation from the personal viewpoint. Int J Hematol 81, 89-93 (2005).

52 Till, J. E. & Mc, C. E. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14, 213-222 (1961).

53 Becker, A. J., McCulloch, E. A., Siminovitch, L. & Till, J. E. The Effect of Differing Demands for Blood Cell Production on DNA Synthesis by Hemopoietic Colony-Forming Cells of Mice. Blood 26, 296-308 (1965).

54 Till, J. E., McCulloch, E. A. & Siminovitch, L. A STOCHASTIC MODEL OF STEM CELL PROLIFERATION, BASED ON THE GROWTH OF SPLEEN COLONY-FORMING CELLS. Proc Natl Acad Sci U S A 51, 29-36 (1964).

55 Pluznik, D. H. & Sachs, L. The cloning of normal "mast" cells in tissue culture. Journal of cellular physiology 66, 319-324 (1965).

56 Issaad, C., Croisille, L., Katz, A., Vainchenker, W. & Coulombel, L. A murine stromal cell line allows the proliferation of very primitive human CD34++/CD38- progenitor cells in long-term cultures and semisolid assays. Blood 81, 2916-2924 (1993).

57 Dexter, T. M., Allen, T. D. & Lajtha, L. G. Conditions controlling the proliferation of haemopoietic stem cells in vitro. Journal of cellular physiology 91, 335-344, doi:10.1002/jcp.1040910303 (1977).

58 Liu, M., Miller, C. L. & Eaves, C. J. Human long-term culture initiating cell assay. Methods in molecular biology (Clifton, N.J.) 946, 241-256, doi:10.1007/978-1-62703-128-8_15 (2013).

59 Sutherland, H. J., Eaves, C. J., Lansdorp, P. M., Thacker, J. D. & Hogge, D. E. Differential regulation of primitive human hematopoietic cells in long-term cultures maintained on genetically engineered murine stromal cells. Blood 78, 666-672 (1991).

60 Whitlock, C. A. & Witte, O. N. Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc Natl Acad Sci U S A 79, 3608-3612 (1982).

61 Lemischka, I. R., Raulet, D. H. & Mulligan, R. C. Developmental potential and dynamic behavior of hematopoietic stem cells. Cell 45, 917-927 (1986).

62 Copley, M. R., Beer, P. A. & Eaves, C. J. Hematopoietic stem cell heterogeneity takes center stage. Cell stem cell 10, 690-697, doi:10.1016/j.stem.2012.05.006 (2012).

63 Ema, H., Morita, Y. & Suda, T. Heterogeneity and hierarchy of hematopoietic stem cells. Exp Hematol 42, 74-82, doi:DOI 10.1016/j.exphem.2013.11.004 (2014).

64 Dykstra, B. et al. Long-Term Propagation of Distinct Hematopoietic Differentiation Programs In Vivo. Cell stem cell 1, 218-229, doi:10.1016/j.stem.2007.05.015 (2007).

65 Morita, Y., Ema, H. & Nakauchi, H. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. The Journal of experimental medicine 207, 1173-1182, doi:10.1084/jem.20091318 (2010).

66 Morrison, S. J., Wright, D. E., Cheshier, S. H. & Weissman, I. L. Hematopoietic stem cells: challenges to expectations. Curr Opin Immunol 9, 216-221 (1997).

67 Wagers, A. J. & Weissman, I. L. Differential expression of alpha2 integrin separates long-term and short-term reconstituting Lin-/loThy1.1(lo)c-kit+ Sca-1+ hematopoietic stem cells. Stem Cells 24, 1087-1094, doi:10.1634/stemcells.2005-0396 (2006).

68 Wilson, N. K. et al. Combined Single-Cell Functional and Gene Expression Analysis Resolves Heterogeneity within Stem Cell Populations. Cell stem cell 16, 712-724, doi:10.1016/j.stem.2015.04.004 (2015).

69 Challen, G. A., Boles, N. C., Chambers, S. M. & Goodell, M. A. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. Cell stem cell 6, 265-278, doi:10.1016/j.stem.2010.02.002 (2010).

70 Nestorowa, S. et al. A single-cell resolution map of mouse hematopoietic stem and progenitor cell differentiation. Blood 128, e20-e31, doi:10.1182/blood-2016-05-716480 (2016).

71 Weksberg, D. C., Chambers, S. M., Boles, N. C. & Goodell, M. A. CD150- side population cells represent a functionally distinct population of long-term hematopoietic stem cells. Blood 111, 2444-2451, doi:10.1182/blood-2007-09-115006 (2008).

72 Kiel, M. J., Yilmaz, O. H. & Morrison, S. J. CD150- cells are transiently reconstituting multipotent progenitors with little or no stem cell activity. Blood 111, 4413-4414; author reply 4414-4415, doi:10.1182/blood-2007-12-129601 (2008).

73 Sutherland, H. J., Eaves, C. J., Eaves, A. C., Dragowska, W. & Lansdorp, P. M. Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood 74, 1563-1570 (1989).

74 Bhatia, M., Bonnet, D., Murdoch, B., Gan, O. I. & Dick, J. E. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. Nature medicine 4, 1038-1045, doi:10.1038/2023 (1998).

75 Anjos-Afonso, F. et al. CD34(-) cells at the apex of the human hematopoietic stem cell hierarchy have distinctive cellular and molecular signatures. Cell stem cell 13, 161-174, doi:10.1016/j.stem.2013.05.025 (2013).

76 Baum, C. M., Weissman, I. L., Tsukamoto, A. S., Buckle, A. M. & Peault, B. Isolation of a candidate human hematopoietic stem-cell population. Proc Natl Acad Sci U S A 89, 2804-2808 (1992).

77 Craig, W., Kay, R., Cutler, R. L. & Lansdorp, P. M. Expression of Thy-1 on human hematopoietic progenitor cells. The Journal of experimental medicine 177, 1331-1342 (1993).

78 Majeti, R., Park, C. Y. & Weissman, I. L. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell stem cell 1, 635-645, doi:10.1016/j.stem.2007.10.001 (2007).

79 Hao, Q. L., Shah, A. J., Thiemann, F. T., Smogorzewska, E. M. & Crooks, G. M. A functional comparison of CD34 + CD38- cells in cord blood and bone marrow. Blood 86, 3745-3753 (1995).

80 Bhatia, M., Wang, J. C., Kapp, U., Bonnet, D. & Dick, J. E. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci U S A 94, 5320-5325 (1997).

81 Notta, F. et al. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science 333, 218-221, doi:10.1126/science.1201219 (2011).

82 Attar, E. C. & Scadden, D. T. Regulation of hematopoietic stem cell growth. Leukemia 18, 1760-1768, doi:10.1038/sj.leu.2403515 (2004).

83 Rossi, L. et al. Less is more: unveiling the functional core of hematopoietic stem cells through knockout mice. Cell stem cell 11, 302-317, doi:10.1016/j.stem.2012.08.006 (2012).

84 Sherr, C. J., Beach, D. & Shapiro, G. I. Targeting CDK4 and CDK6: From Discovery to Therapy. Cancer discovery 6, 353-367, doi:10.1158/2159-8290.cd-15-0894 (2016).

85 Cheung, T. H. & Rando, T. A. Molecular regulation of stem cell quiescence. Nature reviews. Molecular cell biology 14, 329-340, doi:10.1038/nrm3591 (2013).

86 Dyson, N. The regulation of E2F by pRB-family proteins. Genes & development 12, 2245-2262 (1998).

87 Laurenti, E. et al. CDK6 levels regulate quiescence exit in human hematopoietic stem cells. Cell stem cell 16, 302-313, doi:10.1016/j.stem.2015.01.017 (2015).

88 Cheng, T. et al. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. Science 287, 1804-1808 (2000).

89 Rossi, D. J. et al. Cell intrinsic alterations underlie hematopoietic stem cell aging. Proc Natl Acad Sci U S A 102, 9194-9199, doi:10.1073/pnas.0503280102 (2005).

90 Suda, T., Takubo, K. & Semenza, G. L. Metabolic regulation of hematopoietic stem cells in the hypoxic niche. Cell stem cell 9, 298-310, doi:10.1016/j.stem.2011.09.010 (2011).

91 Reya, T., Morrison, S. J., Clarke, M. F. & Weissman, I. L. Stem cells, cancer, and cancer stem cells. Nature 414, 105-111, doi:10.1038/35102167 (2001).

92 Chen, E. et al. Distinct clinical phenotypes associated with JAK2V617F reflect differential STAT1 signaling. Cancer cell 18, 524-535, doi:10.1016/j.ccr.2010.10.013 (2010).

93 Campbell, P. J. & Green, A. R. The myeloproliferative disorders. The New England journal of medicine 355, 2452-2466, doi:10.1056/NEJMra063728 (2006).

94 van Galen, P. et al. The unfolded protein response governs integrity of the haematopoietic stem-cell pool during stress. Nature 510, 268-272, doi:10.1038/nature13228 (2014).

95 Wilson, A. et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. Cell 135, 1118-1129, doi:10.1016/j.cell.2008.10.048 (2008).

96 Foudi, A. et al. Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. Nature biotechnology 27, 84-90, doi:10.1038/nbt.1517 (2009).

97 Oguro, H., Ding, L. & Morrison, S. J. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. Cell stem cell 13, 102-116, doi:10.1016/j.stem.2013.05.014 (2013).

98 King, K. Y. & Goodell, M. A. Inflammatory modulation of HSCs: viewing the HSC as a foundation for the immune response. Nature reviews. Immunology 11, 685-692, doi:10.1038/nri3062 (2011).

99 Liu, Y. et al. p53 regulates hematopoietic stem cell quiescence. Cell stem cell 4, 37-48, doi:10.1016/j.stem.2008.11.006 (2009).

100 Matsumoto, A. et al. p57 is required for quiescence and maintenance of adult hematopoietic stem cells. Cell stem cell 9, 262-271, doi:10.1016/j.stem.2011.06.014 (2011).

101 Zou, P. et al. p57(Kip2) and p27(Kip1) cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. Cell stem cell 9, 247-261, doi:10.1016/j.stem.2011.07.003 (2011).

102 Yuan, Y., Shen, H., Franklin, D. S., Scadden, D. T. & Cheng, T. In vivo self-renewing divisions of haematopoietic stem cells are increased in the absence of the early G1-phase inhibitor, p18INK4C. Nature cell biology 6, 436-442, doi:10.1038/ncb1126 (2004).

103 Viatour, P. et al. Hematopoietic stem cell quiescence is maintained by compound contributions of the retinoblastoma gene family. Cell stem cell 3, 416-428, doi:10.1016/j.stem.2008.07.009 (2008).

104 Kozar, K. et al. Mouse development and cell proliferation in the absence of D-cyclins. Cell 118, 477-491, doi:10.1016/j.cell.2004.07.025 (2004).

105 Bonizzi, G., Cicalese, A., Insinga, A. & Pelicci, P. G. The emerging role of p53 in stem cells. Trends in molecular medicine 18, 6-12, doi:10.1016/j.molmed.2011.08.002 (2012).

106 Forsberg, E. C. et al. Differential expression of novel potential regulators in hematopoietic stem cells. PLoS Genet 1, e28, doi:10.1371/journal.pgen.0010028 (2005).

107 Garaycoechea, J. I. et al. Alcohol and endogenous aldehydes damage chromosomes and mutate stem cells. Nature 553, 171-177, doi:10.1038/nature25154 (2018).

108 Lacorazza, H. D. et al. The transcription factor MEF/ELF4 regulates the quiescence of primitive hematopoietic cells. Cancer cell 9, 175-187, doi:10.1016/j.ccr.2006.02.017 (2006).

109 Park, I. K. et al. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 423, 302-305, doi:10.1038/nature01587 (2003).

110 Kalaszczynska, I. et al. Cyclin A is redundant in fibroblasts but essential in hematopoietic and embryonic stem cells. Cell 138, 352-365, doi:10.1016/j.cell.2009.04.062 (2009).

111 Yamazaki, S. et al. TGF-beta as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. Blood 113, 1250-1256, doi:10.1182/blood-2008-04-146480 (2009).

112 Yilmaz, O. H. et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature 441, 475-482, doi:10.1038/nature04703 (2006).

113 Zhang, J. et al. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature 441, 518-522, doi:10.1038/nature04747 (2006).

114 Juntilla, M. M. et al. AKT1 and AKT2 maintain hematopoietic stem cell function by regulating reactive oxygen species. Blood 115, 4030-4038, doi:10.1182/blood-2009-09-241000 (2010).

115 Miyamoto, K. et al. Foxo3a is essential for maintenance of the hematopoietic stem cell pool. Cell stem cell 1, 101-112, doi:10.1016/j.stem.2007.02.001 (2007).

116 Tothova, Z. et al. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell 128, 325-339, doi:10.1016/j.cell.2007.01.003 (2007).

117 Singbrant, S. et al. Smad5 is dispensable for adult murine hematopoiesis. Blood 108, 3707-3712, doi:10.1182/blood-2006-02-003384 (2006).

118 Wang, X. et al. TGF-beta1 Negatively Regulates the Number and Function of Hematopoietic Stem Cells. Stem cell reports 11, 274-287, doi:10.1016/j.stemcr.2018.05.017 (2018).

119 Yamazaki, S. et al. Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. Cell 147, 1146-1158, doi:10.1016/j.cell.2011.09.053 (2011).

120 Karlsson, G. et al. Smad4 is critical for self-renewal of hematopoietic stem cells. The Journal of experimental medicine 204, 467-474, doi:10.1084/jem.20060465 (2007).

121 Yamazaki, S. et al. Cytokine signals modulated via lipid rafts mimic niche signals and induce hibernation in hematopoietic stem cells. EMBO J. 25, 3515-3523, doi:10.1038/sj.emboj.7601236 (2006).

122 Lev, S., Blechman, J. M., Givol, D. & Yarden, Y. Steel factor and c-kit protooncogene: genetic lessons in signal transduction. Crit Rev Oncog 5, 141-168 (1994).

123 Boitano, A. E. et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. Science 329, 1345-1348, doi:10.1126/science.1191536 (2010).

124 Audet, J., Miller, C. L., Eaves, C. J. & Piret, J. M. Common and distinct features of cytokine effects on hematopoietic stem and progenitor cells revealed by dose-response surface analysis. Biotechnol. Bioeng. 80, 393-404, doi:10.1002/bit.10399 (2002).

125 Peters, S. O., Kittler, E. L., Ramshaw, H. S. & Quesenberry, P. J. Ex vivo expansion of murine marrow cells with interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor leads to impaired engraftment in irradiated hosts. Blood 87, 30-37 (1996).

126 Molineux, G., Migdalska, A., Szmitkowski, M., Zsebo, K. & Dexter, T. M. The effects on hematopoiesis of recombinant stem cell factor (ligand for c-kit) administered in vivo to mice either alone or in combination with granulocyte colony-stimulating factor. Blood 78, 961-966 (1991).

127 Migliaccio, G. et al. Stem cell factor induces proliferation and differentiation of highly enriched murine hematopoietic cells. Proc. Natl. Acad. Sci. U.S.A. 88, 7420-7424 (1991).

128 Metcalf, D. & Nicola, N. A. Direct proliferative actions of stem cell factor on murine bone marrow cells in vitro: effects of combination with colony-stimulating factors. Proc. Natl. Acad. Sci. U.S.A. 88, 6239-6243 (1991).

129 Holyoake, T. L. et al. Ex vivo expansion with stem cell factor and interleukin-11 augments both short-term recovery posttransplant and the ability to serially transplant marrow. Blood 87, 4589-4595 (1996).

130 Knapp, D. J. H. F. et al. Dissociation of Survival, Proliferation, and State Control in Human Hematopoietic Stem Cells. Stem cell reports 8, 152-162, doi:10.1016/j.stemcr.2016.12.003 (2017).

131 Schuettpelz, L. G. & Link, D. C. Regulation of hematopoietic stem cell activity by inflammation. Frontiers in immunology 4, 204, doi:10.3389/fimmu.2013.00204 (2013).

132 Essers, M. A. et al. IFNalpha activates dormant haematopoietic stem cells in vivo. Nature 458, 904-908, doi:10.1038/nature07815 (2009).

133 Baldridge, M. T., King, K. Y., Boles, N. C., Weksberg, D. C. & Goodell, M. A. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. Nature 465, 793-797, doi:10.1038/nature09135 (2010).

134 Dybedal, I., Bryder, D., Fossum, A., Rusten, L. S. & Jacobsen, S. E. Tumor necrosis factor (TNF)-mediated activation of the p55 TNF receptor negatively regulates maintenance of cycling reconstituting human hematopoietic stem cells. Blood 98, 1782-1791 (2001).

135 Pronk, C. J., Veiby, O. P., Bryder, D. & Jacobsen, S. E. Tumor necrosis factor restricts hematopoietic stem cell activity in mice: involvement of two distinct receptors. The Journal of experimental medicine 208, 1563-1570, doi:10.1084/jem.20110752 (2011).

136 Zhao, Y., Ling, F., Wang, H. C. & Sun, X. H. Chronic TLR signaling impairs the long-term repopulating potential of hematopoietic stem cells of wild type but not Id1 deficient mice. PloS one 8, e55552, doi:10.1371/journal.pone.0055552 (2013).

137 Lechman, E. R. et al. Attenuation of miR-126 activity expands HSC in vivo without exhaustion. Cell stem cell 11, 799-811, doi:10.1016/j.stem.2012.09.001 (2012).

138 Guo, S. et al. MicroRNA miR-125a controls hematopoietic stem cell number. Proc Natl Acad Sci U S A 107, 14229-14234, doi:10.1073/pnas.0913574107 (2010).

139 Wojtowicz, E. E. et al. Ectopic miR-125a Expression Induces Long-Term Repopulating Stem Cell Capacity in Mouse and Human Hematopoietic Progenitors. Cell stem cell 19, 383-396, doi:10.1016/j.stem.2016.06.008 (2016).

140 Yu, F. et al. let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 131, 1109-1123, doi:10.1016/j.cell.2007.10.054 (2007).

141 Copley, M. R. et al. The Lin28b–let-7–Hmga2 axis determines the higher self-renewal potential of fetal haematopoietic stem cells. Nature cell biology 15, 916, doi:10.1038/ncb2783 (2013).

142 Muller-Sieburg, C. E., Cho, R. H., Thoman, M., Adkins, B. & Sieburg, H. B. Deterministic regulation of hematopoietic stem cell self-renewal and differentiation. Blood 100, 1302-1309 (2002).

143 Weissman, I. L. Stem cells: units of development, units of regeneration, and units in evolution. Cell 100, 157-168 (2000).

144 Huang, X., Cho, S. & Spangrude, G. J. Hematopoietic stem cells: generation and self-renewal. Cell Death Differ 14, 1851-1859, doi:10.1038/sj.cdd.4402225 (2007).

145 Christ, O. et al. Improved purification of hematopoietic stem cells based on their elevated aldehyde dehydrogenase activity. Haematologica 92, 1165-1172 (2007).

146 Rodriguez-Fraticelli, A. E. et al. Clonal analysis of lineage fate in native haematopoiesis. Nature 553, 212, doi:10.1038/nature25168 (2018).

147 Cheshier, S. H., Morrison, S. J., Liao, X. & Weissman, I. L. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. Proc Natl Acad Sci U S A 96, 3120-3125 (1999).

148 Kiel, M. J. et al. Haematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU. Nature 449, 238-242, doi:10.1038/nature06115 (2007).

149 Takizawa, H., Regoes, R. R., Boddupalli, C. S., Bonhoeffer, S. & Manz, M. G. Dynamic variation in cycling of hematopoietic stem cells in steady state and inflammation. The Journal of experimental medicine 208, 273-284, doi:10.1084/jem.20101643 (2011).

150 Muller-Sieburg, C. E., Cho, R. H., Karlsson, L., Huang, J. F. & Sieburg, H. B. Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. Blood 103, 4111-4118, doi:10.1182/blood-2003-10-3448 (2004).

151 Benz, C. et al. Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. Cell stem cell 10, 273-283, doi:10.1016/j.stem.2012.02.007 (2012).

152 Benveniste, P. et al. Intermediate-term hematopoietic stem cells with extended but time-limited reconstitution potential. Cell stem cell 6, 48-58, doi:10.1016/j.stem.2009.11.014 (2010).

153 Cho, R. H., Sieburg, H. B. & Muller-Sieburg, C. E. A new mechanism for the aging of hematopoietic stem cells: aging changes the clonal composition of the stem cell compartment but not individual stem cells. Blood 111, 5553-5561, doi:DOI 10.1182/blood-2007-11-123547 (2008).

154 Gekas, C. & Graf, T. CD41 expression marks myeloid-biased adult hematopoietic stem cells and increases with age. Blood 121, 4463-4472, doi:10.1182/blood-2012-09-457929 (2013).

155 Dykstra, B., Olthof, S., Schreuder, J., Ritsema, M. & de Haan, G. Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. The Journal of experimental medicine 208, 2691-2703, doi:10.1084/jem.20111490 (2011).

156 Sudo, K., Ema, H., Morita, Y. & Nakauchi, H. Age-associated characteristics of murine hematopoietic stem cells. The Journal of experimental medicine 192, 1273-1280 (2000).

157 Yamamoto, R. et al. Large-Scale Clonal Analysis Resolves Aging of the Mouse Hematopoietic Stem Cell Compartment. Cell stem cell 22, 600-607 e604, doi:10.1016/j.stem.2018.03.013 (2018).

158 Bernitz, J. M., Kim, H. S., MacArthur, B., Sieburg, H. & Moore, K. Hematopoietic Stem Cells Count and Remember Self-Renewal Divisions. Cell 167, 1296-1309.e1210, doi:10.1016/j.cell.2016.10.022 (2016).

159 Moignard, V. & Gottgens, B. Transcriptional mechanisms of cell fate decisions revealed by single cell expression profiling. Bioessays 36, 419-426, doi:10.1002/bies.201300102 (2014).

160 Ramos, C. A. et al. Evidence for diversity in transcriptional profiles of single hematopoietic stem cells. Plos Genetics 2, 1487-1499, doi:ARTN e15910.1371/journal.pgen.0020159 (2006).

161 Pina, C. et al. Inferring rules of lineage commitment in haematopoiesis. Nature cell biology 14, 287-+, doi:10.1038/ncb2442 (2012).

162 Moignard, V. et al. Characterization of transcriptional networks in blood stem and progenitor cells using high-throughput single-cell gene expression analysis (vol 15, pg 363, 2013). Nature cell biology 15, 544-544 (2013).

163 Glotzbach, J. P. et al. An Information Theoretic, Microfluidic-Based Single Cell Analysis Permits Identification of Subpopulations among Putatively Homogeneous Stem Cells. PloS one 6, doi:ARTN e2121110.1371/journal.pone.0021211 (2011).

164 Wohrer, S. et al. Distinct Stromal Cell Factor Combinations Can Separately Control Hematopoietic Stem Cell Survival, Proliferation, and Self-Renewal. Cell Rep 7, 1956-1967, doi:10.1016/j.celrep.2014.05.014 (2014).

165 Kondo, M., Weissman, I. L. & Akashi, K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91, 661-672 (1997).

166 Akashi, K., Traver, D., Miyamoto, T. & Weissman, I. L. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404, 193-197, doi:10.1038/35004599 (2000).

167 Adolfsson, J. et al. Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell 121, 295-306, doi:10.1016/j.cell.2005.02.013 (2005).

168 Arinobu, Y. et al. Reciprocal activation of GATA-1 and PU.1 marks initial specification of hematopoietic stem cells into myeloerythroid and myelolymphoid lineages. Cell stem cell 1, 416-427, doi:10.1016/j.stem.2007.07.004 (2007).

169 Mildner, A. & Jung, S. Development and function of dendritic cell subsets. Immunity 40, 642-656, doi:10.1016/j.immuni.2014.04.016 (2014).

170 Naik, S. H. et al. Diverse and heritable lineage imprinting of early haematopoietic progenitors. Nature 496, 229-232, doi:10.1038/nature12013 (2013).

171 Drissen, R. et al. Distinct myeloid progenitor-differentiation pathways identified through single-cell RNA sequencing. Nat Immunol 17, 666-676, doi:10.1038/ni.3412 (2016).

172 Hoppe, P. S. et al. Early myeloid lineage choice is not initiated by random PU.1 to GATA1 protein ratios. Nature 535, 299-302, doi:10.1038/nature18320 (2016).

173 Paul, F. et al. Transcriptional Heterogeneity and Lineage Commitment in Myeloid Progenitors. Cell 163, 1663-1677, doi:10.1016/j.cell.2015.11.013 (2015).

174 Guo, G. et al. Mapping cellular hierarchy by single-cell analysis of the cell surface repertoire. Cell stem cell 13, 492-505, doi:10.1016/j.stem.2013.07.017 (2013).

175 Karsunky, H., Inlay, M. A., Serwold, T., Bhattacharya, D. & Weissman, I. L. Flk2+ common lymphoid progenitors possess equivalent differentiation potential for the B and T lineages. Blood 111, 5562-5570, doi:10.1182/blood-2007-11-126219 (2008).

176 Yamamoto, R. et al. Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. Cell 154, 1112-1126, doi:10.1016/j.cell.2013.08.007 (2013).

177 Grover, A. et al. Single-cell RNA sequencing reveals molecular and functional platelet bias of aged haematopoietic stem cells. Nat Commun 7, 11075, doi:10.1038/ncomms11075 (2016).

178 Roch, A., Trachsel, V. & Lutolf, M. P. Brief Report: Single-Cell Analysis Reveals Cell Division-Independent Emergence of Megakaryocytes From Phenotypic Hematopoietic Stem Cells. STEM CELLS 33, 3152-3157, doi:doi:10.1002/stem.2106 (2015).

179 Carrelha, J. et al. Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. Nature 554, 106-111, doi:10.1038/nature25455 (2018).

180 Sanjuan-Pla, A. et al. Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. Nature 502, 232-236, doi:10.1038/nature12495 (2013).

181 Notta, F. et al. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. Science 351, aab2116, doi:10.1126/science.aab2116 (2016).

182 Velten, L. et al. Human haematopoietic stem cell lineage commitment is a continuous process. Nature cell biology 19, 271-281, doi:10.1038/ncb3493 (2017).

183 Laurenti, E. & Gottgens, B. From haematopoietic stem cells to complex differentiation landscapes. Nature 553, 418-426, doi:10.1038/nature25022 (2018).

184 Karamitros, D. et al. Single-cell analysis reveals the continuum of human lympho-myeloid progenitor cells. Nat Immunol 19, 85-97, doi:10.1038/s41590-017-0001-2 (2018).

185 Hamey, F. K., Nestorowa, S., Wilson, N. K. & Gottgens, B. Advancing haematopoietic stem and progenitor cell biology through single-cell profiling. FEBS letters 590, 4052-4067, doi:10.1002/1873-3468.12231 (2016).

186 Dahlin, J. S. et al. A single-cell hematopoietic landscape resolves 8 lineage trajectories and defects in Kit mutant mice. Blood 131, e1-e11, doi:10.1182/blood-2017-12-821413 (2018).

187 Hamey, F. K. et al. Reconstructing blood stem cell regulatory network models from single-cell molecular profiles. Proc Natl Acad Sci U S A 114, 5822-5829, doi:10.1073/pnas.1610609114 (2017).

188 Williams, D. E. et al. Identification of a ligand for the c-kit proto-oncogene. Cell 63, 167-174 (1990).

189 Huang, E. et al. The hematopoietic growth factor KL is encoded by the SI locus and is the ligand of the c-kit receptor, the gene product of the W locus. Cell 63, 225-233, doi:10.1016/0092-8674(90)90303-V (1990).

190 Witte, O. N. Steel locus defines new multipotent growth factor. Cell 63, 5-6, doi:10.1016/0092-8674(90)90280-R (1990).

191 Lennartsson, J. & Rönnstrand, L. Stem cell factor receptor/c-Kit: from basic science to clinical implications. Physiol. Rev. 92, 1619-1649, doi:10.1152/physrev.00046.2011 (2012).

192 Wilkinson, A. C. & Yamazaki, S. The hematopoietic stem cell diet. Int J Hematol 107, 634-641, doi:10.1007/s12185-018-2451-1 (2018).

193 Copeland, N. G. et al. Mast cell growth factor maps near the steel locus on mouse chromosome 10 and is deleted in a number of steel alleles. Cell 63, 175-183 (1990).

194 Zsebo, K. M. et al. Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. Cell 63, 213-224 (1990).

195 Bennett, D. Developmental analysis of a mutation with pleiotropic effects in the mouse. J. Morphol. 98, 199-233, doi:10.1002/jmor.1050980202 (1956).

196 Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P. & Bernstein, A. The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. Nature 335, 88-89, doi:10.1038/335088a0 (1988).

197 Geissler, E. N., Ryan, M. A. & Housman, D. E. The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. Cell 55, 185-192 (1988).

198 Geissler, E. N., McFarland, E. C. & Russell, E. S. Analysis of pleiotropism at the dominant white-spotting (W) locus of the house mouse: a description of ten new W alleles. Genetics 97, 337-361 (1981).

199 Kitamura, Y., Go, S. & Hatanaka, K. Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation. Blood 52, 447-452 (1978).

200 Yang, S. et al. Persistent distention of colon damages interstitial cells of Cajal through Ca2+-ERK-AP-1-miR-34c-SCF deregulation. J Cell Mol Med 21, 1881-1892, doi:10.1111/jcmm.13108 (2017).

201 Linnekin, D. Early signaling pathways activated by c-Kit in hematopoietic cells. Int. J. Biochem. Cell Biol. 31, 1053-1074 (1999).

202 Jin, K., Mao, X. O., Sun, Y., Xie, L. & Greenberg, D. A. Stem cell factor stimulates neurogenesis in vitro and in vivo. The Journal of clinical investigation 110, 311-319, doi:10.1172/JCI15251 (2002).

203 Arakawa, T. et al. Glycosylated and unglycosylated recombinant-derived human stem cell factors are dimeric and have extensive regular secondary structure. J. Biol. Chem. 266, 18942-18948 (1991).

204 Flanagan, J. G., Chan, D. C. & Leder, P. Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the Sld mutant. Cell 64, 1025-1035 (1991).

205 Lu, H. S. et al. Amino acid sequence and post-translational modification of stem cell factor isolated from buffalo rat liver cell-conditioned medium. J. Biol. Chem. 266, 8102-8107 (1991).

206 Lev, S., Yarden, Y. & Givol, D. Dimerization and activation of the kit receptor by monovalent and bivalent binding of the stem cell factor. J. Biol. Chem. 267, 15970-15977 (1992).

207 Zhang, Z., Zhang, R., Joachimiak, A., Schlessinger, J. & Kong, X.-P. Crystal structure of human stem cell factor: Implication for stem cell factor receptor dimerization and activation. PNAS 97, 7732-7737, doi:10.1073/pnas.97.14.7732 (2000).

208 Liu, H., Chen, X., Focia, P. J. & He, X. Structural basis for stem cell factor-KIT signaling and activation of class III receptor tyrosine kinases. EMBO J. 26, 891-901, doi:10.1038/sj.emboj.7601545 (2007).

209 Yuzawa, S. et al. Structural basis for activation of the receptor tyrosine kinase KIT by stem cell factor. Cell 130, 323-334, doi:10.1016/j.cell.2007.05.055 (2007).

210 Blechman, J. M., Lev, S., Givol, D. & Yarden, Y. Structure-function analyses of the kit receptor for the steel factor. Stem Cells (Dayton, Ohio) 11 Suppl 2, 12-21, doi:10.1002/stem.5530110804 (1993).

211 Rönnstrand, L. Signal transduction via the stem cell factor receptor/c-Kit. Cell. Mol. Life Sci. 61, 2535-2548, doi:10.1007/s00018-004-4189-6 (2004).

212 Miyazawa, K. et al. Membrane-bound Steel factor induces more persistent tyrosine kinase activation and longer life span of c-kit gene-encoded protein than its soluble form. Blood 85, 641-649 (1995).

213 Ho, C. C. M. et al. Decoupling the Functional Pleiotropy of Stem Cell Factor by Tuning c-Kit Signaling. Cell 168, 1041-1052.e1018, doi:10.1016/j.cell.2017.02.011 (2017).

214 Bradley, H. L., Hawley, T. S. & Bunting, K. D. Cell intrinsic defects in cytokine responsiveness of STAT5-deficient hematopoietic stem cells. Blood 100, 3983-3989, doi:10.1182/blood-2002-05-1602 (2002).

215 McCulloch, E. A., Siminovitch, L., Till, J. E., Russell, E. S. & Bernstein, S. E. The cellular basis of the genetically determined hemopoietic defect in anemic mice of genotype Sl-Sld. Blood 26, 399-410 (1965).

216 Russell, E. S. & Bernstein, S. E. Proof of whole-cell implant in therapy of W-series anemia. Arch. Biochem. Biophys. 125, 594-597 (1968).

217 McCulloch, E. A., Siminovitch, L. & Till, J. E. SPLEEN-COLONY FORMATION IN ANEMIC MICE OF GENOTYPE WW. Science (New York, N.Y.) 144, 844-846 (1964).

218 Thoren, L. A. et al. Kit regulates maintenance of quiescent hematopoietic stem cells. Journal of immunology (Baltimore, Md. : 1950) 180, 2045-2053 (2008).

219 Bernstein, I. D., Andrews, R. G. & Zsebo, K. M. Recombinant human stem cell factor enhances the formation of colonies by CD34+ and CD34+lin- cells, and the generation of colony-forming cell progeny from CD34+lin- cells cultured with interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor. Blood 77, 2316-2321 (1991).

220 Andrews, R. G. et al. Recombinant human stem cell factor, a c-kit ligand, stimulates hematopoiesis in primates. Blood 78, 1975-1980 (1991).

221 de Vries, P., Brasel, K. A., Eisenman, J. R., Alpert, A. R. & Williams, D. E. The effect of recombinant mast cell growth factor on purified murine hematopoietic stem cells. The Journal of experimental medicine 173, 1205-1211 (1991).

222 Tong, J. et al. In vivo administration of recombinant methionyl human stem cell factor expands the number of human marrow hematopoietic stem cells. Blood 82, 784-791 (1993).

223 Zsebo, K. M. et al. Radioprotection of mice by recombinant rat stem cell factor. Proc. Natl. Acad. Sci. U.S.A. 89, 9464-9468 (1992).

224 Gardner, R. V., Oliver, P. & Astle, C. M. Stem cell factor improves the repopulating ability of primitive hematopoietic stem cells after sublethal irradiation (and, to a lesser extent) after bone marrow transplantation in mice. Stem Cells (Dayton, Ohio) 16, 112-119, doi:10.1002/stem.160112 (1998).

225 Costa, J. J. et al. Recombinant human stem cell factor (kit ligand) promotes human mast cell and melanocyte hyperplasia and functional activation in vivo. The Journal of experimental medicine 183, 2681-2686 (1996).

226 Li, C. L. & Johnson, G. R. Stem cell factor enhances the survival but not the self-renewal of murine hematopoietic long-term repopulating cells. Blood 84, 408-414 (1994).

227 Bradley, H. L., Couldrey, C. & Bunting, K. D. Hematopoietic-repopulating defects from STAT5-deficient bone marrow are not fully accounted for by loss of thrombopoietin responsiveness. Blood 103, 2965-2972, doi:10.1182/blood-2003-08-2963 (2004).

228 Bunting, K. D., Galipeau, J., Topham, D., Benaim, E. & Sorrentino, B. P. Transduction of murine bone marrow cells with an MDR1 vector enables ex vivo stem cell expansion, but these expanded grafts cause a myeloproliferative syndrome in transplanted mice. Blood 92, 2269-2279 (1998).

229 Ogawa, M., Yonemura, Y. & Ku, H. In vitro expansion of hematopoietic stem cells. Stem Cells 15 Suppl 1, 7-11; discussion 12, doi:10.1002/stem.5530150803 (1997).

230 Bodine, D. M., Karlsson, S. & Nienhuis, A. W. Combination of interleukins 3 and 6 preserves stem cell function in culture and enhances retrovirus-mediated gene transfer into hematopoietic stem cells. Proc Natl Acad Sci U S A 86, 8897-8901 (1989).

231 Audet, J., Miller, C. L., Rose-John, S., Piret, J. M. & Eaves, C. J. Distinct role of gp130 activation in promoting self-renewal divisions by mitogenically stimulated murine hematopoietic stem cells. Proc. Natl. Acad. Sci. U.S.A. 98, 1757-1762, doi:10.1073/pnas.98.4.1757 (2001).

232 Yonemura, Y., Ku, H., Lyman, S. D. & Ogawa, M. In vitro expansion of hematopoietic progenitors and maintenance of stem cells: comparison between FLT3/FLK-2 ligand and KIT ligand. Blood 89, 1915-1921 (1997).

233 Miller, C. L. & Eaves, C. J. Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. Proc Natl Acad Sci U S A 94, 13648-13653 (1997).

234 Miller, C. L., Audet, J. & Eaves, C. J. Ex vivo expansion of human and murine hematopoietic stem cells. Methods Mol Med 63, 189-208, doi:10.1385/1-59259-140-X:189 (2002).

235 Fares, I. et al. Cord blood expansion. Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. Science 345, 1509-1512, doi:10.1126/science.1256337 (2014).

236 Walasek, M. A., van Os, R. & de Haan, G. Hematopoietic stem cell expansion: challenges and opportunities. Ann N Y Acad Sci 1266, 138-150, doi:10.1111/j.1749-6632.2012.06549.x (2012).

237 Sauvageau, G., Iscove, N. N. & Humphries, R. K. In vitro and in vivo expansion of hematopoietic stem cells. Oncogene 23, 7223-7232, doi:10.1038/sj.onc.1207942 (2004).

238 Lutolf, M. P., Doyonnas, R., Havenstrite, K., Koleckar, K. & Blau, H. M. Perturbation of single hematopoietic stem cell fates in artificial niches. Integr Biol (Camb) 1, 59-69, doi:10.1039/b815718a (2009).

239 Fares, I., Rivest-Khan, L., Cohen, S. & Sauvageau, G. Small molecule regulation of normal and leukemic stem cells. Curr Opin Hematol 22, 309-316, doi:10.1097/MOH.0000000000000151 (2015).

240 North, T. E. et al. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. Nature 447, 1007-1011, doi:10.1038/nature05883 (2007).

241 Cutler, C. et al. Prostaglandin-modulated umbilical cord blood hematopoietic stem cell transplantation. Blood 122, 3074-3081, doi:10.1182/blood-2013-05-503177 (2013).

242 Lutolf, M. P. & Blau, H. M. Artificial stem cell niches. Adv Mater 21, 3255-3268, doi:10.1002/adma.200802582 (2009).

243 Wells, R. G. The role of matrix stiffness in regulating cell behavior. Hepatology 47, 1394-1400, doi:10.1002/hep.22193 (2008).

244 Ivanovska, I. L., Shin, J. W., Swift, J. & Discher, D. E. Stem cell mechanobiology: diverse lessons from bone marrow. Trends Cell Biol 25, 523-532, doi:10.1016/j.tcb.2015.04.003 (2015).

245 Shin, J. W. et al. Contractile forces sustain and polarize hematopoiesis from stem and progenitor cells. Cell stem cell 14, 81-93, doi:10.1016/j.stem.2013.10.009 (2014).

246 Cox, T. R. & Erler, J. T. Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer. Dis Model Mech 4, 165-178, doi:10.1242/dmm.004077 (2011).

247 Holst, J. et al. Substrate elasticity provides mechanical signals for the expansion of hemopoietic stem and progenitor cells. Nature biotechnology 28, 1123-1128, doi:10.1038/nbt.1687 (2010).

248 Lee, J., Abdeen, A. A., Tang, X., Saif, T. A. & Kilian, K. A. Matrix directed adipogenesis and neurogenesis of mesenchymal stem cells derived from adipose tissue and bone marrow. Acta Biomater, doi:10.1016/j.actbio.2016.06.037 (2016).

249 Kourouklis, A. P., Kaylan, K. B. & Underhill, G. H. Substrate stiffness and matrix composition coordinately control the differentiation of liver progenitor cells. Biomaterials 99, 82-94, doi:10.1016/j.biomaterials.2016.05.016 (2016).

250 Connelly, J. T. et al. Actin and serum response factor transduce physical cues from the microenvironment to regulate epidermal stem cell fate decisions. Nature cell biology 12, 711-718, doi:10.1038/ncb2074 (2010).

251 Trappmann, B. et al. Extracellular-matrix tethering regulates stem-cell fate. Nat Mater 11, 642-649, doi:10.1038/nmat3339 (2012).

252 Kent, D. G. et al. Self-renewal of single mouse hematopoietic stem cells is reduced by JAK2V617F without compromising progenitor cell expansion. PLoS Biol 11, e1001576, doi:10.1371/journal.pbio.1001576 (2013).

253 Hu, Y. & Smyth, G. K. ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. J Immunol Methods 347, 70-78, doi:10.1016/j.jim.2009.06.008 (2009).

254 Chen, E. Y. et al. Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC bioinformatics 14, 128, doi:10.1186/1471-2105-14-128 (2013).

255 Kuleshov, M. V. et al. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic acids research 44, W90-97, doi:10.1093/nar/gkw377 (2016).

256 Bowie, M. B., Kent, D. G., Copley, M. R. & Eaves, C. J. Steel factor responsiveness regulates the high self-renewal phenotype of fetal hematopoietic stem cells. Blood 109, 5043-5048, doi:10.1182/blood-2006-08-037770 (2007).

257 Kent, D. et al. Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway. Clinical cancer research : an official journal of the American Association for Cancer Research 14, 1926-1930, doi:10.1158/1078-0432.ccr-07-5134 (2008).

258 Watts, K. L., Adair, J. & Kiem, H. P. Hematopoietic stem cell expansion and gene therapy. Cytotherapy 13, 1164-1171, doi:10.3109/14653249.2011.620748 (2011).

259 Ma, A. D. & Abrams, C. S. Pleckstrin induces cytoskeletal reorganization via a Rac-dependent pathway. J Biol Chem 274, 28730-28735 (1999).

260 Sloan, D. C., Wang, P., Bao, X. & Haslam, R. J. Translocation of pleckstrin requires its phosphorylation and newly formed ligands. Biochemical and biophysical research communications 293, 640-646, doi:10.1016/s0006-291x(02)00260-7 (2002).

261 Roll, R. L., Bauman, E. M., Bennett, J. S. & Abrams, C. S. Phosphorylated pleckstrin induces cell spreading via an integrin-dependent pathway. The Journal of cell biology 150, 1461-1466 (2000).

262 Hasegawa, S. et al. Effects of in vivo deletion of GATA2 in bone marrow stromal cells. Exp Hematol 56, 31-45.e32, doi:10.1016/j.exphem.2017.08.004 (2017).

263 Bhattacharya, R., Frankfurter, A. & Cabral, F. A minor β-tubulin essential for mammalian cell proliferation. Cell Motility 65, 708-720, doi:doi:10.1002/cm.20292 (2008).

264 Lewis, S. A., Gu, W. & Cowan, N. J. Free intermingling of mammalian beta-tubulin isotypes among functionally distinct microtubules. Cell 49, 539-548 (1987).

265 Maceckova, M. et al. Bone marrow-derived macrophages exclusively expressed caveolin-2: The role of inflammatory activators and hypoxia. Immunobiology 220, 1266-1274, doi:10.1016/ j.imbio.2015.06.018 (2015).

266 Smith, A. M. et al. Integration of Elf-4 into stem/progenitor and erythroid regulatory networks through locus-wide chromatin studies coupled with in vivo functional validation. Molecular and cellular biology 32, 763-773, doi:10.1128/mcb.05745-11 (2012).

267 Trumpp, A., Essers, M. & Wilson, A. Awakening dormant haematopoietic stem cells. Nature Reviews Immunology 10, 201, doi:10.1038/nri2726 (2010).

268 Carlini, M. J., Shrivastava, N. & Sosa, M. S. Epigenetic and Pluripotency Aspects of Disseminated Cancer Cells During Minimal Residual Disease. Advances in experimental medicine and biology 1100, 1-18, doi:10.1007/978-3-319-97746-1_1 (2018).

269 Yoshida, K. et al. Targeted disruption of gp130, a common signal transducer for the interleukin 6 family of cytokines, leads to myocardial and hematological disorders. Proc Natl Acad Sci U S A 93, 407-411 (1996).

270 Beerman, I. et al. Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. Proc Natl Acad Sci U S A 107, 5465-5470, doi:10.1073/pnas.1000834107 (2010).

271 Santaguida, M. et al. JunB protects against myeloid malignancies by limiting hematopoietic stem cell proliferation and differentiation without affecting self-renewal. Cancer cell 15, 341-352, doi:10.1016/j.ccr.2009.02.016 (2009).

272 Passegue, E., Wagner, E. F. & Weissman, I. L. JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. Cell 119, 431-443, doi:10.1016/j.cell.2004.10.010 (2004).

273 Kruse, E. A. et al. Dual requirement for the ETS transcription factors Fli-1 and Erg in hematopoietic stem cells and the megakaryocyte lineage. Proc Natl Acad Sci U S A 106, 13814-13819, doi:10.1073/pnas.0906556106 (2009).

274 Ciau-Uitz, A., Wang, L., Patient, R. & Liu, F. ETS transcription factors in hematopoietic stem cell development. Blood cells, molecules & diseases 51, 248-255, doi:10.1016/j.bcmd.2013.07.010 (2013).

275 Moroy, T., Vassen, L., Wilkes, B. & Khandanpour, C. From cytopenia to leukemia: the role of Gfi1 and Gfi1b in blood formation. Blood 126, 2561-2569, doi:10.1182/blood-2015-06-655043 (2015).

276 Reddi, A. H., Gay, R., Gay, S. & Miller, E. J. Transitions in collagen types during matrix-induced cartilage, bone, and bone marrow formation. Proc Natl Acad Sci U S A 74, 5589-5592 (1977).

277 Moeendarbary, E. et al. The soft mechanical signature of glial scars in the central nervous system. Nat Commun 8, 14787, doi:10.1038/ncomms14787 (2017).

278 Isern, J. et al. The neural crest is a source of mesenchymal stem cells with specialized hematopoietic stem cell niche function. Elife 3, e03696, doi:10.7554/eLife.03696 (2014).

279 Caliari, S. R. & Burdick, J. A. A practical guide to hydrogels for cell culture. Nature methods 13, 405-414, doi:10.1038/nmeth.3839 (2016).

280 Lynam, D. et al. Augmenting protein release from layer-by-layer functionalized agarose hydrogels. Carbohydrate polymers 103, 377-384, doi:10.1016/j.carbpol.2013.12.069 (2014).

281 Berg, J. M., Tymoczko, J. L. & Stryer, L. Biochemistry. (Palgrave Macmillan, 2012).

282 Ema, H. et al. Quantification of self-renewal capacity in single hematopoietic stem cells from normal and Lnk-deficient mice. Dev Cell 8, 907-914, doi:10.1016/j.devcel.2005.03.019 (2005).

283 Deneault, E. et al. A functional screen to identify novel effectors of hematopoietic stem cell activity. Cell 137, 369-379, doi:10.1016/j.cell.2009.03.026 (2009).

284 Pabst, C. et al. Identification of small molecules that support human leukemia stem cell activity ex vivo. Nature methods 11, 436-442, doi:10.1038/nmeth.2847 (2014).

285 Tzeng, Y.-S., Kang, Y.-L., Chen, W.-C., Cheng, W.-C. & Lai, D.-M. Loss of CXCL12/SDF-1 in adult mice decreases the quiescent state of hematopoietic stem/progenitor cells and alters the pattern of hematopoietic regeneration after myelosuppression. Blood, doi:10.1182/blood-2010-01-266833 (2010).

286 Barrett, O., Sottocornola, R. & Lo Celso, C. In vivo imaging of hematopoietic stem cells in the bone marrow niche. Methods in molecular biology (Clifton, N.J.) 916, 231-242, doi:10.1007/978-1-61779-980-8_18 (2012).

287 Smith, A. G. et al. Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 336, 688, doi:10.1038/336688a0 (1988).

288 Silva, J. et al. Promotion of reprogramming to ground state pluripotency by signal inhibition. PLoS Biol 6, e253, doi:10.1371/journal.pbio.0060253 (2008).

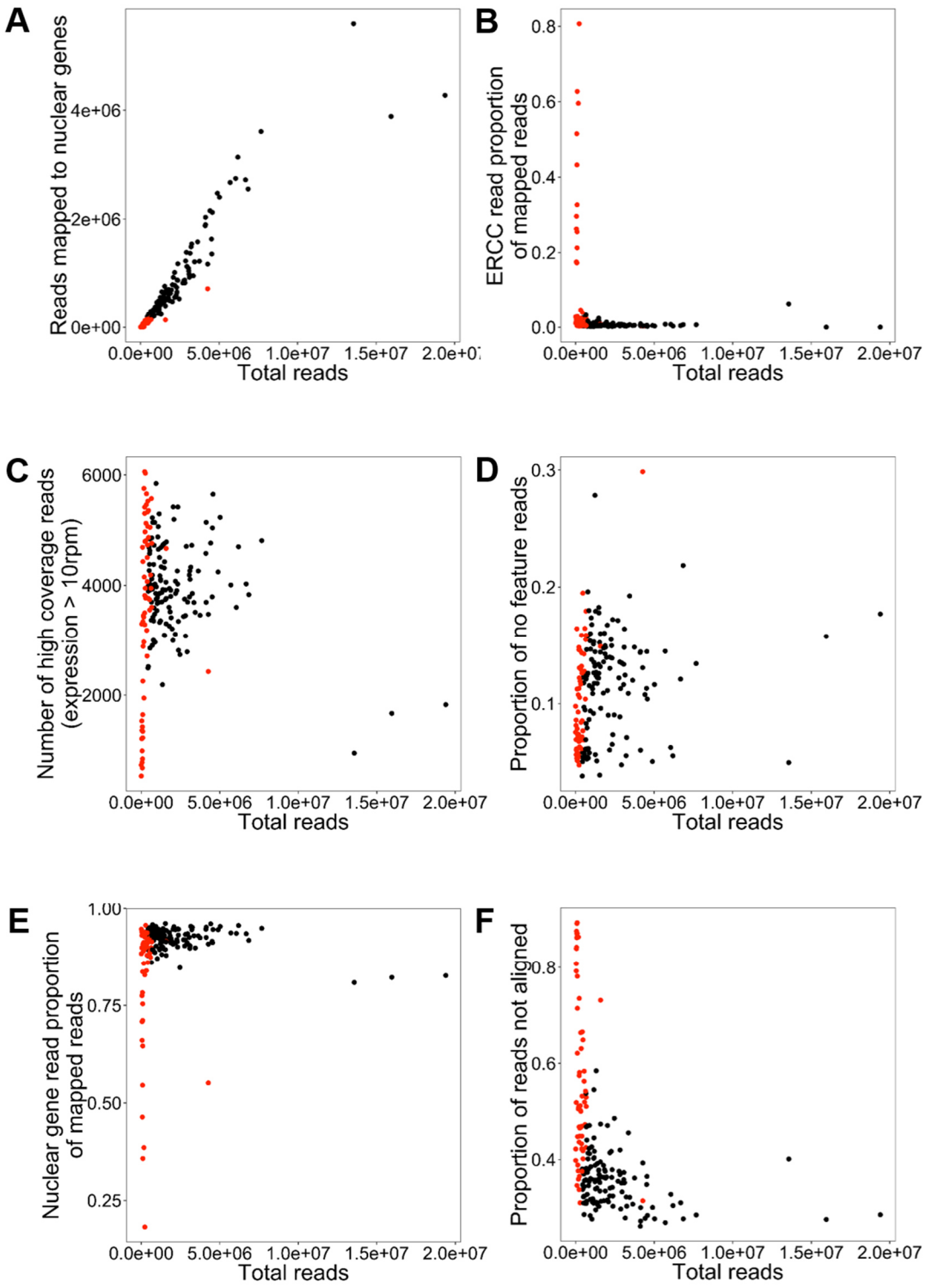
289 Dick, J. E., Magli, M. C., Huszar, D., Phillips, R. A. & Bernstein, A. Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/Wv mice. Cell 42, 71-79 (1985).

290 Kent, D. G. et al. Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential. Blood 113, 6342-6350, doi:10.1182/blood-2008-12-192054 (2009).

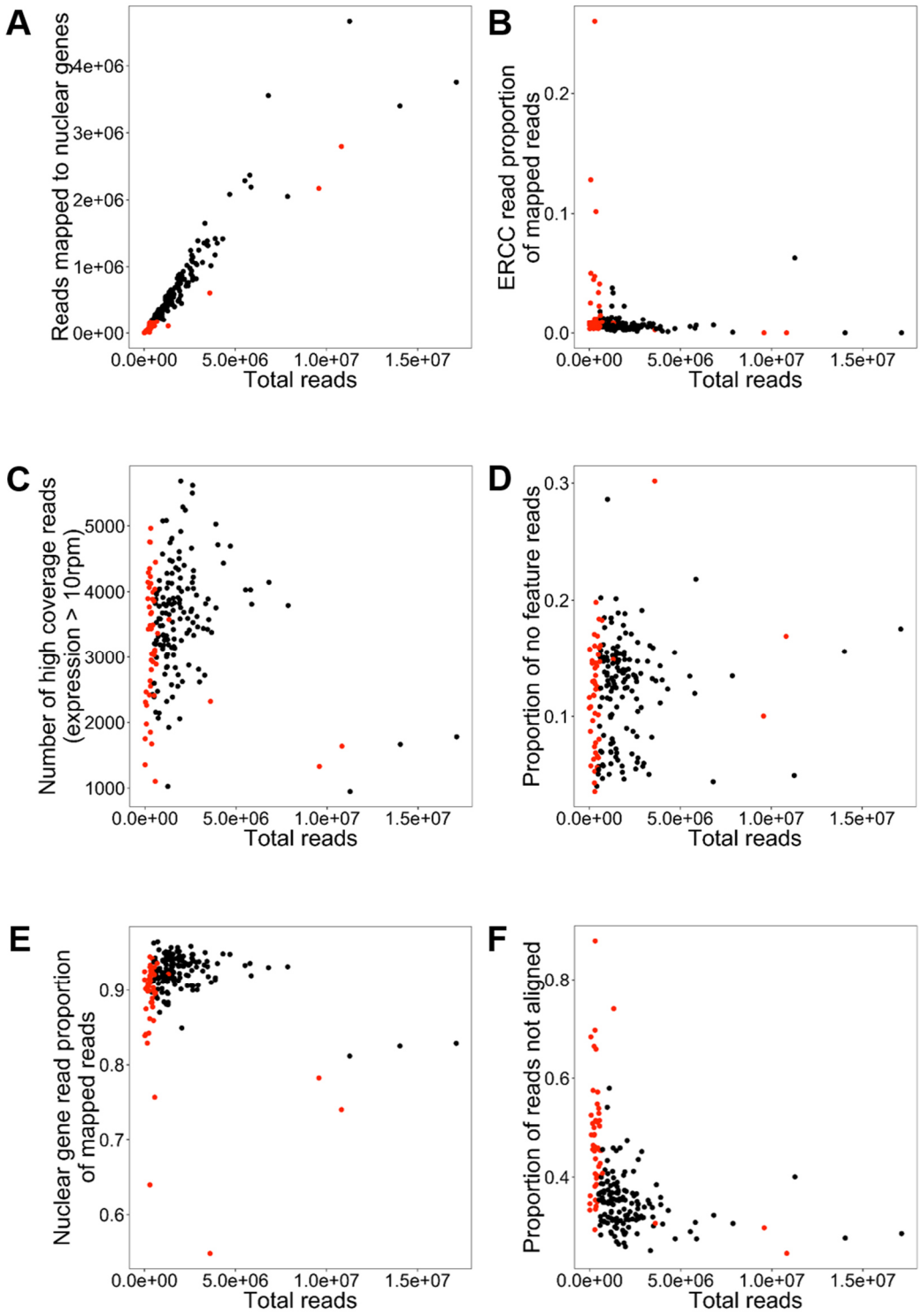
291 Lee-Six, H. et al. Population dynamics of normal human blood inferred from somatic mutations. Nature 561, 473-478, doi:10.1038/s41586-018-0497-0 (2018).

292 Cholewinski, E., Dietrich, M., Flanagan, T. C., Schmitz-Rode, T. & Jockenhoevel, S. Tranexamic acid--an alternative to aprotinin in fibrin-based cardiovascular tissue engineering. Tissue engineering. Part A 15, 3645-3653, doi:10.1089/ten.TEA.2009.0235 (2009).

Appendix A



Appx-A Figure 1: Quality control parameters for single HSCs processed in batch SLX-12565. Red dots depicts cells excluded from analysis (62) following thresholds set for mapped reads (>$2*10^5$), reads mapping to genes (>0.2), and reads mapping to mitochondrial genes (<0.2). Black dots depicts cells that passed quality control (129). Additional parameters (individual Y axis) were not applied as these would not have excluded further cells and aided in ensuring quality control. Total reads were 322,955,793.



Appx-A Figure 2: Quality control parameters for single HSCs processed in batch SLX-12566. Red dots depicts cells excluded from analysis (50) following thresholds set for mapped reads (>$2*10^5$), reads mapping to genes (>0.2), and reads mapping to mitochondrial genes (<0.2). Black dots depicts cells that passed quality control (141). Additional parameters (individual Y axis) were not applied as these would not have excluded further cells and aided in ensuring quality control. Total reads were 343,499,048.

Appendix B

Appx-B Table 1: Gene ontology terms significantly enriched in HSCs stimulated with 30 ng/mL SCF compared to freshly isolated HSCs.

Gene ontology biological process	P-value	Adjusted P-value
DNA metabolic process (GO:0006259)	3.52E-16	4.93E-13
DNA-dependent DNA replication (GO:0006261)	1.38E-12	9.7E-10
DNA replication (GO:0006260)	2.09E-12	9.76E-10
DNA replication initiation (GO:0006270)	4.93E-08	1.15E-05
G1/S transition of mitotic cell cycle (GO:0000082)	3.04E-12	1.07E-09
cell cycle G1/S phase transition (GO:0044843)	9.2E-10	2.58E-07
positive regulation of DNA-directed DNA polymerase activity (GO:1900264)	0.000102	0.009544
DNA strand elongation involved in DNA replication (GO:0006271)	9E-05	0.009544
nucleotide-excision repair, DNA incision, 5'-to lesion (GO:0006296)	0.000213	0.015936
regulation of DNA-directed DNA polymerase activity (GO:1900262)	0.000102	0.009544
error-prone translesion synthesis (GO:0042276)	0.000216	0.015936
DNA repair (GO:0006281)	5.65E-06	0.000879
cellular macromolecule biosynthetic process (GO:0034645)	8.23E-06	0.001153
regulation of transcription involved in G1/S transition of mitotic cell cycle (GO:0000083)	3.91E-05	0.004567
nucleotide-excision repair, DNA gap filling (GO:0006297)	2.13E-05	0.002719
error-free translesion synthesis (GO:0070987)	0.000177	0.015496

Appx-B Table 2: Gene ontology terms significantly enriched in HSCs stimulated with 300 ng/mL SCF compared to freshly isolated HSCs.

Gene ontology biological process	P-value	Adjusted P-value
DNA replication (GO:0006260)	5.1E-13	2.02E-10
regulation of transcription involved in G1/S transition of mitotic cell cycle (GO:0000083)	1E-10	2.38E-08
DNA metabolic process (GO:0006259)	2.86E-14	3.41E-11
strand displacement (GO:0000732)	1.6E-07	2.12E-05
G1/S transition of mitotic cell cycle (GO:0000082)	6.91E-14	4.11E-11
DNA-dependent DNA replication (GO:0006261)	1.25E-10	2.47E-08
DNA replication checkpoint (GO:0000076)	5.4E-07	6.42E-05
cell cycle G1/S phase transition (GO:0044843)	7.18E-11	2.14E-08
cellular response to DNA damage stimulus (GO:0006974)	2.38E-09	3.54E-07
DNA biosynthetic process (GO:0071897)	1.87E-06	0.000186
mitotic cell cycle phase transition (GO:0044772)	3.46E-10	5.88E-08
DNA repair (GO:0006281)	6.43E-07	6.96E-05
cellular macromolecule biosynthetic process (GO:0034645)	2.21E-06	0.000202
regulation of transcription from RNA polymerase II promoter in response to oxidative stress (GO:0043619)	0.00303	0.069333
regulation of double-strand break repair (GO:2000779)	1.8E-05	0.001258
positive regulation of mitophagy in response to mitochondrial depolarization (GO:0098779)	0.001789	0.048394
positive regulation of cell cycle arrest (GO:0071158)	1.32E-05	0.001041
DNA replication initiation (GO:0006270)	8.2E-05	0.004644
photoperiodism (GO:0009648)	0.001104	0.033671
double-strand break repair via homologous recombination (GO:0000724)	8.68E-05	0.004696
DNA replication-dependent nucleosome assembly (GO:0006335)	0.000128	0.005939
DNA recombination (GO:0006310)	1.4E-05	0.001041

Appx-B Table 3: Genes upregulated in HSCs treated with 300 ng/mL SCF compared to both 30 ng/mL SCF and freshly isolated HSCs.

Gene ID	ENSEMBL ID
Cav2	ENSMUSG00000000058
Kdelr2	ENSMUSG00000079111
Rnf187	ENSMUSG00000020496
Msn	ENSMUSG00000031207
Gem	ENSMUSG00000028214
Vps35	ENSMUSG00000031696
2810474O19Rik	ENSMUSG00000032712
Plek	ENSMUSG00000020120
Ctnna1	ENSMUSG00000037815
Lyn	ENSMUSG00000042228
Bzw1	ENSMUSG00000051223
Hmgcr	ENSMUSG00000021670
Naa40	ENSMUSG00000024764
Tubb6	ENSMUSG00000001473
Ash2l	ENSMUSG00000031575
Zdhhc13	ENSMUSG00000030471
Galk1	ENSMUSG00000020766
Pidd1	ENSMUSG00000025507
Lypla1	ENSMUSG00000025903
Tmem120a	ENSMUSG00000039886