

Understanding clonal dynamics in normal and malignant haematopoietic stem cells

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Abstract
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Ever since their discovery, haematopoietic stem cells (HSCs) have become the paradigm for understanding somatic stem cell systems. Throughout my doctoral research, I explored questions in HSC clonal dynamics by high-throughout *in vitro* and *in vivo* single-cell assays, state-of-the-art computational analysis and powerful mouse models of myeloid malignancies. In Chapter 3, I dissected clonal evolution, functional and molecular profiles of single HSCs residing within the marrow of different bones. Phylogenetic analysis demonstrated that HSCs stay embedded their seeded location from embryogenesis. At the same time, single-cell molecular and *in vivo* transplantation analysis revealed that biological HSC properties are largely dictated by the tissue they reside in, thereby challenging some of the classical views on HSC migration and impact of anatomical location.

To continue in Chapter 4, I conveyed the power of somatic mutations to study preleukaemic competition dynamics. Leveraging on HSC *ex vivo* expansion and split cultures, a novel *in vitro* model of clonal competition unveiled the *TET2-super competitor clones*. These were highly efficient at impairing the growth of their wild-type counterparts. These findings imply that supercompetitive behaviour could be one of the fundamental mechanisms driving clone expansion in myeloid cancers.

Given the multifaceted nature of cancer, in chapter 5 I profiled the synergy between JAK2 and TET2 mutations and IP-10, a key inflammatory molecule. By generating an allelic series of MPN mouse models, we found that, albeit mildly, loss of IP-10 rescued the severe red cell phenotype driven by JAK2 V617F, and partially restored erythroid development. The paramount importance of dissecting the role of different inflammatory cytokines is therefore reinforced by this study.

In summary, this doctoral research has shed new light on HSC clonal dynamics, clonal competition and the bone marrow microenvironment in health and disease, hopefully accelerating the burgeoning HSC biology field.

Declaration

I declare that this thesis is the result and presentation of my original work, and I am the sole author, and includes nothing that has been the outcome of work done in collaboration, except as stated in the Preface section or specified in the text. All sources consulted for this thesis are acknowledged as References.

I therefore further state this doctoral thesis has not been, or is being concurrently submitted for any such degree, and thereby has not been presented for an award at the University of York or any other institution.

It does not exceed the prescribed word limit for the Biomedical Sciences PhD programme at the Department of Biology, University of York.

Publications that have arisen from this thesis:

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Preface

Description of contributions by chapter

Chapter 3

All work presented in this chapter was supervised by Prof. David Kent and carried out by myself, with the following contributions: Ellie Bennet and Joanna Milek assisted with all animal work and sample processing. Single-HSC isolation by flow cytometry was performed by Dr. Karen Hogg and myself, with Dr. Graeme Park, Sukhveer Mann and Karen Hodgkinson (Technology Facility, University of York). Dr. Sally James, Lesley Gilbert and Samantha Donninger (Technology Facility) prepared the libraries using the SmartSeq2 protocol for scRNA-seq. I was involved in scRNA-seq bioinformatic analysis, which was mainly performed by Monika Gonka using the Seurat Pipeline (Hao et al, 2021), together with Dr. Sandy MacDonald (Technology Facility) and Dr. Daniel Bode (Kent Lab, now Forbion). Dr. Michael Spencer Chapman (Wellcome Sanger Institute) helped plan and analyse whole genome sequencing (WGS) data and constructed the phylogenetic trees from both mice. Laura O'Neill coordinated reception, library preparation and WGS (Illumina) of single-cell derived colonies performed as per the Sanger Institute Core Pipelines.

Chapter 4

All work presented in this chapter was supervised by Prof. David Kent and carried out by myself, with the following contributions: Juan Rubio-Lara and Nicola Wilson (Cambridge Stem Cell Institute) performed the initial split culture experiment, and corresponding flow cytometry, transplants, and scRNA-seq data analysis. Dr. Juan Rubio-Lara and myself conceived of and designed the clonal competition assays. Ellie Bennett and Joanna Milek assisted with all animal work and sample processing; Sophie Davies provided help with tissue preparation. Single-cell and bulk sorts were performed by Dr. Karen Hogg and myself, with support from Dr. Graeme Park, Sukhveer Mann and Karen Hodgkinson. Dr. Sally James, Samantha Donninger and Lesley Gilbert performed library preparation and bulk poly(A) RNA sequencing of the samples; RNA-seq analysis was performed by myself, Dr. Sandy MacDonald, and Dr. Fabiano Pais. Dr. Maria Jassinskaja and myself performed sample collection and processing for Data-Independent Acquisition Mass Spectrometry (DIA-MS) with help from Chloe Baldreki and Chris Taylor (Proteomics, Technology Facility); DIA-MS data analysis was performed by Dr. Maria Jassinskaja, Chloe Baldreki, Dr. Adam Dowle.

Chapter 5

All work presented in this chapter was supervised by Prof. David Kent and performed by myself, with the following contributions: Nina Friesgaard Øbro performed the initial MPN

patient serum cytokine screening (Øbro et al, 2020); Miriam Belmonte carried out serum cytokine analysis in JAK2 and TET2-mutant patients and in MPN mouse models, and she initially characterised the phenotype of the JAK2/IP10 strain. Karen Hogg assisted with flow cytometry experiments, as well as with sample preparation, experimental design, and plate analysis for all Luminex assays of mouse serum samples. Ellie Bennett and Joanna Milek assisted with phenotyping and sample processing for the 10-plex Luminex assay.

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Abbreviations

AML	Acute myeloid leukaemia
ASXL1	Additional sex comb-like transcriptional regulator 1
BL6	Black 6
BM	Bone marrow
BSA	Bovine serum albumin
CAR	CXCL12 abundant reticular cell
Cas9	CRISPR-associated enzyme 9
CASIN	Cdc42 activity specific inhibitor
CCL5	Chemokine ligand 5
CFC	Colony-forming cell
CFU	Colony-forming unit assay
CFU-S	Colony-forming unit (spleen)
Chisq	Chi square
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukaemia
CMP	Common myeloid progenitor
Comp	Competitor
Cos2	Square of cosine function
CRAN	Comprehensive R archive network
CRISPR	Clustered regularly interspaced short palindromic repeats
CRU	Competitive repopulating unit
CXC	Cys-X-X-Cys motif
CXCL1	Cytokine CXC Ligand 10 (Keratinocyte chemoattractant)
CXCL10	Cytokine CXC Ligand 10 (IP-10)
DE	Differentially expressed
DEG	Differentially expressed gene
DGE	Differential gene expression
DIA-MS	Data-independent acquisition mass spectrometry
Dim	Dimension
dL	Deci-litre
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3

E	Embryonic day (mouse)
ECM	Extracellular matrix
ELDA	Extreme limiting dilution assay
ELISA	Enzyme-linked immunosorbent assay
ELSK	EPCR-LSK
ESLAM	EPCR-SLAM
ET	Essential thrombocythemia
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
F12	Ham's Nutrient Mixture
FACS	Fluorescence-activated cell sorting
FC	Fold-change
FDR	False discovery rate
FLT3	FMS-like tyrosine kinase 3
FU	Fluoroacil
G-CSF	Granulocyte-colony stimulating factor
G0/G1	Gap 0/Gap 1 phase (cell cycle)
G2M	Gap 2/Mitotic phase (cell cycle)
GM	Granulo-myeloid (cellular fraction)
GM-CSF	Granulocyte-monocyte colony stimulating factor
GMP	Granulo-myeloid progenitor
Hct	Haematocrit
Het	Heterozygous
Hgb	Haemoglobin
Hom	Homozygous
HPLC	High-performance liquid chromatography
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
HSPC	Haematopoietic stem and progenitor cell
IDH1	Isocitrate dehydrogenase 1
IFN-γ	Interferon-γ
IL	Interleukin
IP-10	Interferon-γ induced protein 10 kDa
ITSX	Insulin-Transferrin-Selenium-Ethanolamine

JAK2	Janus kinase 2
kDa	Kilo-Dalton
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KI	Knock-in
KO	Knockout
LDA	Limiting dilution assay
LIF	Leukaemia inhibitory factor
Lin	Lineage
LMPP	Lymphoid-primed multipotent progenitor
LOF	Loss-of-function
LSC	Leukaemic stem cell
LSK	Lin- Sca-1+ c-Kit+
LT	Long-term
LTC-IC	Long-term culture initiating cell
M-CSF	Macrophage colony stimulating factor
MCP-1	Monocyte chemoattractant protein
MDS	Myelodysplastic syndrome
MEP	Megakaryocyte-erythroid progenitor
MFI	Median fluorescence intensity
MIP-2	Macrophage inflammatory protein 2
mL	Millilitre
MoIO	Molecular Overlap
MPN	Myeloproliferative neoplasm
MPP	Multipotent progenitor
MSC	Mesenchymal stem cell
mt	Mitochondrial
NF-κB	Nuclear factor κ B
ng	Nanogram
NGF	β-nerve growth factor
nM	Nanomolar
NoMo	No Molecular Overlap
NSC	N6-[2-(4-Diethylamino-1-methyl-butylamino)-6-methyl-pyrimidin-4-yl]-2-methyl-quinoline-4,6-diamine trihydrochloride

NTC	No-template control
p.adjust	Adjusted p significance value
PB	Peripheral blood
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
Pen/Strep	Penicillin/Streptomycin
pg	Picogram
PLT	Platelet
PMF	Primary myelofibrosis
PV	Polycythemia vera
PVA	Polyvinyl alcohol
QC	Quality control
R	Recombinant allele
r²	R square
rb	Ribosomal
RBC	Red blood cell
RepopSig	Repopulation Signature
Rhosin	Rho inhibitor
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640 medium
S	Synthesis phase (cell cycle)
SBS	Single-base substitution
SBS	Variant allele fraction
sc	single-cell
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
scRNA-seq	Single-cell RNA sequencing
SD	Standard deviation
SEM	Standard error of the mean
SF3B1	Splicing factor 3B subunit 1
SP	Side-population

ST	Short-term
STAT	Signal transducer and activator of transcription
T2S	TET2-super competitor
TET2	Ten-eleven translocation enzyme 2
TNF-α	Tumour necrosis factor α
TPO	Thrombopoietin
TPOR	Thrombopoietin receptor
UMAP	Uniform Manifold Approximation and Projection
WBC	White blood cell
WGS	Whole-genome sequencing
μg	Microgram
μL	Microlitre
μM	Micromolar

"It is the great prerogative of Mankind, above other Creatures, that we are not only able to behold the works of Nature, or barely to sustain our lives by them, but we also have the power of considering, comparing, altering, affiliating, and improving them to various uses".

Robert Hooke in *Micrographia*, 1665.

Chapter 1 - Introduction

1 A brief history of haematopoietic stem cell discovery

1.1 The dawn of stem cell research

The field of stem cell biology rose at the end of the 19th century, in light of Schleiden's and Schwann's recently proposed Cell Theory, along with Rudolf Virchow's principles of cellular pathology, encapsulated in the phrase: "*Omnis cellula e cellula*", denoting the origin of every cell of an organism relying on a previously existing cell (Virchow, 1858; Lendhal, 2022). Ernst Haeckel, a German zoologist, supporter of Darwinian evolution, coined the term "stem cell" (*Stammzellen*, in German) for the first time in the history of the field (Haeckel, 1868; Maehle, 2011; Ramalho-Santos & Willenbring, 2007). His studies on the Monera, one of the most primitive living entities, led to Haeckel's conclusion that stem cells could have evolved from these living 'units', as they were not considered to be cells in themselves, but rather had the capacity to give rise to the *Stammzellen*. Likewise, in his 'biogenetic law', where Haeckel famously asserted how "Ontogeny recapitulates Phylogeny", he considered the fertilised egg to be the "stem father and stem mother" of all cells in the organism (Droscher, 2014; Haeckel, 1877).

Noting the ability of other cell types, specifically those which gave rise to the germ line, Theodor H. Boveri and Valentin Hacker broadened the applicability of the term. Importantly, in his observations of the dynamics of cell divisions in crustaceans, Hacker determined that one of the dividing cells remained a stem cell and the second one entered differentiation (Haecker, 1892; Lendahl, 2022). On the other hand, Boveri depicted the lineages of *Ascaris* as phylogenetic trees, which he termed *Stammbaume*, according to his interpretation of the embryonic development of *Ascaris* (Figure 1.1) (Boveri, 1892; Ramalho-Santos & Willenbring, 2007). These fundamental discoveries by Boveri and Haecker inspired the production of Edmund B. Wilson's written work, "The Cell in Development and Inheritance", which became a pillar for the fields of embryology and genetics, and popularised the term stem cell as it is used today.

Contemporary to the groundbreaking contributions of Haeckel, Haecker, Boveri and others, Ernst Neumann identified nucleated red blood cells in the marrow of rabbits and human beings, noting that they corresponded to the different embryonic stages of cells (Cooper, 2011; Charitos et al, 2021; Neumann, 1868). These findings led Neumann to propose the bone marrow (BM) as the tissue where red blood cells originated and developed, as well as lymphocytes, therefore concluding the existence of a common ancestor cell to all blood cells

(Cooper, 2011). Through the development of innovative histological techniques, Paul Ehrlich's demonstration of the existence of different lineages of white blood cells led to two lines of thought: a common origin for all blood lineages, and committed precursors specific to the myeloid and lymphoid lineages (Ehrlich, 1879). Furthermore, this led to the discovery of the *Mastzellen* (mast cell), aside from other types of cells with a granular surface, which were later termed basophils (Beaven, 2010), marking the beginning of the era of tissue histology.

These early discoveries marked the dawn of an entirely new field in the life sciences - stem cell research. From Haeckel's *stammzellen*, to Neumann's seedbed of blood, an entire body of evidence made strong suggestions of the existence of a common origin for cells of all blood lineages. In a fascinating way, the contributions made during this period comprised a conceptual framework for the unveiling of the first type of tissue stem cell: blood stem cells, formally known as haematopoietic stem cells. In the following sections, I will discuss the series of elegant experiments that led the discovery and prospective quest for the HSC, and how these concepts set up the theoretical basis for my doctoral research.

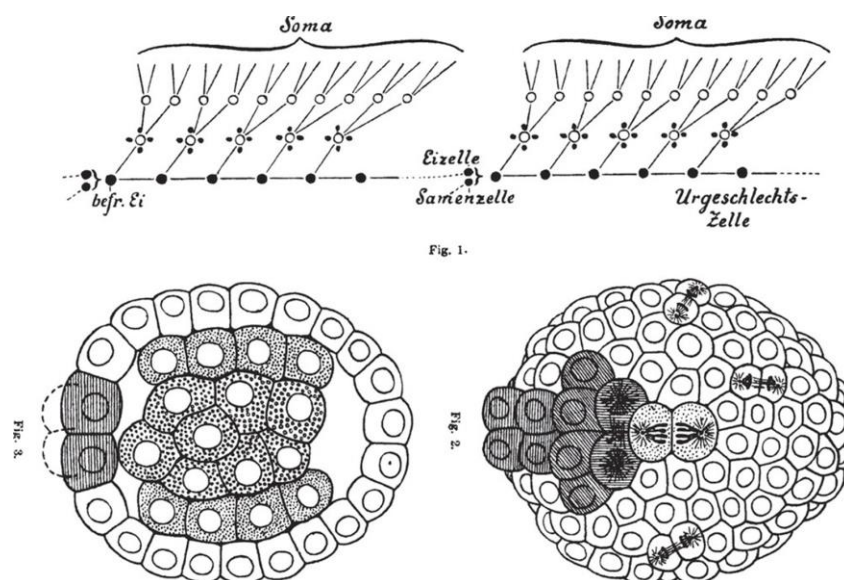


Figure 1.1. Model of embryonic development in *Ascaris megalocephala* as depicted by Theodor Boveri. In 'Fig. 1', self-renewal and differentiation of stem cells (*Stammzellen*) is illustrated, where black circles represent stem cells, and white circles represent the primordial somatic cells (*Urgeschlechtszellen*), all originating from the fertilised egg (left side of the figure). These resemble phylogenetic trees, which Boveri termed *Stammbaume*. In 'Fig. 2' and 'Fig. 3', ventral views of a developing embryo at the 120-cell stage are shown. Adapted from Maehle, 2011, as featured in Boveri, 1892.

1.1.2 The discovery of haematopoietic stem cells and the beginning of the stem cell field

It was not until the beginning of the 20th century that Russian scientist studying lymphocyte biology, Alexander Maximow, established the concept of “Unitarian Haematopoiesis”, stating that all haematopoietic cells derived from a single precursor, a stem cell, found in the marrow stroma, which provided the necessary conditions for haematopoietic cell differentiation (Maximow, 1906, 1909; Konstantinov, 2000). In an effort to describe the haematopoietic process, Artur Pappenheim placed the stem cell at the center of the blood system, highlighting its vital role in the generation of blood lineages (Pappenheim, 1907). Likewise, Wera Dantschakoff referred to the haematopoietic precursors as stem cells (Dantschakoof, 1908). Likewise, Florence Sabin had conducted extensive research on the nature of lymphatic vessels, she studied the embryological origins of blood vessels (Andriole, 1959). It was in this way that Sabin observed the formation of white blood cells (WBCs) from certain areas in connective tissue, at which she marvelled, wondering how the number of WBCs were kept constant in spite of insults, hoping that this would allow a more complete understanding of blood disorders (Sabin et al, 1925).

After three highly productive decades of research leading into the characterisation of the common origins of blood, a world-turning event would result in a breakthrough in the understanding of leukaemia, and with it, the discovery of HSCs.

1.1.2.1 ‘Radiation sickness’ and myeloid malignancies

The tragic atomic bombing of Hiroshima and Nagasaki in 1945 had terrible consequences for the local population and surrounding communities. Most notably, physicians recorded very large numbers of patients suffering from “radiation sickness” (Clevers & Watt, 2018). These patients exhibited low white blood cell counts, and were reported to develop radiation-induced leukaemia or secondary leukaemia. Damage caused by high doses of irradiation would have resulted in an aplastic BM, i.e., unable to continually replenish the blood system (Ichimaru et al, 1991). Chronic myeloid leukaemia (CML) cases were reported in Hiroshima in both male and female patients, but it was particularly in female patients where cases were delayed and the onset occurred around 25 years later, within a 5-year period; moreover, no CML cases were detected in Nagasaki (Folley et al, 1952; Radivoyevitch et al, 2024; Dainiak, 2002). At the end of the 1940s, lead-protected spleens of mice exposed to high irradiation doses did not develop anaemia and had normal blood counts in comparison to unprotected mice (Jacobson, 1950). Moreover, Lorenz and collaborators performed BM transplantations on guinea pigs

post-irradiation, which had a protective effect against the effects of irradiation, and likewise formed haematopoietic centres in other tissues, such as the liver (Lorenz et al, 1951; Clevers & Watt, 2018). Crucially, Ford and colleagues first described the generation of haematopoietic chimaeras in X-ray-exposed recipients, after which they received a dose of spleen cells from heterozygous donors for a chromosome 6 translocation (*T6*). The *T6* marker was found in dividing BM cells from all the recipients, in addition to other haematopoietic tissues, suggesting the presence of a population residing within the BM that held the potential to repopulate an irradiated animal. This was an important finding, as it demonstrated that not only did donor cells have the capacity of reconstituting the haematopoietic system of the host, but that they were also able to replace host cells functionally (Ford et al, 1956). One year later, E Donnall Thomas and colleagues reported the first allogeneic BM transplant in identical human twins, one of them suffering from leukaemia (Thomas et al, 1957; Lendahl, 2022). Due to clinical complications, all six patients died less than four months after receiving a BM transplantation; however, there was still supporting evidence for the existence of a common haematopoietic precursor residing in the BM. In 1951, Dameshek noticed that BM cells did not proliferate as single entities, but they did it *en masse*, namely, as a unit, and these was also the basis for a group of haematological malignancies which he termed 'myeloproliferative disorders' (MPDs) (Dameshek, 1951). Therefore, since aberrant cells proliferate as units, Dameshek speculated about the possibility of finding a common ancestor to these cells.

1.1.2.2 The discovery of the haematopoietic stem cell

Concomitant to the events in Japan in the mid 1940's, Ray Owen carried out experimental anastomoses on fraternal calf twins, from which he derived interesting observations: each of the twins had two distinct blood lineages: their own and that of their twin (Owen, 1945). Thus, determining that the blood cells both twins would produce throughout their lifetime were genetically defined, Owen concluded that there was a cell of origin for the rest of the cells found in the calves' bloodstream (Owen, 1945; Weissman, 2002; Martin, 2016). Around a decade later, inspired by Ford's transplantation experiments in 1956, James Till, Ernest McCulloch and colleagues developed an elegant series of irradiation experiments that would finally demonstrate the existence of an HSC (Steensma & Kyle, 2021; Weissman, 2002). In these experiments, mice received lethal doses of radiation under a cobalt-60 source, and were transplanted with whole BM suspensions immediately afterwards (Till & McCulloch, 1961). Remarkably, when examining the spleens of the irradiated recipients, certain structures were observed, or *nodules*, which Till & McCulloch termed 'colony-forming units in the spleen' (CFU-S). The number of CFU-S present in the spleens was directly proportional to the number of BM cells injected into the bloodstream of the recipients. Furthermore, by histological

examination of the CFU-S nodules allowed the identification of visibly differentiated cells of the myeloid lineage, which served as a strong indication of the multilineage potential of BM cells.

Just a few years later, A. J. Becker along with Till and McCulloch carried out a series of irradiation experiments on mice which also received BM transplants, and the CFU colonies were examined for chromosomal abnormalities (Becker et al, 1963). They found abnormal karyotypes in the colonies, which were the result of radiation damage, and that the chromosomal markers present in one colony were likewise found in the vast majority of cells within the same colony, inferring that this was only possible to have originated from a single cell affected by irradiation. To then demonstrate that these CFUs were not only capable of multilineage potential, but also of generating more cells with the ability of self-renew, Siminovitch profiled single CFUs, both from BM and spleen, and observed that these CFUs gave rise to more colony-forming cells, although at the same time generating cells which would differentiate into distinct lineages (Siminovitch et al, 1963). Hence, these small, rare populations of HSCs in the BM, were assigned two essential properties: 1) the ability to differentiate into cells of multiple lineages, and 2) self-renewal potential. Despite the fact that, it was later demonstrated that these cells were actually progenitors and not long-term self-renewing multilineage stem cells (Worton et al, 1963; Schofield, 1968), these landmark discoveries had thus established the field of stem cell biology, and with it, of HSC biology (Weissman et al, 2002; Boisset & Robin, 2011).

1.2 Adult tissue stem cells

1.2.1 Definition and types of stem cells

Stem cells are immature, unspecialised cells which are present in the embryo and the adult in a myriad of living organisms. These cells do not yet correspond to a determined type of cell since they have not developed the features normally found in differentiated cells, nor do they synthesise proteins specific to any type of tissue (Charitos et al, 2021; Zarewski et al, 2019). Functionally, two main types of stem cells are recognised in mammals: embryonic stem cells and adult tissue (or somatic) stem cells. These can be further classified by their mature cell potential, and by their location and stage of development. Through a series of cleavage divisions, the totipotent fertilised egg progresses into a blastocyst, which encompass two stages of embryonic cell potency: totipotency, which is particular to blastomeres, and pluripotency, which is a property of the epiblast cells, and these stages occur prior to gastrulation (Du & Wu, 2024). Furthermore, in the adult organism, multipotent, oligopotent and

unipotent cells are found. The following list presents types of stem cells by potency and their main characteristics (Zakrzewski et al, 2019; Tabansky & Stern, 2016):

1. Totipotent stem cells - which divide and generate all cells of an entire multicellular organism, both from embryonic and adult lineages (Du & Wu, 2024).
2. Pluripotent stem cells (PSC) - which give rise to cells of the germ line but are not able to generate structures such as the placenta (Singh et al, 2016).
3. Multipotent stem cells - will be able to specialise as discrete cellular units of specific lineages. Somatic stem cells, including HSCs, are an example of these.
4. Oligopotent stem cells - which can differentiate into multiple cell types, and are often called progenitors, such as Common Myeloid Progenitors (CMP, discussed later in this Introduction) (Casado-Diaz, 2022).
5. Unipotent stem cells - these divide repeatedly and possess narrow differentiation potential. Examples of these include megakaryocytes and epidermal cells (Mesfin et al, 2023).

In somatic tissues, adult stem cells are found at the apex of the differentiation hierarchy, and provide the necessary cellular products for tissue regeneration in response to insults, as well as normal tissue growth and renewal (Altshuler et al, 2022; Beumer & Clevers, 2023). Advances in mammalian stem cell research have now demonstrated that there are several kinds of tissue specific stem cells, although ultimately all tissues and organs originate from the totipotent early-stage blastomeres (Posfai et al, 2021; Cui et al, 2023).

On the other hand, researchers have also classified the main types of stem cells by location and stage of development (Charitos et al, 2021):

1. Embryonic stem cells (ESCs), these derive from the inner-cell mass of the embryo and have the ability to generate cells of all somatic tissues in the organism (Dvash et al, 2006; Vazin & Freed, 2010)
2. Amniotic epithelial cells (AECs), whose precursor is the placental amnioblast, which at the same time derives from the 8-day epiblast. AECs possess stem cell-like properties and display paracrine functions (Qiu et al, 2020; Zhang & Lai, 2020)
3. Foetal stem cells (FSCs), possess a greater differentiation potential than adult stem cells (including foetal HSCs, discussed later in this introduction), and can easily be acquired from structures such as the amniotic fluid, Wharton's jelly and placenta (Rosner et al, 2023; O'Donoghue & Fisk, 2004).
4. Umbilical cord epithelial (UCE) stem cells, similarly to FSCs, display multilineage potential and can be accessed directly from the umbilical cord, making them an

important source of several types of cells, including HSCs (Weiss & Troyer, 2006; Hordyjewska et al, 2014).

5. Somatic or adult tissue stem cells, which will be described in further detail below and are the primary subject of this thesis

1.2.2 Characteristics of somatic stem cells

One entangling question arises when observing creatures of different species - how is it that the individuals from the same species exhibit different sizes? And even more so, how is it that there are tissues and organs of different sizes within the same individual? (Clevers and Watt, 2018). What is fascinating is that both solid and liquid tissues in the body (blood) rely on the same mechanism for tissue replenishment of all organ sizes - somatic stem cells, and they all share similar characteristics that allow them to accomplish their function within the organism (Goodell et al, 2015). Somatic stem cells are normally multipotent, producing oligo- and uni-potent progenitors with limited differentiation potential (Tweedell, 2017). Importantly, somatic stem cells do not normally enter organogenesis along the entirety of an individual's lifetime and they make their residence in specialised anatomic sites known as niches (Tweedell, 2017). Within the specialised cellular microenvironment (often referred to as the "stem cell niche"), distinct cell populations - which constitute the stem cell pool - aid in preserving the main stem cell properties, and importantly regulate stem cell behaviour and function (Cui et al, 2023). Moreover, stem cells undergo asymmetric cell division, in which yields two types of cells: an identical daughter cell and a cell committed towards a differentiation pathway, which would only divide a few more times before total differentiation (Altshuler et al, 2022). This is known as the hierarchical model (Nakamuta et al, 2022). However, it has recently become evident that in the majority of solid tissues, stem cell populations are maintained through symmetric divisions, which would generate equipotent daughter cells, which could either enter differentiation or remain stem cells, in what is now known as the neutral competition (NC) model (Visvader & Clevers, 2016; Nakamuta et al, 2022).

In addition to the two main properties which have traditionally been ascribed to somatic stem cells, there are several other hallmarks or features which distinguish stem cells from the rest of the cell populations found in the adult organism: quiescence, transplantability, plasticity, and niche dependence (Beumer & Clevers, 2023). The trait of transplantability and the niche will be discussed in full detail later in this Introduction. Below, I present a brief overview of quiescence and plasticity.

1.2.2.1 Quiescence

The term quiescence refers to a cellular state in which cell cycle exit is prolonged, and re-entry into cell cycle is made possible by a programmatic set of instructions for the cell to start dividing once more (de Morree & Rando, 2023). Quiescence serves as a vital mechanism for preserving genome integrity and prevents the stem cell pool from exhaustion and depletion, which could have catastrophic consequences for tissue integrity and homeostasis (Beumer & Clevers, 2023). Equally important, quiescent stem cells resist environmental insults, and are afterwards able to give rise to their whole lineage after stress (Urban & Cheung, 2021). Contrary to many presuppositions that quiescence is an 'inactive' cellular state, it has been proven in multiple cell types that quiescent stem cells are metabolically active and interact with their extracellular matrix (ECM), which are likewise important for cell cycle regulation (Urban & Cheung, 2021). Several stem cell systems depend on quiescence for protection of the stem cell pool, including HSCs, Muscular stem cells (MuSC), Hair Follicle Stem Cells (HFSC), Neural Stem Cells (NSC) and Intestinal Stem Cells (ISC) (Tumpel & Rudolph, 2019; Khoa et al, 2024). All quiescent cells remain in the G₀ phase of the cell cycle, however, this process is reversible unlike in senescent or in terminally differentiated cells, where they are unable to re-enter the cell cycle after their exit (Cho et al, 2019).

1.2.2.2 Plasticity

Under homeostatic conditions, stem cells residing specifically within their corresponding tissue would generate lineages native to such tissue, i.e., they are not expected to transition from one tissue type to another (Lakshmiathy & Verfaillie, 2005). Nevertheless, under stress, stem cells can modify their phenotypes and exit the canonical differentiation process, allowing them to transition onto another cell type that differs from their normal differentiation programme (Qin & Tape, 2024). These changes in cellular identity are collectively known as cellular plasticity or transdifferentiation, and have mainly been identified in HSCs and neural stem cells (Wagers & Weissman, 2004; Mills et al, 2019). Although rather controversial, there have been a number of studies reporting transdifferentiation of neural stem cells into haematopoietic progenitors, and BM HSCs giving rise to hepatic oval cells (Bjornson et al, 1999; Petersen et al, 1999). A core concept to understand stem cell plasticity is dedifferentiation, which refers to the process by which partially or terminally differentiated cells can reversely transition into a less differentiated cell state, and therefore generate cells from a different lineage to their native one (Yao & Wang, 2020). For instance, cells of the small intestine have been found to contribute to repair of the colonic epithelium after tissue injury, whereas intestinal epithelial

cells of the colon have been described to maintain the stem cell pool of the colonic crypts (Azofeifa et al, 2019; Ishibashi et al, 2018; Beumer & Clevers, 2023).

1.2.3 Types of somatic stem cells

Whilst somatic stem cells for virtually every organ system in the body, HSCs are the most comprehensively characterised type of somatic stem cell to date, since their formal discovery in the 1960s. However, other stem cell systems such as the intestinal, neural, dermic, gastric, muscular, skeletal and glandular amongst others, have likewise gained attention and more knowledge concerning these types of cells continues to be generated (Goodell et al, 2015; Tweedell, 2017). Out of such types of adult stem cells, skin epithelial and intestinal stem cells reside in anatomically defined niches, in a similar way to HSCs, and the reason for which they are more informative for the results that I will present in Chapters 3 and 5 of this doctoral dissertation. Moreover, the turnover rate for each of these stem cell types is of fundamental importance to the regeneration of skin and intestinal epithelium - respectively - upon injury. Likewise, HSCs together with skin and intestinal epithelial stem cells, exhibit the highest cellular turnover rates among tissues (Sender & Milo, 2021; Banjac et al, 2023):

- HSCs = 10^9 newly produced cells per day
- Skin epithelial stem cells = 10^7 newly produced cells per day
- Intestinal stem cells = 10^8 newly produced cells per day

Therefore, I will briefly describe the main properties of these two types of somatic stem cells, before a more extensive description of HSCs.

1.2.3.1 Epithelial stem cells

1.2.3.1.1 Skin epithelial stem cells

The skin is considered to be the largest organ in the body, functioning as a vital physical barrier in all mammals, which prevents water loss, injuries to soft tissues, solar radiation damage and infections (Tang et al, 2024). The skin comprises a myriad of tissue and cell layers, which work harmoniously together and are constantly exposed to damage from the environment, and therefore require a rapid response for regeneration. It is in the epidermis and its array of appendages (hair follicles, sebaceous glands, sweat glands, scales, feathers, fins, beaks and nails) where progenitors are harboured for long-term tissue maintenance (Gonzales & Fuchs, 2017). There are different populations of skin epithelial stem cells, which can be distinguished from each other by their surface markers. The process in which the role of epithelial stem cells has been more extensively investigated is wound healing, which consists

of three stages: inflammation, regeneration and remodelling of the epidermis (Figure 1.2A) (Aragona et al, 2017; Diaz-Garcia et al, 2021). Upon injury, blood vessels will be disrupted, prompting the formation of blood clots in order to prevent blood loss (Diaz-Garcia et al, 2021). During the first phase of healing, immediately after the wound has been inflicted (Figure 1.2A-1), long-lived stem cells the follicles would be recruited to the injured area to promote re-epithelialisation; on the other hand, short-lived follicle stem cells are able to migrate to wound sites and generate progeny within only a month (Yang et al, 2020). Generation of new epidermal cells occurs in the second phase of healing, which is also called proliferation phase, where fibroblasts are recruited to the proliferating zone to secrete collagen, forming a layer of scar tissue known as scab (Pena & Martin, 2024) . The third and final phase - the remodelling and resolution phase - lasts the longest. Here, the wound edges begin to close until they confront each other, accompanied by the re-formation of extracellular matrix in addition to the regeneration of cells that were lost in the wounding (Pena & Martin, 2024; Aragona et al, 2017).

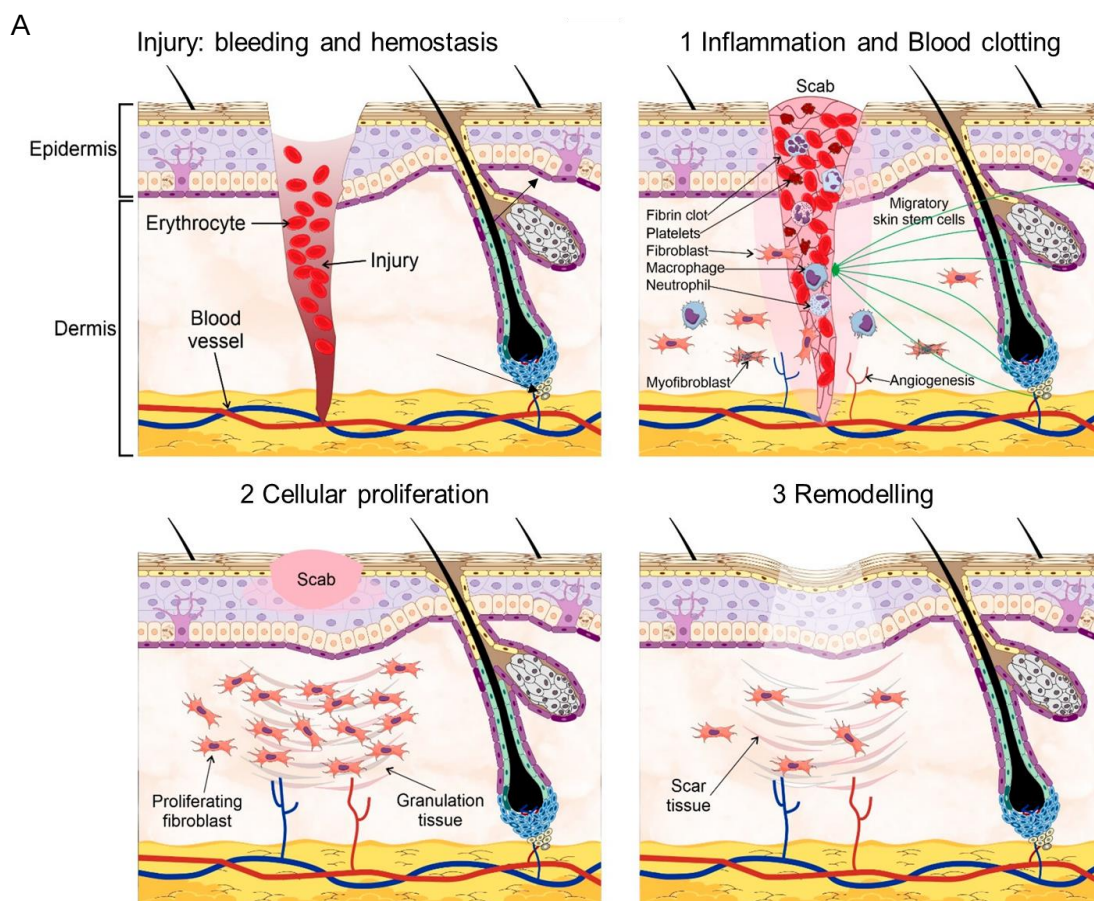
In a similar fashion, HSCs are able to regenerate the entire blood system of an individual upon different kinds of insult, such as haemorrhage, infection, cancer or irradiation, resulting in BM damage or blood loss. This is clearly illustrated by treatment of a vast array of haematological diseases by allogeneic haematopoietic stem cell transplantation (HSCT), whereby the BM of patients is destroyed commonly by high doses of irradiation, and replaced with the healthy haematopoietic stem and progenitor fraction of healthy donors (Hatzimichael & Tuthill, 2010; Bittencourt & Ciurea, 2020). The principles of haematopoietic transplantation and how they have led to the functional characterisation of HSCs will be discussed later in this Chapter.

1.2.3.1.2 Stem cells of the gastrointestinal tract

1.2.3.1.2.1 Intestinal stem cells (ISC)

The physiological importance of the digestive system has largely been limited to the processing of food and nutrients and their supply to the different tissues and organs of the body. Nonetheless, its functions are complex and varied, as it interacts with several other systems, such as the immune, the nervous and endocrine systems. The epithelial cell layer lining the gastrointestinal tract resists the vehement forces of intestinal movements during digestion and is exposed to numerous potential pathogens that travel through the tract every time the individual ingests food. Hence, the intestinal epithelium is one of the tissues with the highest turnover rate in the organism, which is intricately regulated by intestinal stem cells (ISCs) (Choi & Augenlicht, 2024). ISCs are Lgr5⁺, which is a target gene of *Wnt* in human

colon cancer cell lines (Barker et al, 2007). These cells reside in specialised structures called crypts or crypt-villus, which are self-renewing units composed of villi - protrusions - of the intestinal wall and maximise the surface area of the epithelium for nutrient absorption (Figure 1.2B) (Clevers, 2013). Stem cell activity in the intestinal crypts is mostly governed by the Wnt signaling pathway, promoting proliferation of crypts *in vitro* and *in vivo* (Clevers & Nurse, 2012; Kim et al, 2005; Sato et al, 2009). Along with Wnt, EGF signalling is crucial for maintaining stemness within the crypt; Wnt and EGF are secreted by Paneth cells, physically located at the bottom of the intestinal crypt, and it has been observed that secretion of these niche signals is done in a circadian manner (Baulies at al, 2020). A small population of slow-cycling cells, known as reserve stem cells, which are vital for the maintenance of the stem cell pool in the adult intestine upon injury, and helps keep the high turnover rate, which is required for epithelial renewal on a weekly basis in the intestine (Bankaitis et al, 2020).



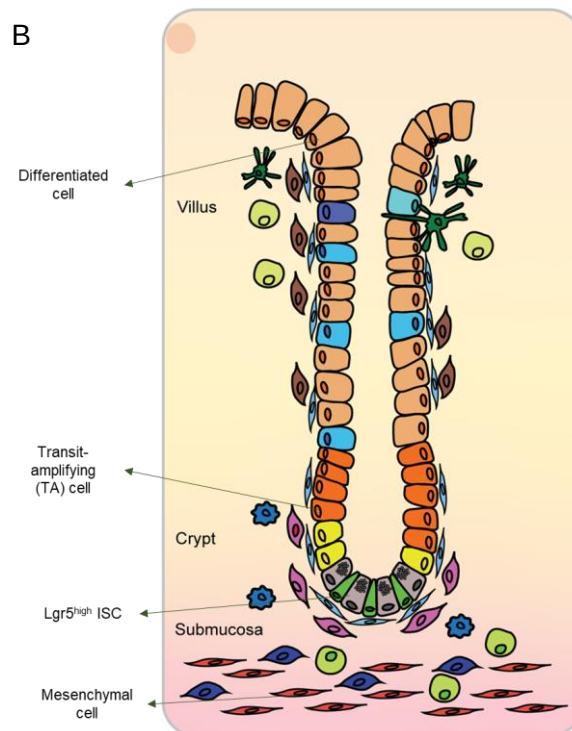


Figure 1.2 A model of wound healing in the skin (A) and the intestine (B). A) Upon injury, platelets will be recruited to the wound area where they will begin the process of hemostasis, forming a blood clot which plugs the injury and avoids excessive blood loss. Long- and short-term lived stem cells residing in the follicles and surrounding areas will migrate to the wound area during the first phase of healing (1), known as the inflammation phase. During the proliferation phase (2), fibroblasts will be recruited to the proliferating zone to secrete collagen and form a scab, which is a type of scar tissue. Finally, in the remodelling and resolution phase (3), wound will close, and the remodelling of the extracellular matrix, as well as regeneration of cells which were lost during injury will be initiated, which could last from weeks to months, or in some instances, even longer, depending on the severity of the injury. Adapted from Diaz-Garcia et al (2021). B) The intestinal epithelium is regenerated on a daily basis by the intestinal stem cells (ISCs) residing at the base of the crypt-villus axes. LGR5^{high} ISCs are found at the base of the crypt, adjacent to Paneth cells, which secrete Wnt3, in addition to mesenchymal cells - located at the submucosa - regulating Wnt signalling, which is crucial for the balance between self-renewal and differentiation of ISCs during homeostasis and regeneration of the epithelium. As ISCs differentiate, they give rise to progenitors, which exit the crypt, and go through the transit-amplifying zone where they will terminally differentiate. Adapted from Dubrovsky & Dunn, (2023); Choi & Augenlicht, (2024).

1.3 Haematopoietic stem cells and haematopoiesis

1.3.1.1 HSC purification and prospective isolation

Around fifteen years after Till and McCulloch's series of seminal discoveries of the colony forming unit, spleen (CFU-S) assay in irradiated mice, it was observed that the spleen was not the primary source for HSCs in the mouse (Schofield, 1978; Bryder et al, 2006). Studies were greatly accelerated by the advent of multiparametric fluorescence-activated cell sorting (FACS)(Hullet et al, 1969) and the development of the highly specific monoclonal antibodies

by hybridoma technology (Milstein and Kohler, 1975), ultimately leading to the prospective isolation of HSCs (Spangrude et al, 1988; Osawa et al, 1996; Weissman, 2002). Building on these technological advances, Muller and colleagues identified two populations of cells while studying lymphocyte development: a population which lacked the expression of B220 and Thy-1, markers for B- and T-cells, respectively, and another population which was positive for Thy-1 but did not express any of the B-cell markers (Muller et al, 1986). Only two years after this work was published, members of the same research group purified and defined murine haematopoietic stem cells as Thy-1.1^{lo}Lin⁻Sca-1⁺, which were able to reconstitute all blood lineages of myeloablated recipients, i.e., they possessed multilineage potential (Spangrude et al, 1988). Furthermore, this population represented 0.05% of total BM cells, and were identified as the only cellular subset of the adult mouse BM - specifically, C57BL/Ka-Thy-1.1 adult mice - with the ability to self-renew and restore the entire blood system of a recipient (Uchida & Weissman, 1992). A series of limiting-dilution transplant assays demonstrated that single haematopoietic stem cells (Thy1^{lo}Lin⁻Sca-1⁺) were able to restore haematopoiesis for up to 7 weeks, contributing to T-, B- and granulo-myeloid cell production (Smith et al, 1990).

The prospective quest for HSCs continued in the 1990s with the addition of different surface markers to flow cytometry panels with the aim of obtaining more enriched populations of stem and progenitor cells. Importantly, the addition of stem cell factor receptor, CD117 or c-Kit, allowed the discovery of three subsets of the Thy-1.1^{lo}Sca-1^{high}Lin^{lo} progenitor pool (Morrison & Weissman, 1996). All of the populations were c-Kit⁺, but differed in the expression of two selected lineage markers: CD11b (or Mac-1) and CD4, which were classified as short-term HSCs (ST-HSC), long-term HSCs (LT-HSCs) and multipotent progenitor cells (MPPs). Towards the optimisation of single-cell transplantation assays, the search for HSC markers was also encouraged. Osawa and colleagues incorporated CD34 - a marker classically used in human HSC biology - into a new gating strategy to characterise single-cell function *in vitro* and *in vivo* (Osawa et al, 1996). A large proportion of the Lin⁻Sca-1⁺c-Kit⁺CD34⁻ cells formed multilineage colonies *in vitro*; in addition, around 20% of mice transplanted with a single HSC showed long-term reconstitution (at least three months post-injection), implying that - unlike human HSCs - functional mouse HSCs did not express CD34.

Furthermore, in the search to solve the great heterogeneity within the haematopoietic stem and progenitor cell (HSPC) compartment, other HSC purification strategies integrated the measurement of dye efflux activity, such as Rhodamine-123 (Rho) - for mitochondria visualisation - or Hoechst 33342 - for DNA staining - along with surface marker expression, which showed to be efficient for HSC isolation (Goodell et al, 1996; Kim et al, 1998; Zijlmans et al, 1995; Eaves, 2015; Bastani et al, 2023). Likewise, the separation of progenitor

populations by Rho staining resulted in the isolation of an enriched HSC subset, which was low in Rho retention activity, as demonstrated by *in vitro* and in serial transplantation studies (Uchida et al, 2003).

As HSC research was consolidating, so did other fundamental techniques in molecular biology and genetics, which were instrumental in determining regulators of HSC activity and function. In a seminal study by Kiel and colleagues, the molecular profiles of HSCs and MPPs were compared in order to identify novel markers associated with the primitive nature of HSCs (Kiel et al, 2005). By microarray analysis, the authors identified several genes strongly associated with HSC identity, of which *Slamf1* (CD150), the founding member of the SLAM family of receptors, had previously been implicated in lymphocyte function (Sidorenko & Clark, 2003). A more thorough profiling study of SLAM marker expression in HSPC subsets: HSCs, MPPs and CD45⁺ BM cells, found that only HSCs expressed CD150 or SLAM, but not MPPs, and conversely, MPPs expressed CD244 (*Slamf4*), another member of the SLAM family, but HSCs did not (Kiel et al, 2005). Lastly, the expression of the CD48 (*Slamf2*) member was not found in either MPPs or HSCs, but rather on restricted progenitors of the BM with limited self-renewal and multilineage potential. In a later study, Forsberg and colleagues profiled the expression of self-renewal regulators in ST-HSC, LT-HSC and MPP populations by microarray technology (Forsberg et al, 2005). In particular, the study found that the *Slamf1* ligand was upregulated in the LT-HSC fraction, and that the same could be subdivided into two further fractions by the expression of *Slam1* and CD34 (positive and negative for each marker, respectively). Hence, the expression of CD150 and CD48 could thus be integrated into the HSC purification strategies along with some of the classically used makers to obtain an HSC population with durable reconstitution potential (Eaves, 2015; Oguro et al, 2013; Challen et al, 2008).

In line with this, a large microarray experiment of different types of stem cells, including both embryonic and somatic stem cells, and HSCs in particular had a very distinctive profile from the rest of the BM cell populations, with *Procr* (CD201 or EPCR, Endothelial Protein C receptor) as one of the genes detected at higher expression levels in the HSC fraction (Ramalho-Santos et al, 2002). EPCR was then shown to be associated with potent reconstitution capacity, since EPCR⁺ cells displayed 1000-fold enrichment of HSC activity, indicating that EPCR expression-based fractionation provided a more accurate strategy for isolating the most functional haematopoietic stem cells (Balazs et al, 2006). It was later demonstrated that single CD150⁺ HSCs in the EPCR⁺CD48⁻CD45⁺ (E-SLAM) fraction contributed to long-term multilineage reconstitution in congenic recipients, with more than 50% of recipients displaying detectable engraftment for at least 16 weeks post-injections (Kent et

al, 2009). Together these studies have provided instrumental methodologies for the identification and prospective isolation of HSCs, mainly by phenotypic expression of specific surface markers, which have facilitated the development of *in vitro* and *in vivo* assays that are continually evolving and furthering our understanding of HSC biology.

1.3.1.2 Heterogeneity within the haematopoietic stem cell population

Somatic stem cells, including HSCs, had long been considered to be uniform populations; nonetheless, it is now known that there exists a high degree of heterogeneity within the HSC pool (Goodell, et al, 2015; Jurecic, 2019; Copley & Eaves, 2013). Some of the factors that contribute to HSC heterogeneity include genetic and epigenetic reprogramming during development and adulthood, and potential differences in BM niches across bones in the body (Jurecic, 2019). Heterogeneity of HSCs is essential for several biological processes such as cell fate decisions and tissue regeneration under homeostatic conditions, although it can also play an important role in ageing and cancer (Cui et al, 2023).

Early observations of lineage biased output of HSCs were made evident from *in vitro* clonal assays, and HSC clones were likewise observed to produce clones of varying sizes, as well as producing mixed colony types (Humphries et al, 1999; Suda et al, 1984). Similar deductions were made from *in vivo* evaluation of HSC activity; for instance, transplantation of CD34^{low}Lin⁻Sca-1⁺ cells showed that these displayed a great degree of variation regarding their contribution to myeloid and lymphoid lineages (Williams et al, 1984; Keller et al, 1985; Pierre et al, 1988; Osawa et al, 1996). Furthermore, transplant of *in vitro* haematopoietic stem cell clones revealed the presence of HSC clones which differed in their lineage output: balanced, myeloid biased or lymphoid biased, which supported earlier findings and observations of the heterogeneous behaviour of HSCs (Muller-Sieburg et al, 2002). A landmark study in the field characterised the lineage contributions of nearly 400 HSCs, a large portion of which were highly purified single cells, and a smaller portion consisted of 4-day *in vitro* clones (Dykstra et al, 2007), of which about 25% could be defined as long-term HSCs. Analysis of their lineage output revealed four types of cells with unique differentiation programmes: α type, which are largely myeloid-based; β type, which displayed a balanced output (contributing to the myeloid and lymphoid lineages alike); γ type, with a higher skewing towards B-cell production; and δ type, which displayed larger contribution to T-cell production. In addition, the α and β types were characterised as the most robust, and they were able to display similar patterns in serial transplantation into secondary and tertiary recipients, suggesting that these features were defined at the clonal level.

Over the last decade, studies have benefitted from the availability of retroviral labelling methods, which allow the detection of megakaryocytic/platelet fraction in transplant recipients, which was not possible in previous years due to platelets, megakaryocytes and erythroid cells not expressing CD45, which limits their detection through traditional immunophenotypic methods. Thus, it has been demonstrated that a small fraction of HSCs are biased towards the megakaryocytic lineage, and reduced self-renewal potential, in addition to varying c-Kit expression levels (Yamamoto et al, 2013; Shin et al, 2014; Haas et al, 2014; Goodell, 2015). Given that von Willebrand factor (*vWf*) expression has previously been reported in a small number of HSCs, in a seminal study, Sanjuan-Pla and colleagues transplanted different fractions of HSCs according to their *vWf* expression levels using a transgenic reporter (Sanjuan-Pla et al, 2013). This demonstrated that higher expression of *vWF* correlated with megakaryocytic lineage bias. In addition, it was found that thrombopoietin regulated the maintenance of platelet-primed *vWF*⁺ HSCs, and that these were also placed upstream of lymphoid-contributing HSCs, identifying a new type of platelet-biased HSCs, with myeloid potential (Sanjuan-Pla et al, 2013).

Moreover, other antibody-based fractionation methods have identified different subtypes of HSCs according to the expression of molecules such as N-cadherin, which is expressed in osteoblasts surrounding HSCs in their BM microenvironment. (Haug et al, 2008; Graf & Stadtfeld, 2008). These findings have thus challenged the classical view of haematopoiesis as a hierarchy of progenitors which lose self-renewal potential as they run down the differentiation roadmaps, but it is evident that a more dynamic, complex interplay of transcription factors, epigenetic changes and microenvironmental cues heavily influence the fundamental properties and functional capabilities of the different subsets of the HSC pool. This heterogeneity could be traced back to the developmental origins of HSCs, as they transit through different specialised microenvironments and attain residence within the foetal BM towards the later stages of development, into post-conception and until the end of life. However, the question remains of whether the bone to which HSCs localise impact their transcriptional and differentiation programmes, which will be one of the scientific questions I undertook throughout Chapter 3 of this doctoral thesis.

In the following sections, I will provide a review of the fundamentals of haematopoiesis, the process of blood formation, how novel, high-throughput techniques have shaped our current understanding of this process, and how HSC clonal dynamics can be traced back in time by applying state-of-the-art genomic and computational approaches.

1.3.2 Haematopoiesis - the process of blood formation

1.3.2.1 Blood

Blood is one of the main specialised tissues in the body, it is fundamentally a form of connective tissue whose main functions are the transport and supply of nutrients, oxygen and energy for all organs throughout the body, as well as the removal of waste products and the transport of immune cells to sites of infection or damage (Farber, 2021; Kamrani et al, 2023; Betts et al, 2022). Along with lymphatic fluid, blood is considered to be a liquid or fluid tissue. Blood consists of two main components: cellular and liquid or non-cellular. The non-cellular component is subdivided into: a) Plasma (~ 55% of total blood), which is the liquid base for whole blood, containing coagulants (fibrinogen), plasma proteins which aid in maintaining homeostasis through regulation of osmotic pressure; ionic components including sodium, potassium, bicarbonates, chloride and calcium, all essential for preserving the pH of blood at physiological levels (pH 7.3-7.4); and other biomolecules such as enzymes, hormones and cofactors. b) Serum, which is the liquid fraction that does not contain any fibrinogen (Matthew et al, 2023; Kamrani et al, 2023).

The cellular fraction (~45% of total blood) comprises erythrocytes or red blood cells (RBCs), leukocytes or white blood cells (WBCs), and thrombocytes or platelets. These constitute the cellular blood mosaic, which fulfil different functions within the organism: Erythrocytes transport oxygen to and remove carbon dioxide from tissues; thrombocytes participate in the process of blood clotting and therefore prevent excessive blood loss (haemorrhage), as well as having a crucial role in wound healing; cells from the myeloid and lymphoid lineages execute their protective functions in tissues where an insult has occurred (Liggett and Sankaran, 2020).

1.3.3 Fundamentals of haematopoiesis

From the Greek, αἷμα (*haema*, blood) and ποιέω (*poeio*, *poiesis*, to make, to do, to craft), the term 'haematopoiesis' refers to the highly regulated, stepwise process through which cells from blood and immune lineages are generated. Haematopoiesis is an astonishingly dynamic process, as it produces around 10^{13} blood cells on a daily basis (Cosgrove et al, 2021), and it is highly adaptable to organismal demands and hormonal signals which inform the BM and other haematopoietic sites about the necessity to generate specific cell types, for instance, in response to insults or haemorrhage. As reviewed by Kent & Eaves, early life forms have constituted useful models for the study of the haematopoietic process, such as *Drosophila*,

zebrafish and frogs; however, the mouse as well as human model systems have been the most widely used, due to their accessibility and - in the case of mouse models - reproducibility and similarity to the human blood cell production (Figure 1.3) (Kent & Eaves, 2016). In addition, it is from mouse models that most of the data regarding numbers and lifespan of stem, progenitor and mature cells of the blood has been generated and published (Cosgrove et al, 2021).

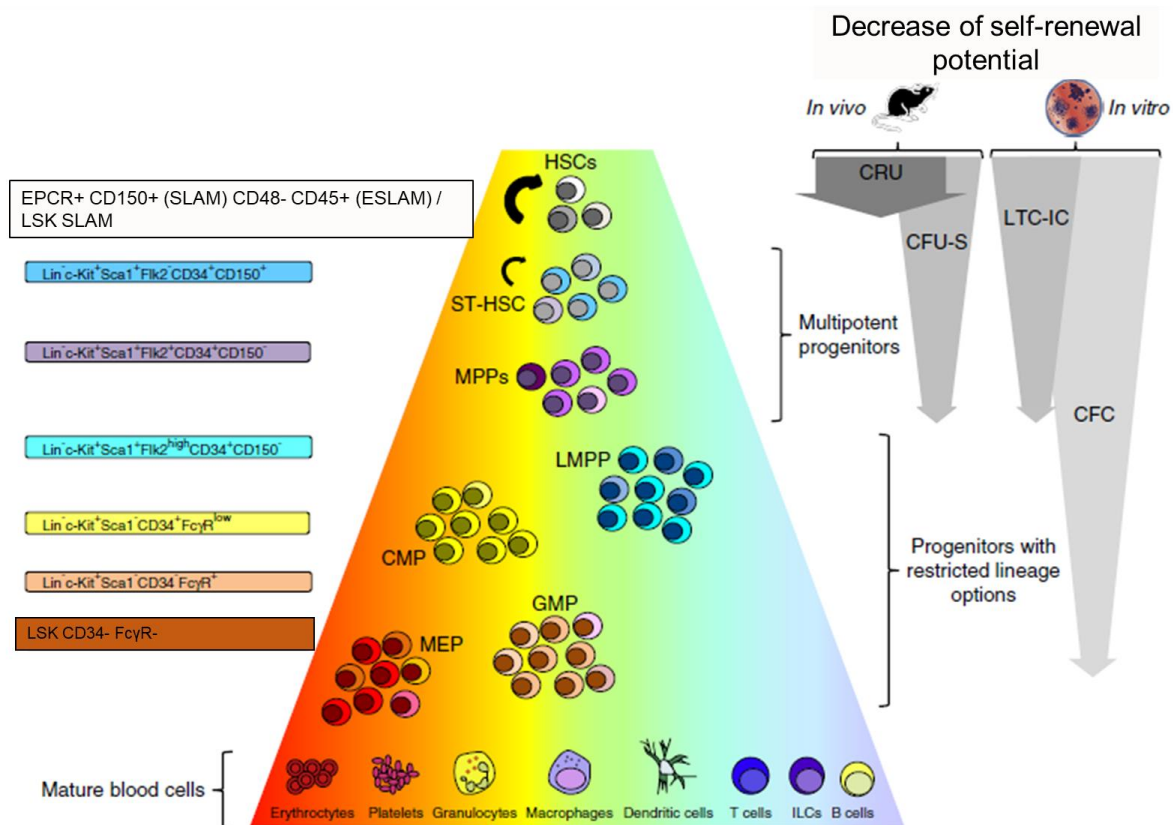


Figure 1.3. Haematopoietic differentiation as a hierarchical model. Primitive HSCs (defined as EPCR⁺CD45⁺CD48⁻CD150⁺, “ESLAM”, or Lin⁻c-Kit⁺Sca-1⁺CD48⁻CD150⁺, “LSK SLAM”), are found at the apex of the hierarchy. HSCs are multipotent and can therefore generate cells of all blood lineages; they also possess extraordinary self-renewal capacity, which they preserve throughout many divisions along an individual’s lifetime, as per demonstrated by stringent *in vivo* functional assays. Short-term HSCs (ST-HSCs) and MPPs are still multipotent, however, their self-renewal potential grows gradually restricted, as they give rise to derivatives committed towards the lymphoid (LMPPs) or myeloid/erythroid lineages (CMPs, MEPs, GMPs). Due to the large heterogeneity within the HSC pool, the system should be better understood as a continuum of cell fate choices, rather than a linearised process (see section 1.3.2.1.2). To the left side of the panel are shown different combinations of cell surface markers for the purification of each of the corresponding progenitor cells by flow cytometry. To the right side of the panel, is the nomenclature for long-term self-renewing cells as per *in vitro* and *in vivo* functional data. Figure adapted from Kent & Eaves, 2016.

1.3.2.1 Redefining haematopoiesis

As previously mentioned, the historical view of haematopoiesis describes it as a hierarchy with HSCs at the top, which through oligopotent progenitors, replenish the entire blood cell system, giving rise to erythrocytes, megakaryocytes, monocytes, neutrophils, and lymphocytes (Brown et al, 2018; Orkin & Zon, 2008; Safina & van Galen, 2024). This unidirectional view of haematopoiesis has been challenged by the wide repertoire of cell fate choices available to HSCs, and how these affect the progression of multipotent and oligopotent progenitors down the haematopoietic pathway (Ceredig et al, 2009). In this section, I will discuss several different models proposed for haematopoiesis in light of more recent advances in the blood stem cell field.

1.3.2.1.2 A continuum of cell fate choices

One of the first studies highlighting the need to revise the haematopoietic lineage commitment model, analysed the lineage contribution of human Lin⁻Sca-1⁺c-Kit⁺CD34⁺ (LSK(CD34⁺)) cells, expressing Flt3, a tyrosine kinase receptor with essential functions for haematopoietic cells (Adolfsson et al, 2005). LSKFlt3⁺ cells had myeloid-lymphoid potential, but lacked megakaryocytic/erythroid potential, which suggested that lineage restriction did not result in progenitors with exclusive myeloid and lymphoid potential, but that HSCs with erythroid and megakaryocytic potential must have resulted from asymmetric divisions of LSK(CD34)Flt3⁻ HSCs in the first instance, which started to challenge the classical tree-like model of haematopoiesis. Other studies have also reported myeloid CD150⁻Flt3⁺ progenitors with lymphoid potential (Chi et al, 2011), as well as CD127⁺ lympho-myeloid multipotent progenitors (LMPPs CD127⁺) more efficient than common lymphoid progenitors (CLPs) at generating innate lymphoid cells (ILCs) and T-cells, which has suggested an independent origin of ILCs from the haematopoietic hierarchy (Ghaed et al, 2016). These findings, together with the existence of thymic progenitors with both lymphoid and myeloid potential, provide evidence for HSC plasticity and that haematopoietic cell fate choice is not irreversible (Ceredig et al, 2009). Studying foetal and adult haematopoiesis stem and progenitor cells, Notta and coworkers proposed a redefinition of the haematopoietic hierarchy, as most of the cells found within the foetal stem and progenitor compartments showed multilineage potential, whereas in the BM, multipotent progenitors were restricted to the stem cell compartment, and unipotent progenitors were more abundant in the progenitor compartment (Notta et al, 2016).

Indeed, molecular profiling of different types of multipotent and oligopotent progenitors have shed light on the *continuum* of fate choices available to HSCs. Velten and colleagues profiled

single human HSCs and MPPs (Lin⁻CD34⁺CD38⁻) and committed progenitors (Lin⁻CD34⁺CD38⁺) by scRNA-seq, flow index-sorting and *in vitro* functional assays (Velten et al, 2017). HSCs and their immediate progeny were molecularly ‘interconnected’, i.e., they shared a number of molecular similarities that distinguished them from populations in the Lin⁻CD34⁺CD38⁺ compartment, implying that HSCs and MPPs are part of a ‘continuum’ of haematopoietic stem and progenitor cells (HSPCs), and that discrete populations only begin to emerge at later stages in the differentiation roadmap. A parallel study reported the transcriptional profiling of over 4000 HSPCs isolated from human cord blood, including LMPPs, multi-lymphoid progenitors (MLPs), common myeloid progenitors (CMPs), granulocyte-myeloid progenitors (GMPs), megakaryocyte-erythroid progenitors (MEPs) and B/Natural killer (NK) cell progenitors (Karamitros et al, 2018). Accordingly, these three types of progenitors constituted a transcriptional continuum, where GMPs and MLPs were distanced from each other, but intersected with the LMPPs. *In vitro* functional assessment revealed multiple differentiation potentials for each of the progenitor types, which likewise suggested that they were not confined to a single-lineage choice.

Whereas most of the groundbreaking research was being performed using human HSPC models, experimental findings with murine HSCs have likewise significantly contributed to the renewed view of the haematopoietic continuum. Grinenko and colleagues traced the progeny of stem and progenitor cells in transplantation (Grienenko et al, 2018). These experiments revealed that HSC can develop and undergo differentiation into ST-HSCs, MPPs and myeloid progenitors while in the G0/G1 phase of the cell cycle, namely, before cell division occurs. A similar approach was undertaken in a study of thrombocyte development in zebrafish, where the authors coupled scRNA-seq data with flow index-sorting data of CD41⁺ thrombocytes (equivalent to megakaryocytes in humans) and delineated myeloid differentiation by ordering the transition of progenitors according to their transcriptional programmes, offering a redefined view of haematopoiesis in vertebrates (Macaulay et al, 2018).

1.3.2.1.3 Haematopoiesis revisited

“The path followed by the ball, as it rolls down towards the spectator, corresponds to the developmental history of a particular part of the egg. There is first an alternative, towards the right or the left. Along the former path, a second alternative is offered; along the path to the left, the main channel continues leftwards, but there is an alternative path which, however, can only be reached over a threshold.”

C.H. Waddington in *The Strategy of the Genes*, 1957.

In the aim of describing development in algebraic terms, Waddington wrote these words to refer to his multidimensional “epigenetic landscape” of the “phase-space” diagram explaining development, in which each dimension corresponds to an organ or anatomical region of the body. These remarks by Waddington now underlie the newly proposed “Waddington-continuum” model of haematopoiesis (Haas et al, 2018), where cell states are transitory and not stationary, and these are determined by an interplay between transcriptional and epigenetic factors, in which cells continually acquire lineage biases multi-directionally, without necessarily undergoing discrete differentiation states (Cheng et al, 2020). Importantly, as reviewed in Laurent & Gottgens, there are currently two-dimensional and tri-dimensional ways to visualise haematopoiesis:

- 1) Two-dimensional - in the early stages of haematopoiesis, HSCs together with MPPs follow distinct continuous trajectories. These cells and progeny pass through different compartments defined by cell surface marker expression, where most of the progenitors are unilineage, but a small fraction have been found to be bi- or tri-potent.
- 2) Tri-dimensional - the differentiation journey of a single HSC can be modelled by considering its cell division, history and degree of progenitor expansion throughout the myeloid, lymphoid, erythroid and megakaryocytic lineages.

Nonetheless, a new ‘multi-track’ landscape of murine haematopoiesis has recently been proposed (Lin et al, 2024). The authors used a multi-stage barcoding approach, in which they labelled early HSPCs, including HSCs and MPPs, as well as oligopotent progenitor cells; the different types of progenitors were transplanted into sublethally irradiated recipients and their progeny was analysed at different timepoints. The results showed that cells traverse between defined cell states, and that time is a strong clonal determinant of cell fate choice, suggesting that clonal potency decreases with time. Thus, from this data it is possible to understand that haematopoietic differentiation can also occur in ‘tracks’ of cell states; across a series of multiple tracks, there are progenitor cells which can be multipotent, but it is also equally possible that some of them have already committed to a specific differentiation trajectory. A recent review by the van Galen group, highlights the strength of this new model, although in native haematopoiesis (non-transplantation setting), transition down canalised tracks might transform into horizontal track transitions of cell states (Safina & van Galen, 2024).

These new models account for the vast heterogeneity observed in the HSC and MPP pools, as well as differences in multilineage potential and output, and are accurately informed by transcriptomic snapshots correlating with time (Laurenti & Gottgens, 2018). Nevertheless, as understanding of the haematopoietic landscape dynamically evolves, the tree-like or branch

model is not to be definitively dispelled, as the existence of hierarchical arrangement to haematopoiesis still holds, and it helps conceptualise the fundamental principles of blood cell production.

1.3.3 Developmental origins of haematopoietic stem cells

1.3.3.1 Emergence of HSCs in the mammalian embryo

Mammalian haematopoiesis occurs in partially overlapping waves and across different anatomical sites; it is mostly driven by conserved molecular mechanisms found in almost all vertebrate species (Calvanese & Mikkola, 2023; Ivanovs et al, 2017; Ciau-Uitz et al, 2014). The first committed erythroid-myeloid progenitors (EMPs) and mature erythrocytes emerge from the yolk sac blood islands, around day 7-7.5 post-conception in mice and around the day 15-16 of gestation in humans, which corresponds to the primitive wave of haematopoietic development (Robin et al, 2002; Palis & Yoder, 2001; Dziezark & Speck, 2008). However, cells with durable self-renewal potential can be isolated from mouse fetal liver at day 14-14.5 (E14-14.5) or from foetal BM around day 18 post-conception (E18) (Holyoake et al, 1999; Benz et al, 2012; Copley & Eaves, 2015). During the second wave of haematopoietic development - the transient definitive wave - multipotent EMPs emerge, with the potential to differentiate into mature erythrocytes, macrophages and megakaryocytes (Weijts et al, 2021).

Moreover, the aorta-gonada-mesonephros (AGM) is an embryonic structure of critical importance for the emergence of HSC prior to migration and colonisation of the embryonic liver (Medvinsky & Dzierzak, 1988; Ciau-Uitz et al, 2014); in addition, work by the Medvinsky laboratory has shown that HSC activity in the AGM increases at day 11 of mouse embryonic development (Taylor et al, 2010). Likewise, studies in zebrafish have directed attention to the dynamics of blood flow, as well as the released compounds that regulate blood flow, and showed that these factors influence expansion of HSCs in the AGM region (North et al, 2009). Thus, the AGM has been noted to be the site of emergence of definitive HSCs, which sustain haematopoietic cell production throughout adult life; however, recent studies have reported a minimal contribution to embryonic haematopoiesis as it had been previously hypothesized (Ulloa et al, 2021). As they emerge, developmental HSCs are required to undergo a process of maturation, which would eventually lead them into their future niches, where they will engraft and expand rapidly (Mikkola & Orkin, 2006). Following from the generation of HSCs in the AGM region, the chorioallantoic placenta provides a temporary niche for the establishment of the HSC pool in mouse conceptus, with HSCs being supplemented from other sites such as the dorsal aorta and the umbilical cord (Mikkola et al, 2005).

1.3.3.2 HSC colonisation of the foetal liver

During endothelial-to-haematopoietic transition (EHT), haematopoietic clusters of haemogenic endothelial cells - which contain haematopoietic precursors, including HSCs - bud off from the haemogenic endothelium, and enter circulation to then migrate and colonise the foetal liver, starting at late E9 and continuing on to the late gestation period (Yokomizo et al, 2022; Dziezar & Speck, 2008; Lewis et al, 2021). The foetal liver comprises a diverse mixture of cells of haematopoietic and hepatic origin, as well as endothelial cells, which support the development and expansion of HSCs and multipotent progenitors residing in the liver (Lewis et al, 2021). Recently, it has been reported that mesothelial cells (MCs) as an important source of Kit ligand - also stem cell factor, SCF - which is essential for regulation of haematopoiesis and maintenance of the HSC pool (Mesquita-Peixoto et al, 2025; Matsuoka et al, 2023). In the same way, HSCs have been found to reside close to Nestin-expressing stromal cells, which equally contribute to HSC expansion in the foetal liver (Khan et al, 2016).

Once the HSCs have homed to the liver, HSCs massively expand - up to 38-fold - from E12 to E16, in contrast to adult HSCs, which are mostly quiescent (Lewis et al, 2021). Furthermore, functional heterogeneity has been reported in foetal liver HSCs, since they also show the α , β , γ , and δ patterns of long-term repopulating HSCs in adult BM (Benz et al, 2012); nonetheless the alpha subtype is less frequent than in adult BM. Transplant studies of foetal liver HSCs have revealed a two-fold increase in the donor-derived granulo-myeloid fraction in comparison to adult BM (4 and 8-12 weeks old) in mice, but similar to 3-week old BM, which suggests that HSCs undergo a developmental switch towards adulthood, where their proliferation potential decreases and they acquire a more quiescent phenotype (Bowie et al, 2007; Copley & Eaves, 2013). These properties of HSCs residing in the murine foetal- liver are vital for the establishment of the pool which would eventually sustain lifelong haematopoiesis, in junction with other haematopoietic precursors (approximately 600-700 precursors, including HSCs) generated in the AGM and the yolk sac (Ganuza et al, 2017). In a similar fashion, HSCs transit through different haematopoietic sites throughout development, from AGM, to the yolk sac and final seeding of the liver, which is governed by molecular programmes which distinguish the development of HSCs from other haematopoietic precursors (Calvanese et al, 2022).

1.4 Clonal tracking and lineage tracing of the blood-forming system

The long-standing question of how tissues form and how the history of cellular progeny can be traced back in time has prompted the development of high-throughput lineage-tracing methods, which range from transplantations for functional characterisation of stem cells to the use of naturally occurring mutations as ‘barcodes’, which are uniquely shared between the lineage. In addition, the advent of single-cell technologies together with powerful computational tools, have accelerated the optimisation of clonal tracking methods, which are increasingly being implemented on the study of haematopoietic as well as other stem cell systems across a wide range of species. These tools make it possible to map cell fate and cellular differentiation pathways, which are all crucial for shaping how we understand the complex dynamics of HSC behaviour and haematopoiesis.

1.4.1 The diversity of lineage tracing methods

Table 1.1 summarises landmark methods throughout lineage tracing history (Chen et al, 2022; Han-Deng et al, 2025; VanHorn & Morris, 2021; Gabbutt et al, 2022; Hsu, 2016; Eaves, 2015; Purton & Scadden, 2007), by categorising techniques into three groups: 1) Direct observation 2) Chimaeras and genetic mosaics and 3) Molecular biology methods, including key contributions to the field of HSC biology, as well as wider applications in the fields of stem cell, tissue and developmental biology.

The earliest lineage tracing experiments were developed at the end of the 19th century, and by the first decade of the 20th century, Charles Whitman’s group published the first report on embryological cell tracing, applying light microscopy techniques to carefully study embryos of different invertebrate species (Child, 1905; Kretzchmar & Watt, 2012). Furthermore, the nematode *Caenorhabditis elegans* gained enormous popularity as a model system in biology in the 20th century, contributing to tremendous discoveries such as apoptosis as a tissue sculpting mechanism during development (Putcha & Johnson, 2004). Importantly, the first complete embryonic lineage tracing was performed on *C. elegans* at the beginning of the 1980s (Sulston et al, 1983), and two decades later, the first automated lineage tracing method based on tri-dimensional time-lapsing system was created using the developing *C. elegans* as a model (Bao et al, 2006). Other species, such as *Danio rerio* - the zebrafish - have been an advantageous model for direct observation methods thanks to the relative transparency of the embryos, which permits tracking of cellular lineages without the addition of markers or genetic modifications (Kimmel et al, 1990).

In the field of HSC biology, the gold-standard method for the functional characterisation are transplantations, whether at bulk or clonal scales, these have allowed the tracing of cellular lineages and have incorporated the use of barcoding systems and multicolor labelling. At the same time, lentiviral barcodes, transposon and recombinase systems have allowed to track the progeny of cells in native haematopoiesis (i.e., non-transplantation setting). In the next sections, I will discuss the main methods that have enabled lineage tracing and clonal tracking of HSCs.

Table 1.1 Lineage tracing methods					
Category	Method	Model organism	Type of dye/marker/ barcoding/ cell or tissue	Representative examples	Key studies
Direct observation	Light microscopy	Nematodes, amphibians, zebrafish	Marker-free	Light microscopy techniques	Child, 1905; Sulston et al, 1983; Kimmel et al, 1990
	Non-selective dyes	Mice, amphibians, humans, avians	Vital dyes	Carbocyanine, dextrans, peroxyases (HRP)	Serbedzija et al, 1989; Weisblat et al, 1978
	Radioactive labelling	Mice, humans	Radioactive compounds	Bromodeoxyuridine (BrdU)	Pietrzyk et al, 1985
		Mice, humans	Carbon dating	Carbon 14 (^{14}C)	Spalding et al, 2005
	Live imaging		Fluorescent proteins	Bioluminescent proteins - GFP, RFP, YFP	Shimomura et al, 1962; Campbell et al, 2002

Chimeras and genetic mosaics	Transplantation of adult cells and tissues	Mice, humans	Stem cell transplantation (haematopoietic and other tissues)	Bulk and clonal (single-cell) functional assays	Till & McCulloch, 1961; Harrison, 1980; Osawa et al, 1996; Kent et al, 2009
	Transplantation of embryonic tissues	Amphibians, avians	Inter-species transplantation	Embryo transplantation	Spemann & Magold, 1924; Waddington, 1932
	Genetic mosaics	<i>Drosophila melanogaster</i>	Enzymatic markers	Developing embryo and adult tissues	Lawrence & Johnston, 1986; Stowers & Schwarz, 1999
Molecular Biology	Multicolour labelling	Mice	Recombination systems	Rosa26-confetti, <i>Braimbow</i>	Livat et al, 2007; Snippert et al, 2010
	Genetic barcodes	Mice and human	Integration barcodes	<i>Sleeping beauty</i> (transposon, LARRY (lentiviral))	Rodriguez-Fraticelli et al, 2018; Weinreb et al, 2020
			Lox and CRISPR-Cas9-based	<i>Polylox</i> , <i>Polylox express</i> ,	Pei et al, 2017; Pei et al, 2020;

				CARLIN, DARLIN	Bowling et al, 2020; Li et al, 2023
			Natural barcodes	Somatic mutations in cellular genome	Lee-Six et al, 2018; Chapman et al, 2021
				Mitochondria l genome mutations	Ludwig et al, 2019; Weng et al, 2024

1.4.1.1 Haematopoietic lineage tracing in transplantation assays.

Short-term *in vivo* assays for understanding the effects of radiation on BM activity can in principle be attributed to Jacobson and colleagues (Jacobson, 1951), and a decade later to Till and McCulloch (1961), where they elucidated the multilineage reconstitution potential of a population residing within the mouse BM. However, with the immunophenotypic identification of the putative HSC (Spangrude et al, 1988), the prospective isolation of HSCs (Osawa et al, 1996), the development of more advanced flow cytometry techniques, monoclonal antibodies and the generation of mouse strains suitable for transplantation, long-term transplantation assays were established, and thus the burgeoning area of quantitative stem cell research was consolidated (Perry & Li, 2010; Eaves, 2015). Furthermore, it was demonstrated that single HSCs with durable transplantable potential were able to contribute to haematopoiesis for at least 16 weeks at a defined threshold of 1% donor-derived circulating white blood cells (WBCs), namely, the granulocyte, myeloid and lymphoid fractions (Miller & Eaves, 1997; Morita et al, 2000). Hence, long-term transplantations are the most suitable technique to test the durability of active contribution of haematopoiesis by a single cell or a progenitor population.

The competitive repopulation assay was the first type of long-term reconstitution transplantation assays to be developed (Harrison, 1980; Szilvassy et al, 1990). In this assay, the number of donor repopulating units (repopulating HSCs) found in a given BM source are calculated against a known number of competing HSCs in the BM of congenic mice; however, this calculation is not exact and it is not possible to determine the progeny produced by every HSC present in the donor BM source (Purton & Scadden, 2007). A modification of the

competitive repopulation assay, the limiting dilution assay (LDA) was designed in order to overcome the quantitative limitations of the former: in LDA, a number of titrated doses of donor BM cells of unknown HSC content, along with competitive population of BM cells, which becomes the standard for calculating the reconstitution potential of the donor BM cells (Harrison et al, 1993). The mean of donor contribution to multilineage blood production is compared to functional potential of the competitor population, and thus the frequency of HSCs (in this assay, competitive repopulation units, CRU) is calculated by Poisson statistics, which requires a minimum of three doses with positive (repopulated) and negative (non repopulated) recipients, in order to make exact quantitative estimations (Taswell, 1984), which can also be executed using online tools available today (Hu & Smyth, 2009).

In light of the substantial heterogeneity of the HSC pool, and the diversity of functional outcomes of each subtype of reconstituting cell, it is imperative to stringently identify the most primitive HSC which can sustain haematopoiesis (Rosendaal et al, 1979), particularly since it has been reported that, in competitive reconstitution assays, merely one or two clones contribute to overall haematopoiesis (Lemischka et al, 1986). Still to this day, serial transplantation has proven to be the most stringent and reliable method to determine stemness and multilineage reconstitution potential of a given population or single cell - it formally proves that one HSC gives rise to many HSCs that can sustain themselves for the lifetime of the animal. In some cases, multiple rounds of sequential transplantations have been performed, allowing for truly long-term functional profiling (up to 48 months or four sequential transplants) that reaches well beyond the lifespan of a mouse and the identification of the best-performing - functionally speaking - HSCs or HSPCs. This has in consequence a myriad of applications, from the study of healthy (Rundberg Nilsson et al, 2015) and malignant hematopoiesis (Kotani et al, 2019), young and aged haematopoietic stem cells (Kamminga et al, 2005), paired daughter HSC assays (Sieburg et al, 2011); and production of adult HSCs from induced pluripotent stem cells (iPSCs) (Ng et al, 2024).

1.4.1.1.1 Linking transcriptome to function

While it is crucial to dissect the functional heterogeneity of the HSC pool, one cannot negate the fact that it is equally as important to decipher the molecular changes that largely impact the way HSCs mature, which in consequence determines their relative contribution to different blood lineages and underlies the dynamics of haematopoiesis. Single-cell technologies have undoubtedly revolutionised every area of the life sciences (Proserpio & Lonnberg, 2014; Sandberg, 2014), as well as every area of biomedical research since they gained momentum over a decade ago. That said, it is challenging to obtain full transcriptome coverage in single

cells, particularly quiescent HSCs. Nevertheless, methods such as the microfluidics-based C1 fluidigm (DeLaughter, 2018), SmartSeq2 (Picelli et al, 2013), and the droplet-based 10X Genomics platform (Zheng et al, 2016) are high-throughput, large-scale, highly efficient and highly sensitive, covering a wide variety of cell types and tissues. This has evidently had a substantial impact in the study of stem cell ageing (Young et al, 2016; Sun et al, 2014); developmental origins of HSCs (Moignard et al, 2015; Baron et al, 2018), extending to different model systems, such as zebrafish (Athanasiadis et al, 2015).

A landmark study in the field integrated index-sorting (phenotype), transcriptomic (single-cell gene expression and RNA-seq) and functional (single-cell transplants) assays to characterise different HSC and HSPC populations, which were isolated through four different sorting strategies, in addition to a finite-self renewal HSC population (FSR-HSC), along with four progenitor populations - common myeloid progenitors (CMPs), myeloerythroid progenitors (MEPs), granulomyeloid progenitors (GMPs) and lymphoid-primed multipotent progenitors (LMPPs) (Wilson et al, 2015; Qiao & Zandstra, 2015). Data integration allowed the authors to develop a computational approach in which successfully engrafting HSCs were found to share a highly specific gene expression pattern, with higher CD150 and Sca-1 expression inferred from index-sort data. Further analysis revealed a homogeneously expressed gene signature in these cluster, which the authors termed the Molecular Overlap (MoO signature), comprised of 28 genes: main genes for driving data variance were *Procr* (EPCR) and *Ly6a* (Sca-1) in component 1, whereas *vWF* (von-Willebrand factor) influenced component 2 variance, which have all been reported to be associated with stem cell activity (Balazs et al, 2005; Sanjuan-Pla et al, 2013; Morcos et al, 2017). Using sophisticated computational analysis tools, members of the same research group published one of the first transcriptomic atlases of thousands of haematopoietic stem and progenitor molecular profiles at single-cell resolution, linked to surface marker expression profiles, providing interactive visualisation of gene expression across the HSC/HSPC differentiation landscape (Nestorowa et al, 2016), which supported the concept of haematopoiesis as a continuum of cell fate choices throughout time.

A more recent study sought to profile *in vitro* expanded clones derived from single HSCs, in order to equally correlate molecular features with repopulating activity (Che et al, 2022). Using the F12-PVA based *ex vivo* expansion method created by Wilkinson and colleagues (Wilkinson, 2019), the authors classified phenotypic HSCs (pHSCs) in culture by expression of cell surface markers, namely, EPCR⁺Lin⁻Sca-1⁺c-Kit⁺ (ELSK) cells, as well as non-ELSK cells. Results demonstrated a direct correlation between the relative contribution to the granulo-myeloid lineage and the number of HSCs injected per successfully engrafted recipient. In addition, engrafting ELSK cells shared molecular features with freshly isolated HSCs, which derived in

a Repopulation (Repop) signature for expanded HSC clones, highlighting novel molecular features such as high expression of the endothelial cell-selective adhesion molecule (ESAM) (Ueda et al, 2019; Ishibashi et al, 2016). Profiling of distinct cellular states - active vs quiescent and adult vs foetal - showed that RepopSig score was comparable between hibernating HSCs (Oedekoven et al, 2021) and cycling foetal liver HSCs, which was also quite similar to cytokine-stimulated cultured HSCs.

1.4.2 Cellular barcoding

1.4.2.1 Endogenous labelling

Historically, transplantations have typically relied on the CD45 congenic system, where the donor cells would differ from the recipient cells in the CD45.1 or CD45.2 epitope, which allows for the discrimination between donor and recipient cells. Notwithstanding, cells from the erythroid and megakaryocytic fractions do not express the CD45 surface marker (Saunders & Johnson, 2010), which limits the detection of erythrocyte and platelet production in transplant recipients. Methods such as flat bed electrophoresis were useful to detect glucose-6-phosphate isomerase 1 (Gpi1) in donor-derived anucleated cells (i.e., erythrocytes) to discriminate between CD45.1/CD45.2 genotypes (Benveniste et al, 2010; Trevisan et al, 1995). However, considering the complexity of HSC functional heterogeneity, such methods have had to be replaced, thereby prompting the design and development of transplantation assays incorporating state-of-the-art molecular biology techniques, in order to overcome the limitations of the traditional form of the assay and to delineate an all-encompassing perspective of haematopoiesis.

The insertion of lentiviral constructs into HSCs (Dick et al, 1985; Keller et al, 1985) facilitated the distinction between HSCs with transplantable potential from the ones lacking such potential in different haematopoietic tissues. To achieve this, a large number of cells are required, since DNA expression is assessed by Southern blotting or polymerase chain reaction (PCR), for which the cellular input is in the scale of 1×10^6 or higher, which is prominently difficult to achieve with a rare population such as HSCs. Cellular barcoding technologies allow for the tagging or labelling of an insertion site into the host's genome, this *barcode* is heritable, and can therefore be found across the entire progeny of a single cell (Gerrits et al, 2010; Naik et al, 2013; Tak et al, 2021). The Weissman laboratory executed a functional assay with barcoded HSCs (Lu et al, 2011) where 9000 HSCs were transplanted into 7 myeloablated recipients, and clonal tracking was followed for up to 22 weeks. The results demonstrated that around 30-50 barcodes which labelled 10 different types of HSPCs were recovered,

suggesting that 50-80 cells were contributing to haematopoiesis. Contribution of HSCs to a specific lineage was also made possible by another study using cellular barcoding, which showed that the number of barcodes in granulocytes was directly related to the number of HSCs transplanted, but these were reversed in lymphocytes, in which the number of barcodes declined as HSC dose increased (Verovskaya et al, 2013).

Transgenic mouse lines of developmental haematopoiesis (Schmitt et al, 2014), as well as adult haematopoiesis, such as the *Kusabira-Orange* (KuO) line, were key in pioneering studies which for the first time clonally traced the production of multiple lineages, including erythrocytes and platelets, and were shown to have normal haematopoiesis, namely, it was not affected by the transduction procedure (Hamanaka et al, 2013). In a later study likewise conducted by the Nakauchi group, LSK KuO⁺ cells were fractioned into three populations according to their CD150 and CD41 expression (Yamamoto et al, 2013). They showed that progenitors with megakaryocytic potential were of the immediate progeny of HSCs, as these were uniquely found in the CD150⁺CD41⁺ and CD150⁺CD41⁻ fractions, which was evident *in vitro* and *in vivo*.

1.4.2.2 Ex vivo labelling

Moreover, since the discovery of the *Cre* recombinase in bacteriophage virus by Sternberg and colleagues (Sternberg et al, 1986), which revolutionised the field of genetic engineering, researchers have relied on this system for a simplified recombination method of stable barcode integration (Yarmolinsky & Hoess, 2015; Weber et al, 2016). Indeed, one of the most prominent examples of recombination *in situ* is the *polylox* barcoding method (Pei et al, 2017). *Polylox* consists of a cassette of loxP sites carrying DNA elements, which through the cre-excision system, can generate hundreds of thousands of barcode sequences which tag single cells. This was soon followed by the *PolyloxExpress* allele method, which facilitated RNA recombination *in situ* by *Cre*-mediated recombination in HSCs at different stages of development: foetal, newborn and adult, upholding the haematopoietic model as a bifurcating tree (Pei et al, 2020).

1.4.3 Clonal tracking through sequencing technologies

Clonal fate mapping and the capture of cellular states to trace haematopoietic differentiation across time have been made possible by advanced viral barcoding and single-cell sequencing methodologies (Kester & van Ourdenaarden, 2018). Conducting a large study on paired daughter cells, Weinreb and colleagues designed a modified lentiviral heritable barcoding strategy in which barcodes contain an EGFP transgene, which can be detected by scRNA-

seq, which they termed *lineage and RNA recovery* (LARRY), since it did not diminish the ability to capture state and fate of paired daughter cells, *in vitro* and *in vivo*, both in transplant assays and native haematopoiesis (Weinreb et al, 2018).

The CRISPR-Cas9 genome-editing system also offers high-throughput integration of target sequences in the genome, which undergo ‘scarring’ mediated by guide RNAs (gRNA) directed by the bacterial nuclease Cas9 (Schmitt et al, 2014). The genomic scars then constitute a unique barcode which would have been produced during a certain period of time, thus enabling massive parallel tracing of thousands of single cells with minimal overlap, and consequently the construction of ‘trees’ of clonal fates in cellular systems (Spanjaard et al, 2019; Kester & van Ourdennarden). Although the primary applications of CRISPR-Cas9 lineage tracing were initially on cultured cells and invertebrate models such as zebrafish (McKenna et al, 2016) the generation of genetically defined, CRISPR-based mouse models has overcome the technical challenges of incorporating this method to mammalian models. In a mouse model of inducible lineage tracing, the CRISPR array repair lineage tracing (CARLIN) system generated transcribed barcodes which could be induced at any point during embryonic development or adulthood, enabling sequential phylogenetic analysis, clonal tracking of blood progenitors across time, and identification of molecular signatures which correlate with HSC functional heterogeneity (Bowling et al, 2020). A further developed mouse model, DARLIN, an *improved* lineage tracing mouse line, consisting of a Cas9 inducible model, and CRISPR-terminal deoxynucleotidyl transferase (TdT) target sites, resulting in massive barcode generation across multiple tissues and single-cell profiling (Li et al, 2023).

Together, all of these studies represent a new generation of haematopoietic lineage tracing technologies, linking cellular fates with cellular states for the first time in over 70 years of HSC research at unprecedented resolution. However, the inability to detect clonal relationships in detail, in addition to the susceptibility to random integration of sequences/barcodes, array deletions or modifications, and the highly variable number of barcodes generated by these methods (either inducible or heritable) pose technical challenges which as yet have not been completely resolved.

1.4.3.1 Somatic mutations as natural barcodes

Undoubtedly, every human being is unique, with unique physical and personality traits that contribute to the great and wonderful and overwhelming diversity of the human race. What is even more fascinating is that every human is a diverse mosaic of cells which, even within the same tissue, exhibit unique features as single, discrete entities. This diversity can be attributed

in part to somatic mutations, which are genetic events which occur spontaneously during normal cell divisions (Martincorena & Campbell, 2016), constituting the fundamental processes of cell fate choices and tissue formation at the single-cell level (Martincorena, 2019). Paradoxically, the excessive, uncontrollable accumulation of mutations throughout an individual's lifetime is, in principle, the root cause of ageing and disease, which makes the study of mutational processes in healthy tissues vital to understand the genomic changes which lead to malignancy and other age-related disorders (Wijewardhane et al, 2021). Furthermore, it has become increasingly evident that the detection of a specific mutation shared across multiple cells descending from a single clone unveils the process of somatic evolution in complex systems, such as the haematopoietic hierarchy. In this section, I will explain how naturally occurring somatic mutations can be used as cellular barcodes and how - through cutting-edge computational and sequencing technologies - clonal relationships can be inferred from such mutational acquisition and tracking.

1.4.3.2 Mutational processes in normal tissues

Eukaryotic DNA synthesis is tightly regulated and is orchestrated by a collection of high-fidelity enzymes - specifically, DNA polymerases, which accurately replicate DNA copies with each cell division (Bainbridge & Daigaku, 2024). Understandably, as extraordinarily precise as they are, normal DNA polymerases are still prone to error - albeit minimal: 10^{-10} mutations per base pair per cell division (Preston et al, 2011). In conjunction with errors in DNA repair mechanisms, these mutations accumulate in the genome of a cell over time, and will subsequently be present in the genome of all progeny descended from the cell where the original genetic event occurred (Figure 1.4) (Lee-Six & Kent, 2020; Manders et al, 2021; Welch et al, 2012). For more on how this approach can be leveraged to reconstruct the life histories or phylogenies of haematopoietic clones, refer to section 1.4.3.4 and Figure 1.5).

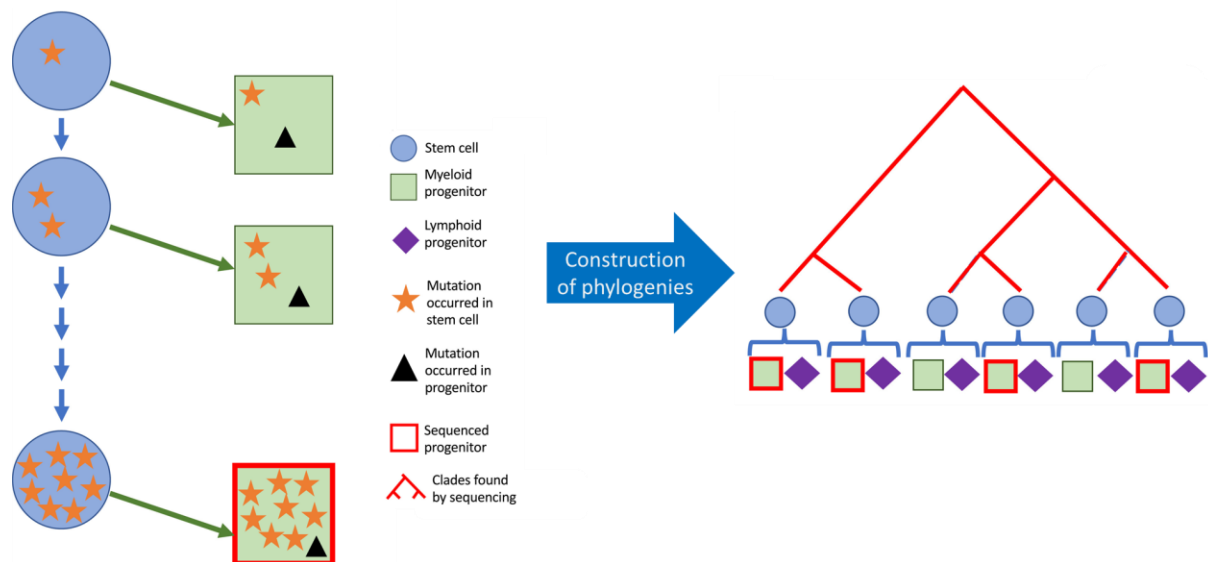


Figure 1.4. Somatic mutations as natural barcodes for clonal tracking. Haematopoietic stem cells accumulate mutations with time in a linear fashion. A constant acquisition rate allows for their use as endogenous genetic barcodes by the means of whole genome sequencing. Mutations acquired in HSCs (left hand side of the panel) will be present in their HSC progeny, as well as downstream haematopoietic progenitors, which are equally prone to acquire mutations. Therefore, sequencing of progenitors will inform about their relatedness to one another, as well as the HSCs from which they descended, representing monophyletic groups or *clades* (refer to section 1.4.3.4 and Figure 1.5 for an explanation of phylogenetic trees). Figure Adapted from Lee-Six & Kent, 2020.

According to their structural nature, post-zygotically acquired mutations manifest as small or large genome sequence changes, ranging from small single-nucleotide variants (SNVs), small insertions and deletions (Indels), to large structural variations (SVs) and chromosomal rearrangements (Ren et al, 2022; Herms & Jones, 2023). These mutations are constantly acquired from the moment of conception to the end of an individual's life, and accumulate linearly with time (Maanders et al, 2021). Given that these mutations are present even when the cellular lineage exhibits normal phenotypic features, a useful terminology to discriminate between them was proposed by Stratton and colleagues, in which they coin the terms *passenger* - also referred to as *neutral* in Herms & Jones, 2022) - and *driver* mutations (Stratton et al, 2009). Driver mutations confer a selective advantage to the cell in which they originated and therefore enable clonal expansion, which contributes to malignant transformation. In contrast, passenger mutations do not confer any advantage over other cells, although they might still be present in the cancer genome as not all mutations have a functional outcome in oncogenesis. Moreover, as a result of relapse from chemotherapy, some cancer cells can also acquire chemotherapy-resistance mutations, which differ from driver mutations as they are not the causal event for oncogenesis, but a consequence of treatment (Nussinov et al, 2021).

The earliest indications of the presence of somatic mutations in non-cancerous tissues were reported in chronically sun-exposed skin, detected by immunostaining techniques (Jonason et al, 1996; Nakazawa et al, 1994). Using deep targeted sequencing approaches, two decades later, Martincorena and coworkers showed the occurrence of mutations in several cancer driver genes in skin biopsies of healthy individuals (Martincorena et al, 2015), in addition to the selective clonal expansion of cells which harboured mutations in these genes. By profiling the mutational landscape of healthy oesophageal epithelium, members of the same research group demonstrated that mutations in *NOTCH1*, a well-known oesophageal cancer driver gene, were more frequent in normal epithelium than in oesophageal cancer cells (Martincorena et al, 2018). Another significant contribution to the new paradigm for somatic mutations in normal tissues, was a large-scale, whole-exome sequencing profiling of 17,182 individuals with no history of haematological malignancies (Jaiswal et al, 2014). In this landmark study, the authors demonstrated that mutations in three well-known driver genes (*DNMT3A*, *TET2*, *ASXL1*) were rare in patients under 40 years of age, in contrast to the remarkable increase in the prevalence of these same mutations in individuals aged 60 and older. This disorder, known as age-related clonal haematopoiesis, increases the risk to leukaemic transformation, however, it can persist for years without developing into leukaemia (Jaiswal et al, 2014). The substantial importance of these studies relies on the fact that the accumulation of somatic mutations increases linearly with age, posing an entirely new paradigm in which mutation acquisition in healthy tissues and somatic evolution was understood (Martincorena et al, 2019; Wijewardhane et al, 2021).

Likewise, adult tissues are maintained by the asymmetric divisions of stem cells residing in specialised niches within their corresponding tissues. Hence, stem cells in tissues with high turnover rate, such as blood (1-120 days), intestinal epithelium (2-3 days), and oesophageal squamous epithelium (~ 11 days) are more susceptible to accumulation of mutations across time (Acha-Sagredo et al, 2022). Accordingly, Moore and coworkers found that the colonic epithelium accumulates a total of 49 single-base somatic substitutions (SBSs), followed by oesophageal epithelium, with lower mutation rates observed in adult pancreatic ducts, for example, as well as germline cells (Moore et al, 2021). Hence, since tissues with higher turnover rates exhibit a higher mutational burden, it is inferred that these mutational events are more likely generated at the stem cell level, and that these genome changes would then constitute unique mutational profiles.

1.4.3.3 Applications of whole genome sequencing in mutation discovery

The completion of the publicly funded Human Genome Project (HGP) led by Francis Collins at the dawn of the 21st century, ushered in an era of high-throughput, low-error, low-cost genome-wide sequencing, which likewise offered shorter turnaround times for data acquisition and analysis, from what used to be weeks (even months) to hours over the last 25 years (Grisham et al, 2000; Giani et al, 2020; Check Hayden, 2014). Together with the previously determined sequences of *C. elegans* (*C. elegans* Sequencing Consortium, 1998) and *S. cerevisiae* (Winzeler et al, 1999), and the publication of the annotated nucleotide sequence - although not fully determined - of *Drosophila melanogaster* by Craig Venter's laboratory (Adams et al), the application of next-generation (NGS) sequencing technologies for mutation screening and discovery, as well as functional annotation of whole eukaryotic genomes made significant contributions to the new genomics era (Tucker et al, 2009). Massively parallel sequencing technologies enable sequencing of the millions of DNA fragments which constitute an individual's entire genome (Lu et al, 2025). Among these, whole genome sequencing (WGS) provides the most comprehensive data on the genetic variation of an individuals from the same species using a reference genome, since as the name implies, since it allows sequencing of both coding and non-coding regions (Ng & Kirkness, 2010; Johnsen et al, 2013; Danovi, 2021).

The utility of WGS in the clinic was demonstrated by Ley and colleagues when they sequenced tumour cells from patients with cytogenetically normal acute myeloid leukaemia (AML), with matched samples from healthy skin cells, corresponding to each individual patient (Ley et al, 2008). Out of over 2 million single-nucleotide variants (SNVs) found in the AML cells, 97.6% were equally found the matched skin cells, which allowed for the discovery of eight new genes with acquired mutations in the patients' tumour genome, including protocadherin/cadherin family members and G-protein coupled receptors (GPCRs). Evidently, such large datasets produced unprecedented amounts of information, demanding highly sophisticated computational tools, as well as infrastructure which could harbor computational clusters with enough capabilities to cope with massive amounts of genomic data. In response to this demand, and in upon the execution of the 1000 Genomes project (The 1000 Project Consortium, 2010), tools such as BreakDancer (Chen et al, 2009) for genetic variant calling, and Sequence Alignment/Map (SAM) tools for alignment to reference genome sequences (Li et al, 2009) were rapidly developed and made available to the scientific community for analysis of population-wide screening data.

Fostered by these new data analysis approaches, it became possible to generate somatic mutation catalogues from individual patients across the cancer type spectrum. In a joint international effort, Nik-Zainal and coworkers sequenced whole genomes of patients representing a total of 21 different breast cancer genomes to generate somatic mutation catalogues (Nik-Zainal et al, 2012). Through a novel mathematical model for multidimensional data factorisation, the authors derived *mutational signatures*, five categories of independent somatic single-nucleotide substitutions mediated by different mutational processes. Subsequently, scientists from the same group sequenced over 7000 cancer genomes (507 genomes) and extracted 21 mutational signatures from ~ 4.5 million mutations by whole genome and whole exome sequencing (WES) (Alexandrov et al, 2013). Somatic mutation prevalence was inferred from all these cancers unveiling the highest mutation prevalence for melanoma and the lowest for astrocytoma. Blood cancers, including AML, acute lymphoblastic leukaemia (ALL) and chronic lymphocytic leukaemia (CLL), showed relatively lower mutation prevalence in comparison to solid tumours, and therefore exhibit distinct mutational signatures.

In line with this, the Catalogue of Somatic Mutations in Cancer (COSMIC) has sought generate mutation catalogues from thousands of cancer genomes from a worldwide population, creating the largest database of single-nucleotide base substitutions (SBS) available today (Bamford et al, 2004). Profiling nearly 22,000 genomes corresponding to almost all cancer types known to date, Alexandrov and colleagues extracted new mutational signatures from just under 80 million mutations, which now add to 53 to the current COSMIC database (Alexandrov et al, 2020). In line with a previous report, two of these mutational signatures, SBS1 and SBS5, correlated positively with age of cancer diagnosis (Alexandrov et al, 2015). These two signatures in particular belong to a class of clock-like processes, which have been shown to account for the observed accumulation of mutations in aged mammalian tissues (Moore et al, 2021; Boysen et al, 2025).

Hence, the relevance of these findings to the study of the mammalian haematopoietic system relies on the fact that somatic mutations are accumulated with age in adult tissues, which implies that these mutations, and thereupon mutational signatures, must exist in the stem cells which maintain those tissues. Moreover, since the acquisition of these mutations follows a selective process - i.e., selected mutations are prevalent throughout the progeny of a specific clone - they represent powerful tools to explore the life history of haematopoietic cells.

1.4.3.4 Phylogenetic tree-building from somatic mutations

“Evolution can only be described in terms of the characters that are changing, and it is convenient to represent such changes in a multi-dimensional space in which the population occupies a position determined by the values of the characters it exhibits”.

Cavalli-Sforza & Edwards in *Phylogenetic Analysis: Models and Estimation Procedures* (1967)

In Darwin's theory of evolution, the diversification of living organisms in the planet is a direct result of *descent with modification* (i.e., evolution), which he depicted as a branching diagram of relationships between species (Darwin, 1859; Gregory, 2008). Darwin coined the term “Tree of Life” to refer to such diagrams, representing the complex dynamics of natural selection driving the evolution and extinction of species. Reasoning that the gene frequencies in a species across times could be represented as branching trees, Cavalli-Sforza and Edwards (1967) in which as branches split, newly generated populations emerge, until they go extinct. Originally coined by Haeckel (1866), the term *phylogenetics* refers to the study of evolutionary relationships between taxa, species, members of a species or individuals, and genes (McLennan, 2010; Gregory, 2008; Hillis, 1997). The standard structure of a phylogenetic tree consists of *lines-of-descent* (lineages), which are connected elements, represented by branch tips, that originally descended from a common ancestor, which is commonly placed at the top of the tree (Baum et al, 2005). Of note, even in non-evolutionary contexts, phylogenetic trees represent historical relationships between the different elements rather than similarities (McLennan, 2010; Baum et al, 2005). Sequencing technologies have now allowed biologists to reconstruct accurate relatedness diagrams - *phylogenetic trees* hereafter - with molecular information inferred from nucleotide or amino acid sequences (Kimura, 1968; Zhang et al, 2018). Furthermore, since the publication of Nowell's theory of Clonal Evolution of Tumours almost five decades ago (Nowell et al, 1976), it has become evident that the organising principle of natural selection in species evolution can likewise be applied to the process of adult tissue formation, which is maintained by stem cells (Merlo et al, 2006; Wang et al, 2024).

In addition to the substantial implications of WGS in the new era of personalised medicine over the last decade, it has become increasingly evident that WGS data has likewise fostered the application of phylogenetics to burgeoning questions, such as the dynamics of complex stem cell systems, which has made significant contributions to the understanding of haematopoietic dynamics. As the technology was optimised, the required DNA input for WGS was reduced from micrograms to the order of nanograms. Still, given the rarity of haematopoietic and other mammalian stem cell populations, it is a great challenge to generate large DNA input amounts from such rare populations. Additionally, since cell fate choices are

executed in cells as individual entities, sequencing single cell genomes was an arduous task, until this was overcome by clonal culture techniques of adult somatic stem cells from different tissues (Blokzijl et al, 2016). Multipotent progenitor cells from the small intestine, colon, and liver were isolated from patients 3-87 years old, in order to generate single-cell derived organoids *in vitro*. In this way, up to 1 ug of genomic DNA was extracted from each of the organoids, which enabled the authors to estimate the mutation acquisition rate in each of the tissues - as expected, small intestine and colon multipotent progenitors acquired a greater number of mutations across time, in contrast to the liver. This, together with the observation of little to no inter-individual variation in the mutational spectrum across tissues, suggested that such mutational patterns were tissue specific. Hence, by implementing a similar approach, Osorio and colleagues clonally expanded HSPCs from two umbilical cord blood (UCB) and five bone marrow human patient samples, determining a mutation acquisition rate of approximately 14 substitutions per year per genome (around 1000 mutations acquired by 60 years of age) (Osorio et al, 2018). Given that mutations are shared between cells from a specific lineage, the authors depicted the genetic relationships between HSPCs from cord blood as a developmental lineage tree, which showed that at birth, HSPCs would have undergone approximately 40 divisions, which is likely that most mutations would have been acquired during embryogenesis.

A more comprehensive profiling of population dynamics in normal haematopoiesis was led by the Campbell and Kent research groups (Lee-Six et al, 2018). Here, the authors isolated and *in vitro*-expanded HSPCs from a 59-year old male individual (from iliac crest bone marrow and peripheral blood) with no history of blood disorders. By leveraging state-of-the-art genomic analysis tools, a phylogenetic tree of the normal adult haematopoietic system was constructed from 129,000+ somatic mutations, with 8676 found in more than one colony. The presence of two mutations that completely partitioned the tree in a matched sample (buccal swab) of the same individual, suggested that the most recent ancestor of blood, which is the mesoderm (Ferreti & Hadjantonakis, 2019), and of the buccal epithelium was the fertilised egg. Thus, the first two mutations must have been acquired during the first cell division, which together with the observation that, after 10 mutations of molecular time - timescale defined by the number of acquired mutations - 33 lineages (termed lines-of-descent in the study) were generated, hence inferring that 1-2 mutations were acquired per cell division before birth. Furthermore, timing of branching points in the tree (coalescence) estimated the population size trajectory from birth to the age of sample collection, pointing to a rapid expansion of the population in early life, reaching a plateau towards adulthood. Furthermore, since the experiment consisted of a capture-recapture design, in which the capture phase HSPCs were collected and profiled, and in the recapture phase, longitudinal peripheral blood samples of the same individual were

collected and subjected to targeted sequencing in order to estimate the relative contribution of stem and progenitor cells to haematopoiesis across time. Bayesian computation of relative contribution to granulocyte (granulocytes harboured most of the mutations found at the top branches of the phylogenetic tree, in relation to T and B lymphocytes) production estimated that the number of active stem cells ranged from 100,000-200,00 at any time during adult life. A schematic representation of the basic structure of a phylogenetic tree for haematopoietic stem and progenitor cells is shown in Figure 1.5 (Podani, 2012; Martin et al, 2010; Novick et al, 2012; Welch et al, 2013; Queiroz, 2013; Challa & Neelapu, 2019; Lee-Six et al, 2018).

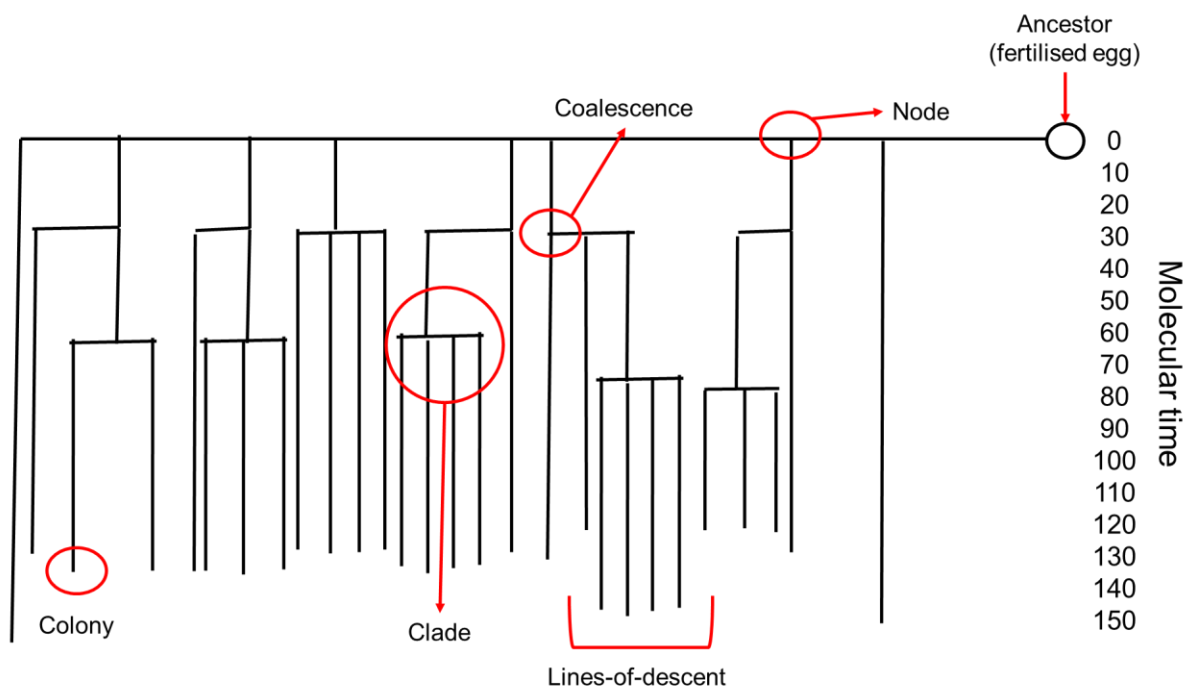


Figure 1.5. Fundamental structure of a phylogenetic tree. To depict the evolutionary history of a population of individuals or - of particular interest for this doctoral thesis work - stem cells, phylogenetic trees are often the method of choice. At the apex of the tree, the common *ancestor* to all cells analysed is placed, namely, the fertilised egg. The vertices originating from the common ancestor are termed *nodes*. Nodes will later begin to form branching points, formally termed *coalescences*. A *clade*, or monophyletic group, contains the descendants - *lines-of-descent* - generated from the same ancestor, inferring therefore that they are more historically related to each other than lines-of-descent from other clades. Each of the tree tips represent a colony, which was derived from single cells grown in culture. Since normal and malignant blood stem cells accumulate mutations as the individual ages, they can be used as a molecular clock, serving as a measure of 'molecular time' in relation to the timing of genetic events occurred *in vivo*.

By using a similar approach, the application of phylogenetic tree building to human blood development was performed by Chapman and coworkers, in which they likewise determined the divergence of embryonic and extraembryonic tissues, including skin, small intestine,

placenta, kidney and cardiac muscle among others (Chapman et al, 2021). In agreement with previous reports (Lee-Six et al, 2018; Osorio et al, 2018), the authors calculated 2.4 mutations acquired in each daughter cell in the 8-cell stage embryo (the equivalent to three cell divisions). Moreover, phylogenetic analysis of divergence revealed that primitive blood cells originate from the extraembryonic mesoderm, derived from the hypoblast, whereas progenitors which give rise to definitive blood later in development derived from the mesoderm posterior to gastrulation.

Further efforts have also been made in the phylogenetics of diseased haematopoiesis. For instance, phylogenetic analysis of blood lineages in individuals aged 0 to 81 years revealed that haematopoiesis in younger individuals (below 65 years of age) was highly polyclonal, gradually growing oligoclonal after the age of 70 (Mitchell et al, 2022). In patients of clonal haematopoiesis, phylogenies of blood were largely dominated by driver genes - *SF3B1*, *U2AF1* and *SRSF2* - which acquired mutations between 25 and 47 years of age, and in some cases up to 70 years of age (Fabre et al, 2022). *U2AF1* and *SRSF2* drove rapid growth in myelodysplastic syndrome (MDS) and AML on an annual rate, in addition to mutations in these two genes being associated with higher risk of malignant transformation. In like manner, Williams and colleagues constructed individual phylogenetic trees of patients of myeloproliferative neoplasms (MPNs), in order to explore disease onset and evolution (Williams et al, 2022). Noticeably, in two with *JAK2V617F*-driven MPN, the mutation was acquired as early as 32.8 weeks after conception, and at 1.4 years of age in the second patient; other three patients acquired *JAK2V617F* mutations before 11 years of age. Across all these patients, mutations in *JAK2* was the first genetic event to occur, followed by mutations in other genes, including *DNMT3A* and *TET2*. The notable delay between the causal genetic event and diagnosis (mean latency to diagnosis was of 34 years in this paper) supports the late disease onset in patients with blood disorders, as it was first discovered in survivors of the Hiroshima and Nagasaki attacks (Radivoyevitch et al, 2023).

These studies comprise prime examples of the wide utility that naturally acquired mutations over time offer to reconstruct clonal relationships between HSCs and their progeny, as well as non-haematopoietic tissues, both in the embryo as in the adult organism, puzzling questions which are otherwise very technically challenging to address. Ergo, these novel approaches to phylogenetic analysis of normal and malignant haematopoiesis, are forging a more comprehensive elucidation of how stem cells selectively compete and what level of contribution to overall haematopoiesis they hold at different stages of an individual's lifetime.

1.5 Somatic mutations in blood malignancies

1.5.1 Early genetic links to cancer

Cancer is often considered through an evolutionary lens, following the principles of natural selection - a genetically related group of cells carrying mutations which confer on those cells a selective advantage over normal, healthy cells, allowing a clone to expand, invade and migrate (Merlo et al, 2006). Although the process of somatic evolution has certainly benefited the preservation of multicellular organisms (Boveri, 1914), in individuals, it can often be the underlying driver of cancer development.

While examining cultured blood samples from patients with CML, Nowell and Hungerford noticed the presence of a *minute*, defective chromosome - chromosome 22 - in two of the patients, and were eventually able to demonstrate the same abnormality in seven CML patients (Nowell & Hungerford, 1960; Nowell & Hungerford, 1961; Nowell, 2007). It was later discovered that this *minute* chromosome was the result of a reciprocal translocation between chromosomes 9 and 22, deemed as an important cytogenetic trait of CML (Nowell, 2007). Cytogenetic analysis of peripheral lymphocytes from CML patients later confirmed that this chromosome was in fact the result of the deletion of chromosome 22 (Rowley, 1973). The discovery of this *minute* chromosome - later called the *Philadelphia* chromosome - marked the first formal demonstration that cancer was the direct result of a genetic abnormality. In addition, with the definition of the hallmarks of cancer, first proposed by Hanahan and Weinberg (2000), it is now understood that genome instability can drive the multistage process of carcinogenesis, along with other cell-intrinsic and extrinsic factors (Hanahan, 2022; Olafsson & Anderson, 2021; Sinkala, 2023). Although it is known that somatic mutations accumulate in normal tissues, cancer *driver* gene mutations are at least two-fold higher than in the germline or adult tissues (Ostroverkhova et al, 2023). Ergo, the detailed study of the implications of mutations on the phenotype in cancer patients is crucial to advance clinical approaches to disease.

1.5.2 Haematological malignancies and pre-leukaemic disorders

Haematological malignancies are the result of the stepwise acquisition of mutations in HSPCs (Robbe & Schuh, 2023) and these cells are considered as the cell of origin in leukaemia (Trumpp & Haas, 2022) following the seminal discovery of AML-initiating cells (Lapidot et al, 1994), and subsequently, leukaemic stem cells (LSCs) by the Dick laboratory (Bonnet & Dick, 1997).

Leukaemia is a heterogeneous group of diseases that arise from altered developing leukocytes - hence the name - which comprise the white blood cell fraction (Chennamadhavuni et al, 2023). According to the cellular lineage of origin, leukaemias can be classified into lymphoid or myeloid, and acute or chronic according to the speed of proliferation of malignant cells (Appelbaum, 2020). In 2022, the Global Cancer Observatory (GLOBOCAN) reported 487,294 new cases of leukaemia, accounting for 3.1% of cancer-related deaths worldwide (GLOBOCAN, 2022). By incidence, AML was reported as the most common form of leukaemia in adults, followed by chronic lymphocytic leukaemia (CLL); whereas in children, acute lymphoblastic leukaemia (ALL) was the most common, seconded by AML (Daltveit et al, 2024). Leukaemia is a highly genetically heterogeneous disease, with over 2000 somatic mutated genes found in a study with 200 AML patients, identifying at least one common driver mutation for all the 11 genetic AML types, as reported in a study by The Cancer Genome Atlas Research Network (2013). In a landmark study led by Papaemmanuil and colleagues, the mutational status of 111 known cancer genes was determined in 738 with myelodysplastic syndrome (MDS) or clinically related disorders, which recognised five classes of mutations: splicing, chromatin regulation, transcription, DNA methylation and cell signalling, in addition to other unclassified mutations (Papaemmanuil et al, 2013). More importantly, the genomic data allowed the correlation of mutational status with clinical features as patient age, sex, and blood counts, and disease subtype. Out of all 111 sequenced genes, mutations in *SF3B1* and *TET2* were the most diversely represented across all disease subtypes, present in just under 200 patients of the cohort, followed by *ASXL1*, *DNMT3A* and *RUNX1*. Remarkably, when combining cytogenetic and sequencing data, oncogenic driver mutations were detected in 78% of the patient cohort - *SF3B1* and *SRSF2* co-mutated genes were different, despite the fact that both these genes participate in splicing processes.

In addition to this complex heterogeneity, pre-leukaemic disorders, including MDS and myeloproliferative syndromes (MPNs) have a high susceptibility to transform into AML (Koeffler & Leong, 2017; Hall et al, 2024). Other forms of pre-leukaemia include clonal haematopoiesis and therapy-related pre-leukaemia (Bowman et al, 2018; Koeffler & Leon, 2017). Given that MDS and MPN (Shastri et al, 2017; Luque Paz et al, 2023) primarily arise in the HSC compartment, they constitute an excellent model to study clonal evolution and disease progression from pre-leukaemia into full-blown leukaemia (Brune et al, 2021).

1.5.2.1 Myeloproliferative neoplasms

Chronic MPNs are a heterogeneous group of acquired clonal disorders, which include three primary types: essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF), CML, chronic neutrophilic leukaemia (CNL), chronic eosinophilic leukaemia and unclassifiable MPNs (Arber et al, 2022). Overall, the BCR-ABL negative MPNs (non-CML) are characterised by overproduction of abnormal cells, particularly erythroid (PV), and megakaryocytic/platelets (ET, PMF); in PMF in particular, the excessive accumulation of megakaryocytes results in fibrosis of the BM, distinguishing MPNs from Philadelphia chromosome-positive CML (McMullin & Anderson, 2020). As discussed, MPN patients are at risk of progressing into leukaemia. The incidence for AML progression has been reported in PMF patients (10-20%), followed by PV (2-4%) and ET (1-2%) (Dunbar et al, 2020).

PV is the MPN primarily studied in my thesis and patients typically present with abnormally high blood parameters, mainly haematocrit, - defined as red blood cell volume in relation to total blood volume (Hct, %) - haemoglobin, - oxygen-transport protein contained in erythrocytes (Hgb, g/dL) - and indeed, red blood cell count (RBC) (Billet, 1990). Under physiological conditions, haematocrit levels for men are around 40-45%, and 36-40% in women; haemoglobin in healthy men are 14-16 g/dL, and 12-14 g/dL in women (Pillai et al, 2023). Diagnostic guidelines established by the World Health Organisation (WHO) consider a haematocrit (Hct) higher than 49% in men and 48% in women, as well as Hgb of 16.5 g/dL indicative of PV (Pillai et al, 2023). Importantly, the presence of JAK2 V617F or JAK2 exon 12 mutations are one of the major criteria within the PV diagnostic algorithm, together with high Hct and Hgb (Tefferi & Barbui, 2023). Thus, abnormal erythrocytosis is the underlying cause for the strong red blood cell phenotype in PV patients, with some patients even exhibiting red skin spots, or developing debilitating conditions such as aquagenic pruritus, which causes a burning sensation on skin after contact with water (Siegel et al, 2013). Additionally, a fundamental hallmark of MPN disease phenotype is chronic inflammation in all patients, regardless of disease subtype, which could be due to enhanced IL-3 and IL-6 signalling-mediated erythrocytosis (Hermouet, 2023), along with cytokines induced by JAK2 V617F mutations, such as IL-1B or CXCL10, particularly in PV (Allain-Maillet et al, 2020).

Fundamentally, recurrent somatic mutations in one of the three driver genes in MPNs - *JAK2*, *CALR*, and *MPL* - is sufficient to alter the signalling pathways JAK2/STAT5, STAT3, PI3K/AKT and MEK/ERK (Viny, 2014; Luque Paz et al, 2023; Maslah et al, 2023). The presence of mutations in one of these genes may or may not be accompanied by a second or third genetic mutation, providing a relatively simple mutational landscape for MPNs and making it an

excellent model for the early stages of disease evolution. Despite this relatively well delineated mutational landscape, large patient heterogeneity still exists, posing one of the greatest challenges for understanding and treating these disorders. Moreover, this also prompts questions regarding how such mutations alter the normal function and behaviour of HSCs, which has important implications for downstream blood production.

1.5.2.1.1 JAK2 V617F mutations in MPNs

JAK2, or *Janus kinase 2*, is a member of non-receptor tyrosine kinase families, whose substrates are the Signal Transducers and activators of Transcription (STAT), and together these molecules play an essential role in regulating proliferation and transcription factor activity (Gnanasambandan & Sayeski, 2016). JAK2 also regulates signal transduction of interleukin 3 (IL-3) receptors, and single-chain receptors such as erythropoietin (EPO) and thrombopoietin (TPO) receptors (Hu et al, 2021). Constitutively active JAK2 signal disrupts normal physiological functions and JAK2 mutations have been implicated in a number of haematological malignancies, such as ALL, CML, MPNs, as well as solid tumours, including breast, prostate, head and neck tumors (Gnanasambandan & Sayeski, 2016).

Haematological malignancy-related JAK2 mutations occur in the pseudokinase domains, located in exon 14, resulting in a valine to phenylalanine substitution at position 617 (Gnanasambandan & Sayeski, 2016). Two decades ago, multiple research groups profiled and reported the incidence of JAK2 V617F mutations in MPN patients (as reviewed in Levine et al, 2007; Levine et al, 2005; Baxter et al, 2005; Kralovics et al, 2005; James et al, 2005). JAK2 V617F mutations were detected across the entire MPN subtype spectrum, identifying it as an acquired mutation in haematopoietic progenitors, which conferred a selective advantage (Levine et al, 2005).

Given that JAK2 V617F is present in all three major types of MPN, although more prevalent in PV patients, lack of JAK2 disease specificity is rather intriguing, as it is many times the only driver mutation found in patients - although it is also accompanied by other mutations, which will be discussed in following sections (Hermouet, 2023; Mascarenhas et al, 2023).

JAK2 V617F mutations were shown to occur in HSCs by examining PV blood samples, and Jamieson et al., showed that a higher proportion of JAK2-mutant cells had an HSC phenotype (Jamieson et al, 2006). Additionally, as JAK2 MPNs are usually persistent, the mutation can also be found in both the lymphoid and myeloid lineages (Larsen et al, 2007). Subsequently, in a later study comprising a large cohort of MPN patients, it was determined that JAK2 V617F does not increase self-renewal potential, but results in an expansion of myeloid, erythroid and

megakaryocytic progenitors (Anand et al, 2011; Grinfield et al, 2017). Equally, JAK2 V617F allele burden correlated positively with increased myeloid expansion in PV and MF patients within that same cohort (Anand et al, 2011). In addition, disease heterogeneity in JAK2-driven MPNs has been attributed to the fact that mutations are acquired in HSCs, as it has been demonstrated by *in vitro* and *in vivo* assays, in which colony-forming and reconstituting cells exhibit the classical hallmarks of multipotency, self-renewal and long-term reconstitution of HSCs, irrespective of the MPN subtype (James et al, 2008).

1.5.2.1.2 MPL and CALR mutations in MPNs

In contrast to JAK2 V617F mutations, which are found in 90% of PV patients, MPL and CALR mutations have not yet been reported in PV, although, they do occur in ET (MPL: 2-3%; CALR: 20-25%), and PMF (MPL: 3-5%, CALR: 20-30%) (Vainchenker & Kralovics, 2017). Myeloproliferative leukaemia protein 10, or MPL, is the receptor for TPO, which regulates HSC maintenance through a myriad of cell signaling pathways, including the JAK/STAT, PI3K/AKT, and hypoxia inducible factor (HIF) pathways, orchestrating metabolism, survival and proliferation (Hiaro; 2014; Hitchcock et al, 2021). Gain of function mutations in the transmembrane domain of MPL, W515L, were first identified in myelofibrotic BM of patients with myeloid dysplasia, which conferred cytokine-independent features to cells with W515L overexpression (Pikman et al, 2006). A screening of 1182 patients identified 20 cases of mutated MPL, including the W515K allele, regardless of the patients' mutational status (Pardanani et al, 2006). Likewise, these data suggested that the 515 codon of MPL coding region was a likely mutational hotspot (Pardanani et al, 2006). Missense MPL mutations were deemed as the cause of disease in congenital amegakaryocytic thrombocytopenia, which in a considerable number of cases drove low platelet counts and progression to pancytopenia (Germeshausen et al, 2006). *In vivo* transplantations of W515L or W515K mutant CD34⁺ cells isolated from PMF patients, demonstrated that MPL mutations were acquired in the haematopoietic stem and progenitor cell compartment of the BM (Chaligne et al, 2007). Heterozygous mutations outside exon 10 of MPL were found in exons 3, 4, 5 and 12, which all led to constitutive activation of the JAK/STAT5 pathway (Milesovic Freenstra, 2016). UT7 cells transfected with these MPL mutations acquired a cytokine-independent phenotype in culture (Cabagnols et al, 2016).

Calreticulin (CALR) is located in the endoplasmic reticulum (ER) and functions as a calcium (Ca²⁺)-binding protein, participating in the folding and secretion of proteins towards the plasma membrane, hence regulating protein homeostasis (Fucikova et al, 2020). The most common CALR mutation is an indel in exon 9 (Vainchenker & Kralovics, 2017). CALR mutations were first detected in 70-84% of JAK2V617F mutant patients by exome sequencing; in total, 148

mutations with 19 variants were reported in this first study (Nangalia et al, 2013). In order to find specific molecular markers in non-JAK2 and non-MPL mutated patients in ET and PMF, Klampfl and colleagues screened a cohort of all MPN subtypes for the presence of CARL 9 exon mutations (Klampfl et al, 2013). Accordingly, the authors found that CARL mutations were absent in PV, but insertions and deletions in CARL were found in 25% of the ET and 35% of the PMF patients. Three years later, the Mullaly group reported that CALR-driven MPNs required mutations in MPL through JAK/STAT constitutive activation, likewise observing that JAK2 inhibition impaired HSC function (Elf et al, 2016).

1.5.2.2 TET2 mutations in MPNs, MDS and AML

The Ten-eleven translocation (TET) family of proteins, including members TET1, 2 and 3, are DNA-deoxygenating enzymes which catalyse the conversion of 5-methylcytosine (5-mc) in DNA to 5-hydroxymethylcytosine (5-hmc), 5-formylcytosine and 5-carboxycytosine, therefore catalysing demethylation of DNA (Gao et al, 2024; Zhang et al, 2023). In particular, the conversion of 5-mc to 5-hmc is essential for the process of DNA methylation, which constitutes an epigenetic mechanism that participates in the regulation of gene transcription, X chromosome inactivation and genomic imprinting (An et al, 2017; Elhamamsy, 2017). Cytosine methylation in most mammalian organisms occurs at the level of CpG dinucleotides in a symmetrical fashion, where cytosines which are part of both strands of the DNA duplex are methylated, in an enzymatic reaction mediated by DNA methyl-transferases (DNMTs) (An et al, 2017). TET enzymes promote DNA demethylation mainly by gradual loss of DNA methylation as cells divide - a mechanism known as passive replication-dependent DNA demethylation (Lio et al, 2019). Thus, TET enzymes regulate haematopoietic differentiation at the epigenetic level by mediating the access of transcription factors to the enhancer regions of target genes, which is also crucial for immune cell activation and expansion (Jiang, 2020; Joshi et al, 2022).

The TET2 gene is ubiquitously expressed in the murine and human HSPC compartment, as well as mature cells of the myeloid and lymphoid lineages (Gao et al, 2024). TET2 is recruited by master transcription factors to enhance regulatory regions to remodel the genomic landscape of 5-mc to 5-hmc, which then enables gene silencing (Jiang, 2020). Structurally, TET2 shares many common features with TET1 and TET3, as they all contain a core catalytic domain contained within the catalytic region which mediates CpG recognition and substrate preference (Lio et al, 2019). Nonetheless, the DNA-binding domain, CXXC located in the N-termini, is separated from the region encoding the catalytic domain in TET2 unlike TET1 and

TET3, which could account for the preferential location of TET2 in gene bodies or enhancer regions (Zhang et al, 2023; An et al, 2017).

In a healthy organism, TET2 can act as a haploinsufficient tumour suppressor gene (Mercher et al, 2012). Nevertheless, TET2 is the most frequently mutated member of the TET family, despite all enzymes accomplishing similar functions, being expressed across the haematopoietic hierarchy and exhibiting similar structural features. TET2 is located on the chromosome 4q24, and mutations are distributed across the coding region, including the Cysteine-rich (cys-rich) and catalytic domains (Huan & Rao, 2014). The vital importance of TET2 mutations in haematological disorders gained widespread attention over 15 years ago, as several groups around the world reported the presence of TET2 loss-of-function mutations primarily in MPNs, MDS and clonal haematopoiesis (Delhommeau et al, 2009; Bowman & Levine, 2017).

The full sequence of TET2 was determined in 320 MPN, MDS and AML patients by Delhommeau and coworkers, where a structural rearrangement of region 4q24 were observed in MDS and AML patients, as well as JAK2 V617F mutant patients (Delhommeau et al, 2009). Furthermore, TET2 LOF mutations were identified in 16% of the CD34⁺CD38⁻ HSC-enriched compartment, and in 54% of CD34⁺CD38⁺ HSPC population, corresponding to less-primitive progenitors. Transplantation of TET2-mutant patient CD34⁺ cells into immunodeficient mice (non-obese severe combined immunodeficiency, NOD-SCID) demonstrated higher long-term reconstitution capacity in comparison with healthy controls. In a concurrent study, recurrent TET2 mutations occurred in 26% of MDS patients (102 profiled patients in total), which were present in 96% of BM cells, mainly in CD34⁺ progenitors, supporting earlier Delhommeau's observations and implying that TET2 mutations were acquired in early stages of disease (Langemeijer et al, 2009).

TET2 mutations are most commonly detected in CML (20-40%), but can be equally found in 12-24% of AML, 19-26% of MDS and 29% of mastocytosis patients (Padda et al, 2021). In a screening study of 239 MPN patients, Tefferi and colleagues performed high-throughput DNA sequencing on DNA extracted from BM, and found that 13% of the patients carried TET2 mutations (Tefferi et al, 2009). Of the 32 different TET2 mutations, 19 were frameshift, 10 nonsense and 10 missense mutations, which mostly occurred in the isoform A (2002 amino acids) of the enzyme. Moreover, these mutations occurred in 17% of patients with a JAK2 V617F gain-of-function mutation, which was significantly correlated with advanced age of the patients, regardless of disease subtype.

The somatic nature of TET2 mutations in haematological patients was demonstrated by single nucleotide polymorphism array (SNP) based karyotyping, in which the absence of loss-of-heterozygosity (LOH) in chromosome 4q was detected in lymphocytes, together with a germline TET2 constellation on the DNA sequence (Jankowska et al, 2009). Furthermore, authors of this same study noted that, in MDS/MPN overlap patients, the majority of TET2 mutations were heterozygous, in comparison to sAML patients (progressed from MDS/MPN), in which TET2 homozygosity accounted for most of the detected mutations, implying that haploinsufficiency of TET2 is enough to sustain disease. Moreover, detection of the mutation in 50% of CML patients, in comparison to other disease subtypes, affirmed the higher frequency of TET2 mutations in myeloid disorders (Kosminder et al, 2009). Impaired DNA demethylation in TET2 mutant BM or blood cells was reported by Ko and colleagues, in which they observed lower 5-hmc levels in TET2-mutant patient BM and blood cells, in contrast to TET2-wild type patient or healthy controls (Ko et al, 2010). Indeed, methylation levels of TET2-mediated transcription factor target genes, such as *ZFX1B* and *RAB3IP* were significantly reduced in TET2-mutant patients, which provided deeper mechanistic insight into how TET2 has significant implications on myeloid disease evolution. In summary, profiling of haematological patient samples generated sufficient evidence that TET2 LOF mutations were acquired at the most primitive compartment of the BM cell population, which together with the high frequency of these mutations in AML and MDS, and co-occurrence with JAK2 V617F in MPNs, suggested substantial alterations in HSC biological functions.

1.5.2.2.1 Co-occurrence of JAK2 and TET2 mutations

Co-occurrence and mutual exclusivity of mutations have been implicated in many cancer types, both solid (Le et al, 2024) and haematological (Papaemmanuil et al, 2013) - specifically, co-occurring mutations activate collaborative oncogenic pathways, largely determining phenotype and disease evolution (El Tekle et al, 2021). TET2 is the most frequently co-mutated gene with JAK2 V617F in myeloid disorders, particularly in MPNs, which has substantial clinical relevance both for treatment and patient survival (Padda et al, 2021). Regardless of JAK2 mutational status, TET2 mutations are found in 15.5% of overall MPN cases: 16.8%, 9.8% and 15.7% in PV, ET and PMF (Cai Chia et al, 2021). The detection of different kinds of TET2 mutations found in four JAK2 mutated PV patients (two sisters and two unrelated female patients) accounted for the dynamics of TET2 expression during *in vitro* expansion of erythroid colonies - when compared to sporadic PV JAK2 only mutant patients, TET2 expression decreased as colonies expanded, but increased in JAK2/TET2 double

mutant patients, particularly in biological sisters, despite harbouring different TET2 mutations (Swierczek et al, 2011).

Timing of co-occurring mutations has been deemed important for the development of disease, in which JAK2-first or TET2-first patients present with different clinical features. Genomic profiling of eight JAK2/TET2 mutant patient-derived *in vitro* expanded colonies revealed three patterns of mutational accumulation: TET2-first, where a fraction of colonies carried only TET2 mutations, whereas others were JAK2 mutant; JAK2-first, with a fraction of JAK2 only mutant colonies, and JAK2/TET2 double mutant colonies; and biclonal disease, in which all colonies were positive for either JAK2 or TET2 mutations, but not both (Schaub et al, 2010). In another study, the temporal order of mutations was analysed in MPN patients with JAK2 V617F mutations co-occurring mostly with TET2, as well as DNMT3A, ASXL1, EZH2 and IDH1 (Lundberg et al, 2014). Mutations in TET2 and DNMT3A were predominantly occurring prior to the acquisition of JAK2 mutations in PV patients. Conversely, mutations in ASXL1 and EZH2 occurred either before, after or independently of JAK2 V617F, whereas in a small fraction of patients, IDH1 occurred exclusively after JAK2 mutations. In patients with 3 or more somatic mutations, JAK2, TET2 and DNMT3A mutations were always acquired first (Lundberg et al, 2014).

These studies were fundamental for bolstering the clinical implications of the temporal acquisition of JAK2/TET2 mutations. By profiling MPN patient-derived colonies and single-cell derived clones, Ortmann/Kent et al., determined that the order of mutation acquisition impacted the biology of MPNs in 24 JAK2/TET2 double-mutant patients (Ortmann et al, 2015). There was an equal distribution of JAK2-first or TET2-first patient-derived colonies, however, in a high proportion of JAK2-first colonies, JAK2 mutations were homozygous. Analysis of 10-day single-cell derived clone size showed a significantly higher size of JAK2 single mutant clones relative to non-mutant or JAK2/TET2 double mutant clones, contrary to TET2 clones, which did not differ in size from their non-mutant or double-mutant counterparts. Accordingly, mutation order substantially influenced clinical features - TET2-first patients were diagnosed at a significantly older age (>10 years) than JAK2-first patients; a higher proportion of JAK2-first patients coursed with PV, but with lower risk of thrombosis from time of diagnosis than TET2-first patients. In addition, JAK2-first double mutant colonies had increased sensitivity to ruxolitinib, a JAK2 inhibitor (Ostojic et al, 2011).

1.5.3 Mouse models of MPNs

Given the remarkable complexity of cancer, experimental modelling of the disease is sometimes limited when working with human patient samples, due to restricted sample availability, difficulty replicating underpinning mechanisms *in vitro* and, specifically for haematological malignancies, the ever-growing need for more robust human HSC purification strategies. As with many other diseases, this issue has been tackled through the generation of mouse models, to faithfully recapitulate aspects of disease phenotype *in vivo*, also enabling drug testing studies and xenograft models (Mullally et al, 2012). Since the discovery of JAK2 V617F mutations in MPNs in 2005 and TET2 in 2009, multiple groups around the world have undertaken considerable effort to model human MPN disease biology in mice mostly, although this has been accompanied by zebrafish models (Lanikova et al, 2019). In order to conduct extensive characterisation of MPN disease biology *in vivo*, four main categories of MPN mouse models are recognised: 1) Retroviral overexpression 2) Transgenic 3) Xenografts and 4) Knock-in (Li et al, 2011; Mullally et al, 2012; Dunbar et al, 2017).

Retroviral transduction of HSPCs facilitated the study of cell signalling of JAK2 V617F mutant MPNs, which resulted in a PV phenotype with myelofibrosis (Stetka & Skoda, 2021; Li et al, 2011; Wernig et al, 2006; Lacout et al, 2006). Additionally, these models provided early indications of the complex interplay between cell intrinsic and extrinsic factors in the severe erythrocytosis exhibited in PV (Bumm et al, 2006), with haematocrit levels close to 80%, almost two-fold higher than wild-type controls (Zaleska et al, 2006).

Stable expression of JAK2 V617F in transgenic mouse lines was achieved by pronuclear injection, which at the same time permitted quantification of JAK2 expression (Li et al, 2011; Lanikova et al, 2019). Shide and coworkers generated two transgenic mouse lines: mice of the first line developed granulocytosis after 4 months, which had also been observed in retroviral models; however, the second line developed severe thrombocytosis and leukocytosis, demonstrating that stable JAK2 expression resulted in PV, ET and PMF phenotypes as it did in human patients (Shide et al, 2008). A mouse model generated by oocyte injection carrying a bacterial artificial chromosome (BAC), which contained exons 1-12 of JAK2 coding sequence (Tiedt et al, 2008). These mice resembled an ET phenotype with significantly elevated platelet counts, while inducible JAK2 transgene expression generated a PV-like phenotype, displaying high haemoglobin and elevated neutrophil count, but normal platelet counts; moreover, both lines displayed BM hypercellularity, indicative of myelofibrosis. Across three transgenic JAK2 mouse lines, Xing et al demonstrated increased numbers of haematopoietic progenitors in the BM, spleen and peripheral blood, accompanied by

significant increase of spleen weight, denoting extramedullary haematopoiesis (EMH), a hallmark of myeloproliferative disorders, as well as other haematological and solid cancers (Xing et al, 2008; Barisas & Choi, 2024).

Since constitutively activated JAK/STAT signalling is one of the most fundamental mechanisms underscoring JAK2 V617F-driven MPNs, there was a latent need to develop mouse models with less variable and robustly transplantable disease phenotype (Jacquelin et al, 2020). In 2010, Li and colleagues created a conditional knock-in mouse model of human JAK2 V617F through homologous recombination of a conditional allele in mouse ESCs (Li et al, 2010). The progeny of JAK2 V617F heterozygous mice were crossed with MxCre1 mice, and JAK2 V617F expression was induced by polyinosinic-polycytidylic acid (plpC). Mice developed an ET-like phenotype, with BM megakaryocytic hyperplasia, elevated platelet counts, as well as haematocrit and haemoglobin in comparison with wild-type controls. At 26 weeks after plpC injection, 10% of mice developed a PV-like phenotype, with abnormal haematocrit counts and erythrocytosis; while one mouse developed BM fibrosis. Serial transplantation demonstrated a reduced serial reconstitution capacity of HSCs, suggesting that JAK2 V617F HSCs were functionally deficient.

Simultaneously, the Ebert group developed a conditional knock-in line of JAK2 V617F through gene editing of mouse ESCs (Mullally et al, 2010). The resulting chimaeric mice were crossed with wild wild-type mice, to generate floxed JAK2 heterozygous animals. Crossing of these animals with *Ell1a-cre* mice (Lakso et al, 1996) resulted in JAK2 V617F germline expression. Mice displayed a characteristic PV phenotype, with abnormally high haematocrit, splenomegaly and increased frequency of CD71⁺TER119⁺ erythroid cells in BM and spleen. Importantly, this phenotype was serially transplantable - primary recipients replicated the PV phenotype from the donors, although haematocrit levels eventually decreased from primary to secondary and tertiary transplant recipients.

In another conditional knock-in line, PV was accompanied by leukocytosis and thrombocytosis in heterozygous mice, with increased severity in homozygous mice (Akada et al, 2010). Marty and colleagues conducted the generation of a knock-in model, in which the endogenous expression of a heterozygous V617F was not lethal to the mouse embryos, resulting in PV, which evolved to fibrosis at 9 months of age of the surviving animals (Marty et al, 2010).

By leveraging the mouse model developed by Li in 2010, Kent and colleagues profiled the self-renewal capacity of JAK2 mutant ESLAM HSCs in functional and clonal *in vitro* assays (Kent et al, 2013). Impairment of HSC functional capacity was evident in competitive and

subsequently, serial transplantation for JAK2 V617F mutants, accompanied by reduced ESLAM frequency of JAK2 mutant BM. *In vitro* clonal assays demonstrated that JAK2 V617F HSCs yielded larger clones with significantly higher CD41 expression than wild-type cells, but division kinetics were hindered with age. One year later, members of the same group published a refined strategy to generate a JAK2 knock-in mouse model through *StellaCre* recombination, yielding progeny of several genotypes, including JAK2 V617F homozygous animals (Li et al, 2014). JAK2 V617F mutations led to abnormally high haematocrit and haemoglobin counts, recapitulating the PV-like phenotype observed in humans, as well as reduced survival as shown by Kaplan-Meier survival curve analysis. Transplantation of JAK2 homozygous HSCs into primary recipients also faithfully resembled the phenotype of the donors, including high haematocrit but low platelet counts. However, engraftment was significantly reduced in JAK2 homozygous HSC recipients than for JAK2 wild-type or heterozygous HSC recipients. Nevertheless, JAK2 homozygous mice are unable to be used as breeders due to the potentially lethal consequences for males due to severe erythrocytosis and twice-fold higher haematocrit levels than in wild-type mice (O'Connell et al, 2015).

1.5.3.1 Mouse models of TET2 mutations

The critical role of TET2 in haematological malignancies such as MDS and AML, and the most commonly mutated gene in MPNs together with JAK2, has led to the development of several mouse knock-out models, which have significantly contributed to understanding how these mutations affect HSC function (Ko et al, 2015). In 2011, three laboratories developed and generated the first three TET2 models - all knock-out since TET2 haploinsufficiency had previously been shown to have clinical relevance but its *in vivo* function had not yet been characterised. Quivoron and coworkers developed two TET2 knock-out lines - one line generated by gene-trap - a mutational insertion approach - and another by a conditional knock-out allele (Quivoron et al, 2011). In these models, loss of TET2 did not affect TET1 and TET3 expression, but resulted in a considerable drop of 5-hmc levels in Lin⁻ BM cells and a significant increase in LSK and myeloid progenitor (contained in the Lin⁻ c-Kit⁺, LK, fraction) frequencies in the BM. Old TET2 knock-out mice developed a lethal phenotype characterised by anaemia, weight loss, high WBC, thrombocytopenia and hepatosplenomegaly. Competitive repopulating unit assays of BM from both knock-out lines demonstrated higher chimaerism in TET2 deficient recipients, which strengthened the observations of an expanded HSPC pool in the BM.

Later on, in the TET2 deficient model developed by the Rao laboratory (Ko et al, 2011), there was no evidence for embryonic lethality, and progeny was generated at a normal Mendelian

ratio, yielding mice of wild-type, heterozygous and homozygous genotypes. Although most of TET2 mutations in human patients are heterozygous, TET2 homozygosity produces a particular HSC phenotype in mice - frequencies of progenitor cells in the BM of TET2 knock-out mice were significantly higher than in TET2 wild-type animals, including LSK, CMP, GMP and MEP populations. Transplant recipients of TET2 knock-out HSCs likewise displayed expansion of LK and LSK cells in the BM, and showed equally higher chimaerism levels in competitive repopulation unit assays, as well as increased multilineage contribution. Li and colleagues undertook a targeted deletion approach of TET2 (Li et al, 2011), generating TET2 knock-out mice with LSK increased frequency in accordance with Ko's previous findings. Strikingly, loss of TET2 resulted in the acquisition of a myriad of haematological phenotypes: more than 50% of the mice developed MDS with elevated erythroid progenitors frequency; equivalent fractions (20%) of mice acquired a CMML or MPN/MDS phenotype; only one mouse of the total studied (21 mice) displayed myeloid-leukaemia like disease (Kogan et al, 2002).

1.5.3.1.1 Modelling concomitant JAK2/TET2 mutations *in vivo*

Thanks to the generation of single-mutant mouse models, the contribution of JAK2 mutations to the onset and evolution of blood cancers has been unveiled, as well as pleiotropic roles for TET2. Nonetheless, it is known that JAK2 and TET2 are the most commonly mutated gene pair in MPNs, and that the temporal order of mutation acquisition has important clinical implications, from diagnosis to treatment response. Hence, genetic lines of mice carrying both JAK2 V617F knock-in and TET2 knock-out mutations have been generated in order to study the impact of mutational synergy in MPNs. Two novel double-mutant mouse models were reported in 2015: By deleting TET2 in JAK2 V617F knock-in mice, Chen and coworkers generated mice which were JAK2 mutant and TET2 nullizygous, or JAK2 wild-type and TET2 nullizygous (Chen et al, 2015). Splenomegaly was significantly elevated in JAK2/TET2 mutant mice than in JAK2 V617F and TET2^{-/-} single mutants; haematopoietic stem and progenitor cell frequency was also elevated in relation to TET2 knock-out mice, but not JAK2 V617F. Furthermore, gene expression profiling in LSK cells revealed upregulated STAT5 targets in JAK2 V617F and JAK2/TET2 mutants, but not TET2. Conversely, a self-renewal signature was upregulated in JAK2/TET2 and TET2-deleted LSK cells only. This was supported by transplantation assays, in which JAK2/TET2 and TET2 recipients showed higher chimaerism and an equally higher contribution to circulating granulocytes than JAK2 V617F single-mutants, implying that JAK2/TET2 co-mutations drive a similar disease phenotype as JAK2 V617F alone, without compromising HSC self-renewal. In a separate study, by crossing JAK2 V617F with TET2-deleted mice, Akeda et al unveiled new roles for TET2 in JAK2-driven MPNs

(Akeda et al, 2015). Kaplan-Meier analysis revealed lower survival of double-mutant animals, with less than 20% of mice surviving beyond 40 weeks of age, close to but still lower than JAK2 V617F alone, in contrast to ~60% and ~80% than TET2 and wild-type mice, respectively. In addition, JAK2/TET2 cooperated to drive cell proliferation in the BM and spleen, while also promoting LSK and LK pool expansion in the BM of transplant recipients, but not in the spleen. As in Chen et al (2015), molecular profiling demonstrated that STAT5 target upregulation was one of the mechanisms by which JAK2 and TET2 sustained disease for longer periods of time.

The application of high-throughput single-cell methods and the generation of a JAK2/TET2 - by crossing the previously reported JAK2 V167F knock-in (Li et al, 2014) with the TET2 knock-out (Ko et al, 2011) lines - double-mutant model were consolidated into a key study conducted by Shepherd et al (2018). Divisional kinetics of JAK2 and JAK2/TET2 single ESLAM cells were significantly faster than for TET2 and wild-type ESLAMs, similar to JAK2/TET2 patient-derived single HSPCs. Functional assays in which whole BM suspensions were transplanted revealed that JAK2 and TET2 cooperated to sustain disease phenotype even up to 24 weeks after injections, likewise indicating enhanced HSC self-renewal. Molecular profiling identified targets such as *Mes1* and *Bmi1* - required for HSC metabolic regulation - in JAK2/TET2 double mutants, whose overexpression in wild-type HSPCs which were then transplanted into JAK2 V617F recipients, produced increased haematocrit and haemoglobin, but not so for wild-type recipients, suggesting that these targets might contribute to disease evolution in JAK2-driven MPNs.

Hence, mouse models represent one of the most powerful tools in the life sciences to study multiple biological processes, as well as the molecular and functional mechanisms which contribute to cancer, amongst other diseases. The synergy of cooperative mutations, such as JAK2 and TET2, can affect cell fate choices, which can only be studied at the single-cell level (Rontautoli et al, 2022), and this has been made possible through the highly accurate purification strategies for mouse haematopoietic stem cells. Nonetheless, despite the great genetic similarity between mice and humans (90%, Breschi et al, 2019), there are evident differences at the biological, social and behavioral levels between mice and humans, which might not permit a direct correlation between findings made using mouse models and human samples. Yet, the mouse blood-forming system, is the best characterised stem cell system across all species, which has bolstered the understanding of how adult tissues are regenerated and how the stepwise acquisition of somatic mutations in HSCs are at the core of haematological malignancies.

1.5 The Bone Marrow niche in health and disease

1.6.1 Historical background - The *niche hypothesis*

“The virtual immortality of the stem cell population”, Raymond Schofield wrote in his 1978 landmark paper, *“is at variance with the evidence that the CFU-S population has an ‘age-structure’”*, by which he meant that the CFU-S was not the most primitive blood-forming cell in the haematopoietic hierarchy. Schofield continues: *“To account for these inconsistencies, a hypothesis is proposed in which the stem cell is seen in association with other cells which determine its behaviour”* (Schofield, 1978). Such hypothesis became the foundational groundwork for the description and characterisation of the complex, specialised microenvironmental structure which is now termed ‘bone marrow niche’ or haematopoietic stem cell niche, since this is where most HSCs and HSPCs reside in mice and humans (Scadden, 2014). Indeed, the *niche hypothesis* was built upon Darlington’s definition of a niche: *“A niche, as I understand it, includes the space(s) an organism occupies, its inorganic environment, and other organisms that affect it”* (Darlington, 1972). Although in this paper Darlington was pondering on parasitism, predation, disease and cooperation as forms of competition between species, his conceptual framework for a *niche* was on-point, since it still made a distinction between the niche itself and the organism that resides within it, albeit a separate entity, was entirely dependent on its niche for survival.

Three years prior to Schofield’s niche hypothesis, Lord and colleagues had analysed the distribution of colony-forming spleen and cultured units (CFU-S and CFU-C, respectively) along different portions of the femur (Lord et al, 1975). Built upon the principle that haematopoietic stem cells find nurture in their surrounding microenvironment, Dexter and colleagues established an *in vitro* method to culture haematopoietic progenitors (here, CFU-S), by providing the necessary growth factors on a weekly basis, attained the maintenance of CFU-S for several months, sustaining granulopoiesis (Dexter et al, 1977). Despite cumulative evidence that the BM microenvironment played an important role in the regulation of HSC function, the concept of the niche was not developed until two decades after Schofield’s first proposal of the *niche hypothesis* (Papayannopolou & Scadden, 2007). Leveraging on *Drosophila* as an experimental model, Xie and Spradling affirmed Schofield’s hypothesis thanks to their findings on the niche surrounding germline stem cells, which unveiled dpp (decapentaplegic) signalling in the microenvironment, essential for stem cell proliferation (Xie & Spradling, 1998). One of the first cellular components of the BM niche to be characterised were mesenchymal stem cells (MSCs) (Friedenstein et al, 1974; Frobél et al, 2021), which produced BM and spleen fibroblast colonies, constituting the stroma of their corresponding

haematopoietic organ. Therefore, understanding the cellular composition of the bone, and in particular the marrow, together with the cell signalling pathways which it regulates, frames the organising principle of niche and HSC interaction.

1.6.2 Composition of the bone marrow

Bone is a type of specialised connective tissue, which is composed of mainly two compartments: trabecular and cortical (Ott, 2018). Long bones, such as the femur, the tibia and humerus have three anatomical regions - epiphysis, metaphysis and diaphysis (Morrison & Scadden, 2014; Comazetto et al, 2021). The vertebrae which compose the spine are considered trabecular. Close to 80% of the total bone mass is contained within the cortical bone, while the remaining 20% concentrates in the trabecular bone. The metaphysis harbours small bone projections called the trabeculae, which support the vasculature which irrigates the bone and hold the anatomical interface between the bone and the marrow - the endosteum (Ott, 2018; Morrison & Scadden, 2014). The composition of trabecular and cortical bone is highly heterogeneous morphologically, albeit with different developmental origins, there is a tight regulation of the number of haematopoietic stem and progenitor cells, as well as committed progenitors, within the niche, which is crucial for healthy haematopoiesis (Pereira et al, 2024). The marrow can be then defined as the semisolid tissue that resides in the cavities of cortical and trabecular bone, which harbours a myriad of cell types, which through highly dynamic interactions with lymphoid tissues and peripheral blood, supports the maintenance and differentiation of haematopoietic stem cells and mesenchymal stem cells (MSCs) (Sovani, 2021; Wei & Frenette, 2018). Almost all non-haematopoietic cells residing in the BM can be classified as stromal cells (Cordeiro-Spinetti et al, 2015), and according to their function and location within the niche, they are grouped into: mesenchymal, endothelial and neural (Pereira et al, 2024). Furthermore, within the BM, HSCs reside within two regions: the central and endosteal niche (Figure 1.6) (Mendez-Ferrer et al, 2020).

1.6.2.1 The mesenchymal compartment of the niche

Mesenchymal stem cells are characterised by their multilineage potential, giving rise mainly to osteoblasts, osteoprogenitors, osteocytes, chondrocytes and adipocytes, (Kandarakov et al, 2022; Gao et al, 2022). They are characteristically positive for CD73, CD90 and CD105, and negative for CD24, CD19, CD34, CD45, as well as for Sca-1 (Huang et al, 2024). MSCs produce essential niche soluble factors such as CXC chemokine ligand 12 (CXCL12), SCF and interleukin 7 (IL-7) which regulate haematopoietic homeostasis in the BM (Sarkaria et al, 2018; Kwon et al, 2024). The CXCL12 abundant reticular (CAR) cells (sometimes known as

Leptin Receptor positive (LepR⁺) cells) possess a spindle-shaped cellular body which allows them to interweave with the diverse cell types of the BM, constituting the structural fabric of the niche (Pereira et al, 2024), although they mostly reside adjacent to the sinusoids (Huang et al, 2024). In addition to CXCL12 and SCF, CAR cells are responsible for the production of Foxc1 and Ebf1/3, which regulate osteogenesis (Galen-Diez & Kustoeni, 2018) , and have been reported to secrete many other molecules including TPO, granulocyte-colony stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), among many others (Sugiyama et al, 2006; Kandarakov et al, 2022).

Osteoblasts are the main type of bone-producing cells, generating vital factors for bone growth such as type I collagen, osteocalcin and alkaline phosphatase (Sarkaria et al, 2018). Despite early studies advocating for the importance of N-cadherin⁺ osteoblasts in the regulation of haematopoiesis through the bone morphogenetic protein (BMP) pathway (Zhang et al, 2003; Morrison & Scadden, 2013), later studies demonstrated that most of HSCs localise near the sinusoids, and not the endosteum where osteoblasts reside (Kiel et al, 2007), and this was further supported by HSC loss upon the deletion of *Scf* in perivascular BM cells expressing LepR⁺ and endothelial cells (Ling et al, 2012). Although it has been suggested the interaction between HSCs and osteoblasts is mediated by Notch signaling (Pereira Lampreia et al, 2017), the mechanism by which osteoblasts help support different aspects of haematopoiesis, as well as HSC homing and mobilisation, remains unclear (Woods & Guez Guez, 2021; Sugiyama et al, 2019). LepR⁺ cells have also been reported to secrete nerve growth factor, thereby promoting the innervation of the BM niche (Gao et al, 2023).

1.6.2.2 Endothelial regulation of HSC activity

The BM is irrigated by a heterogeneous network of arteries and arterioles distributed across the metaphysis, diaphysis and epiphysis of the long adult bones - arterioles form beds of sinusoidal capillaries, characterised by the expression of E-selectin, Icam1, Pdpn and CD105, while arteriolar endothelial cells (ECs) express Sca-1, CD200 and Nestin (Nes) (Boueya et al, 2024); however, both arterioles and sinusoids are high in CD31 expression (Ramalingam et al, 2018). A number of functions described for endothelial cells, which regulate HSC mobilisation, trafficking and homing, as well as regeneration of the BM after irradiation when transplanted along HSCs in human patients (Kwon et al, 2024; Sugiyama et al, 2019; Kandarakov et al, 2022). Moreover, activation of the Akt pathway and recruitment of mTOR in BM endothelial cells has been shown to increase the long-term self-renewal capacity of haematopoietic stem cells (Kobayashi et al, 2010), and deletion of CXCL12 in these cells

resulted in a mild reduction of the long-term reconstitution capacity of HSCs (Greenbaum et al, 2013). Paradoxically, endothelial cells secrete SCF which is essential for HSCs, but not for c-Kit⁺ progenitors, which could be due to the distinct localisation of these cells relative to the endothelial niche (Comazzeto et al, 2021).

1.6.2.3 Neural regulation of HSC function

The BM is richly innervated by the central and peripheral nervous system, which is vital for regulation of HSC trafficking and homing. One of the first anatomical descriptions of BM innervation was published by Kuntz & Richins (1945), noting that nerves enter bone cavities alongside blood vessels, possessing vasomotor functions, but were also likely sensory due to pain inflicted when performing BM aspiration. A later description of BM innervation in laboratory animals - specifically, rat femur - showed the presence of myelinated (motor) and non-myelinated (sensory) nerve fibers (Calvo, 1960; Arcilla & Tadi, 2023). The importance of the sympathetic division of the autonomic nervous system is reliant on the regulation of MSCs, as sympathetic denervation leads to a decrease in HSC survival, which is sometimes associated with cytotoxic drugs which are part of chemotherapy treatment schemes (Jung et al, 2017). The circadian release of HSCs and leukocytes into the circulation is modulated by photic cues in a mechanism orchestrated by the molecular clock of the central nervous system, which downregulates the expression of CXCL12 through the release of noradrenaline into the stroma of the BM niche (Mendez-Ferrer et al, 2008).

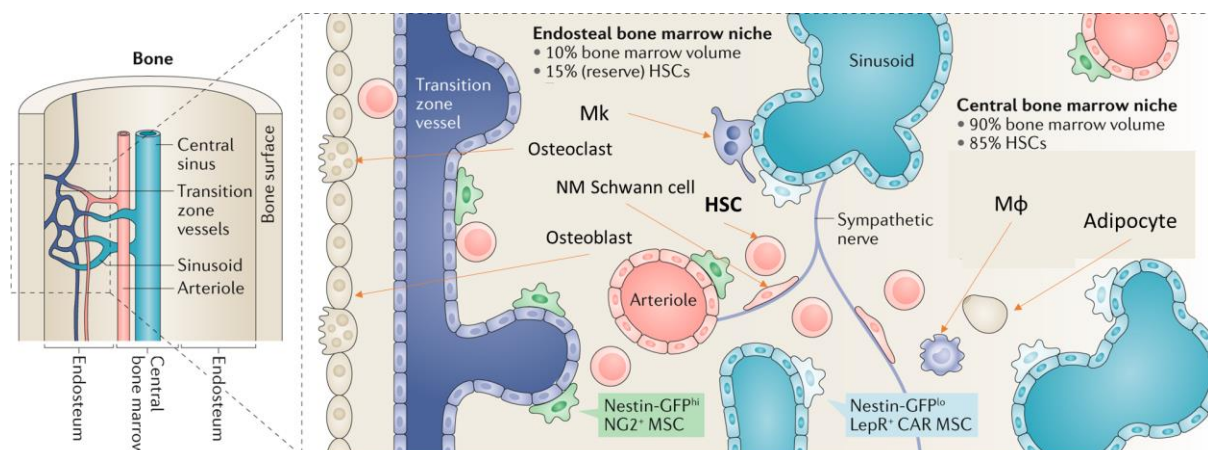


Figure 1.6. Composition of the bone marrow microenvironment surrounding HSCs. Adapted from Mendez-Ferrer et al, 2020. The bone marrow can be understood as a complex ecosystem where various cell populations reside within specific regions. Located in the inner bone marrow, the central niche contains the largest proportion of HSCs, where most of the sinusoids and arterioles irrigated the niche are also located. The central niche is the hotspot for daily blood cell production, which cause the niche to constantly expand by increasing the number of

sympathetic nerve fibres, capillaries surrounded by Nestin⁺ MSCs, proliferation of HSCs and higher rate of myeloid production. Within the endosteal niche, a reserve of HSCs (comprising about 15% of total) which reside in this region are mostly quiescent, thereby contributing to regenerative haematopoiesis in response to different types of stress, and greater production of cells of the lymphoid lineage. Figure adapted from Mendez-Ferrer et al, 2020.

1.6.3 Bone marrow niche in disease

Inflammatory cues in the BM microenvironment can have durable effects on HSCs and HSPCs, as a result of infection, microbiota, or carcinogens (King & Goodell, 2011; Caiado et al, 2021; Cao et al, 2024). Many inflammatory signalling pathways have emerged as critical regulators of physiological HSC activity, including IFN- γ regulation of quiescence (Essers et al, 2009; Baldridge et al, 2010), and developmental emergence of HSCs (Sawamiphak et al, 2014). Likewise, the TNF- α p65 cIAP2 axis has been shown to regulate myeloid differentiation (Yamashita & Passegue, 2019), and its role on HSC formation in the embryonic endothelium (Espin-Palanzon et al, 2014). Nevertheless, acquisition of mutations in HSCs can render them hypersensitive or unresponsive to signalling pathways mediated by such molecules, generating aberrant progeny, which in turn disrupt the normal cellular ecosystem in the niche, accelerating disease (Gleitz et al, 2021).

It is known that leukaemic stem cells are capable of remodelling the niche to their own advantage, thereby promoting leukaemic progression and homeostatic alteration of normal haematopoiesis (Urs et al, 2024). However, the controversy lies on the fact that dysregulation of cell signalling within the niche could also drive malignant transformation of HSCs (Mian et al, 2025). What is clear is that the BM niche does play an essential part in the biology of blood cancer, and especially in myeloid disorders.

1.6.3.1 Bone marrow niche in leukaemia

The importance of the niche for leukaemic progression was first formally demonstrated by deletion of I κ B α - a regulator of the NF- κ B pathway - in perinatal liver cells resulted in the development of myelodysplastic disease in mice, with progression to a secondary AML phenotype (Rupec et al, 2005; Schepers et al, 2015). The upregulation of NF κ B can therefore result in the overproduction of inflammatory cytokines, chemokines, and adhesion molecules within the stromal niche which disrupt normal haematopoiesis (Zhou et al, 2016). A substantial increase in stromal fibroblasts and pericytes has been reported in the preleukaemic BM niche, together with the loss of endothelial cells, which would normally support healthy HSC function (Goda et al, 2024). The stromal niche has also been shown to be altered in CML patients, in

which niche factors such as CXCL14, type I collagen and laminin subunit 4 (encoded by *LAMA4*) are significantly reduced in comparison to healthy volunteers (Dolinska et al, 2023). Overexpression in the NIH3T3 stromal cell line, which was co-cultured with CD34⁺CD38⁻ progenitors, resulted in lower engraftment in transplantation recipients and reduction of progenitor growth *in vitro*.

1.6.3.2 Bone marrow niche in MDS

Importantly, MSCs, osteoblasts and endothelial cells heavily influence normal HSC differentiation and self-renewal, and therefore can exert detrimental effects can contribute to the pathophysiology of MDS through the expansion of MDS clones and the altered production of cytokines and other niche factors (Li & Calvi, 2017). In a study led by Kfoury and colleagues, the mesenchymal niche exhibited increased expression of genes implicated in the epithelial-to-mesenchymal transition (EMT), as well as loss of secreted phosphoprotein 1 (SPP1) or Osteopontin, which is normally secreted by osteoclasts, epithelial, vascular and endothelial cells among others, resulting in increased accelerated MDS disease progression (Kfoury et al, 2023). It has likewise been proposed that the relative spatial location of HSCs in relation to the cytokine or chemokine-producing cells can affect their response to such signals, as being closer in the vicinity of these cells might exacerbate HSC response (Pronk & Raaijmakers, 2019).

1.6.3.3 Bone marrow niche in MPNs

Regarding MPNs and their different subtypes, there is likewise an ever-increasing body of research that highlights the fundamental role of the niche and immune cells in the dysregulation of normal HSC function. The MPN BM niche displays particular hallmarks in contrast to the healthy niche, such increased amount of trabecular bone, increased secretion of IL-6 and IL-1 β , altered transforming-growth factor β (TGF- β) and Notch, and subsequent marrow fibrosis, likely due to LSC induced niche remodelling, as it has previously suggested (Schepers et al, 2013). Alterations to the extracellular matrix surrounding stem and progenitor cells in the marrow have also been reported to be associated with myelofibrosis, since different populations of the niche - including LepR⁺, endothelial cells and fibroblasts - produce elevated quantities of ECM proteins in the fibrotic marrow (Li et al, 2024). The dysfunction of the distinct cellular components of the stromal niche, together with aberrant TPO, Notch, TNF- β signalling, induce a chronic state of inflammation, promoting MPN HSC maintenance, proliferation and survival (Curto-Garcia et al, 2020).

1.6.4 Cellular content by bone marrow location

Early measurements of BM cellularity in humans by radioactive methods estimated a total of 10.4×10^9 nucleated cells per kilogramme (kg) of body weight (Harrison, 1962). In a comprehensive review, Cosgrove and colleagues reported a total of 2×10^8 nucleated cells in normal laboratory mice (Cosgrove et al, 2021; Chervenik et al, 1968). In another review by Nombela-Arrieta & Manz (2017), an estimate of $0.8\text{-}1.5 \times 10^{12}$ total nucleated cells in the human bone marrow were reported, 4-fold higher than the figure calculated by Harrison over four decades ago. In regards to mouse total nucleated BM cells, $2.5\text{-}5.2 \times 10^8$ cells were estimated, consistent with a previous study using radioactive approaches for cell quantification in the whole murine skeleton (Boggs, 1984).

Although it is known that HSCs are a rare population ($<0.01\%$ of total nucleated BM cells) (Challen et al, 2009), whether the number of functional HSCs varies according to BM location (i.e., different bones) is a long-standing question which has not been fully investigated. Historically, the most commonly used bones for haematopoietic stem cell characterisation have been the femur, followed by the tibia, most likely due to ease of access, or cleaner BM preparations obtained from these bones. Early HSC quantification studies profiled femur and tibia BM injections on the recovery from irradiation in mice (Urso & Congdon, 1957) or to determine the number of CFU-S per total BM cells transplanted to prospectively isolate HSCs (Spangrude & Brooks, 1992). Later transplantation studies have estimated the number of repopulating and competitive repopulating units in wild-type mouse hindlimbs - approximately 3.2×10^3 CRUs (defined as Lin⁻ Sca-1⁺ c-Kit⁺ CD34⁻ HSCs) per 10^5 BM cells at 8 weeks of age (Sudo et al, 2000). Quantification of BM cellularity has also been performed on tibia alone (Fruhman, 1964), but there are no reports concerning quantification of HSCs in this individual bone. Besides long bones, other tissues such as the vertebrae in the spine have likewise been extensively used for prospective isolation of HSCs, although there are no thorough studies quantifying the number of functional stem cells in the mouse spine. Among the literature, spine cellularity has been studied with the aim quantifying fibroblast and haematopoietic colony forming assays, and immune cell populations, offering an alternative source of immune and haematopoietic progenitors (Justin et al, 2020).

1.6.4.1 Profiling of haematopoietic stem cell distribution across the mouse skeleton

Nevertheless, even if historically bones have not been meticulously studied one at a time, detailed descriptions of cellularity distributions per BM location have shed light on the concept of HSC distribution across the skeleton. In 1979, Shaposhnikov quantified BM content in bones

of the skull, mandible, upper limbs, clavicle, sternum, rib cage, spine (cervical, thoracic, lumbar, sacral and coccygeal regions), pelvis (hip) and hindlimbs. His results showed that most of the BM was contained in the spine (33.7%), followed by the skull (19.6%), hindlimbs, including femurs and tibias, (11.9%), and pelvis (8.2%). Another comprehensive study was conducted by Colvin and colleagues yielded precise cell counts of BM in 8 different bones across three different strains of wild-type mice to characterise progenitor cell engraftment (Colvin et al, 2004). Their results were similar to those published by Shaposhnikov, in that the spine contained the most BM, but Colvin reported over 50% of total BM content in the spine, followed by the skull, femurs, ribs, ilia (pelvis or hips), humeri and tibias. The authors also performed whole BM transplants from femur, tibia and ilium marrow mixed together to compare against spine BM, resulting in a higher proportion of transplant units in recipients of spine BM than femur, tibia and ilium BM. Nonetheless, since this was not a transplant of purified haematopoietic stem or progenitor cells and data was only collected up to 12 weeks after transplantation, it is difficult to determine whether the overall high BM content in the BM was in fact correlated with the higher number of transplant units in the recipients. Exactly one decade later, the Bystrykh laboratory isolated LSK SLAM stem cells from spatially related bones: sternum and forelimbs, left and right femurs, tibias and ilia, and spine on its own in primary recipients of barcoded HSCs from young and aged mice (Verovskaya et al, 2014). The results demonstrated a highly skewed distribution of barcoded cells, with some clones found in only one BM location, but absent in the others; in addition, mobilisation with G-CSF resulted in a more homogeneous distribution of barcoded cells across the different profiled bone locations.

1.6.4.2 Do HSC functional properties vary according to anatomical bone marrow location?

Although this latter study does not provide information on HSC activity per individual bone, it suggests that there might be intrinsic differences between the microenvironment in each bone, or group of bones, which might influence the multilineage and engraftment potential of HSCs. Likewise, since cell fate is mainly determined by molecular programmes, this could imply that, if HSCs from different bones would actually differ in terms of their functional potential, then it is possible that they could differ molecularly. These questions can only be answered from a single-cell point of view, as HSCs make fate decisions at the single-cell level, in addition to their position as the most primitive elements in the haematopoietic differentiation roadmap, which could be impacted by microenvironmental cues specific to a particular BM niche. The importance of exploring these differences relies on the fact that some of this BM locations could present a more reliable source of functional HSCs for transplantation, which could have

an impact on the clinic, and to even determine how HSCs from distinct bones respond to stress, including infection and inflammation (Wu et al, 2021). However, it is not practical to withdraw BM samples from different anatomical locations on human beings, as this is an invasive clinical procedure. Since the most robust HSC purification strategies have been developed on murine cells, the use of laboratory animals facilitates the profiling of this highly purified population across different BM locations and execute a diverse array of single-cell assays, including transplantation, molecular profiling and single-cell cultures for *ex vivo* expansion of HSCs, and extraction of genomic DNA for sequencing. Thus, questions on the impact on anatomical BM locations on the function and molecular profile of HSCs, as well as their relatedness regarding bone of origin are questions which remain unanswered, and form the basis for the work described in Chapter 3 of this doctoral thesis work.

1.7 *Ex vivo* expansion of haematopoietic stem cells

Through the over six decades of HSC research since Till & McCulloch's seminal work to identify a functional assay for HSCs, there have been numerous culture systems developed in order to characterise diverse aspects of HSC biology *in vitro*. Moreover, due to their potential applications in regenerative medicine for many haematological - and even non-haematological - conditions (Jenq & van den Brink, 2010), the burgeoning field of *ex vivo* HSC expansion has produced highly advanced culture systems, which now allow to execute experiments with large number of HSCs - in the scale of 10^4 or higher, which was not possible before. *Ex vivo* expansion systems can be classified according to the type of self-renewal regulators on which they are based: cell-intrinsic, which is regulated by expression of genes controlling transcription and signalling pathways, such as the Homeobox (Hox) gene family; and cell-extrinsic, regulated by growth factors produced within the BM niche, such as interleukins, SCF and thrombopoietin (Sauvegeau et al, 2004; Walasek et al, 2012). In the following sections, these major HSC culture systems will be discussed, together with the main applications of such systems to study HSC biology.

1.7.1 Semisolid culture systems and the discovery of colony-stimulating factors

The Sachs laboratory developed a culture system to produce mast cells from spleen cells, seeded onto soft agar with a layer of feeder cells, prepared from mouse embryos (Pluznik & Sachs, 1965). Working together in Australia, Metcalf and his colleague Bradley published a landmark method in which they cultured single-cell BM suspension on soft agar or layers of feeder cells (isolated from mouse kidney or embryo) (Bradley & Metcalf, 1966). Here, they demonstrated that only BM cells were able to form granulocytic and monocytic colonies in the

agar, in contrast to cells from thymus or lymph nodes. The feeder cell layer produced certain substances which promoted colony formation, which had also been observed by the laboratory of Sachs (Pluznik & Sachs, 1966). Importantly, time of incubation with feeder cells correlated with the final type of colony formed in culture - in the earlier stages, colonies had a blast cell appearance, but after 15 days of culture, they developed into more granulocyte- and macrophage like colonies (Ichikawa et al, 1966). These 'substances' or 'soluble factors' were later named *colony stimulating factors* (CSF), discovered in serum from different mouse strains, including the AKR leukaemic mouse strain, which interestingly showed the highest colony stimulating activity (Robinson et al, 1967). During the next two decades following these discoveries, four of the mainly recognised CSFs were isolated and characterised: macrophage-stimulating CSF (M-CSF), granulocyte-stimulating (G-CSF), and the multilineage granulo-macrophage stimulating factor (GM-CSF) and interleukin-3 (IL-3) (Metcalf, 2010; Nimer & Uchida, 1995).

These studies constituted a building block for the later development of *in vitro* systems of the long-term maintenance and expansion of HSCs, relying on the addition of soluble factors, not derived from feeder cell layers, but from the different non-haematopoietic populations within the bone marrow niche.

1.7.2 *In vitro* maintenance of HSCs - serum-free cultures and growth factors

Colony forming assays in semisolid agar or specialised methylcellulose media (Purton & Scadden, 2007) are still excellent tools for profiling the functional performance of haematopoietic stem and progenitor cells for a myriad of applications and purposes (Purton & Scadden, 2007; Frisch & Calvi, 2015). Yet, these only allow short-term maintenance of HSPCs, and transplantation becomes technically challenging due to the nature of the semisolid culture media. Although the culture of human blood cells in liquid medium systems, particularly the white blood cell fraction, had been demonstrated previously (Hastings et al, 1961), it was not until the late 1970s that Iscove's group initiated a series of studies with modified culture media. Iscove noted that B and T lymphocytes required nutrients which were not present in fetal calf serum, thus leading to the creation of a modified version of Dulbecco's Modified Eagle Medium by addition of serum albumin, soybean lipid and transferrin (Iscove & Melchers, 1978). This media, termed Iscove's Modified Dulbecco's Medium (IMDM) was also shown to support growth of erythroid colony forming units, supplemented with other nutrients such as lecithin and cholesterol (Iscove et al, 1980; Iscove, 1984).

In the 1990s, the groups of Dick and Eaves made substantial contributions to the development of liquid culture systems of human haematopoietic stem cells. The discovery of cell-intrinsic factors to enhance HSC expansion *ex vivo* was one of the first methods to produce expanded cells with transplantable potential (Bazhilov et al, 2023). For instance, overexpression of Homeobox 4 (HOXB4) in murine BM cells resulted in increased haematopoietic stem and progenitor cells with 50-fold higher self-renewal capacity *in vitro* and *in vivo* serial transplantation experiments (Sauvageau et al, 1994). Petzer and coworkers established single-cell cultures from CD34⁺CD38⁻ cells in IMDM, and a combination of growth factors - which were known to be molecules produced within the BM - SCF, FMS-like tyrosine kinase 3 ligand (Flt3), interleukins 3 and 6, G-CSF, and β -nerve growth factor (NGF) (Petzer et al, 1995). This allowed for the identification of long-term culture initiating cells (LTC-ICs), which were able to expand and be re-expanded, with an expansion yield of up to 50-fold after three weeks of culture. The combination of such growth factors equally promoted the characterisation of haematopoietic progenitors isolated from healthy and CML patients in semisolid culture media (Petzer et al, 1996; Petzer et al, 1997). Likewise, Bhatia and colleagues isolated CD34⁺CD38⁻ and CD34⁺CD38⁺ progenitors and cultured them in the presence of Flt3, SCF, G-CSF, and interleukins 3 and 6, resulting in cells with long-term repopulating capacity (Bahtia et al, 1997).

The discovery that TPO supported HSC expansion came first from *in vitro* experiments of HSCs cultured in the presence or absence of TPO (Bozhilov et al, 2023), demonstrating increased long-term repopulating activity of expanded HSCs in TPO-enriched culture media (Yagi et al, 1999), as well as human HSPC cultures (Ueda et al, 2000). *In vivo* observations supported these findings - an important study showed that transplant recipients of *Tpo*^{-/-} BM cells displayed significantly reduced survival rates than those of *Tpo*^{+/+} (wild-type) cells (Fox et al, 2002).

In addition to growth factors, small molecules such as UM171 or UM279 have demonstrated to support cord-blood (CB) derived HSPC expansion. Both molecules are pyrimidoindole derivatives, and potent agonists of HSC expansion, which have been highlighted for their potent effects on the increase of self-renewal in cord blood progenitors, which offer alternative options for HSCT due to minimum residual disease and reduced need of human leukocyte antigen (HLA) matching (Fares et al, 2014; Cohen et al, 2018; Subramaniam et al, 2020). Due to its implications in the regulation of haematopoiesis (Singh et al, 2009), the aryl hydrocarbon receptor (AHR) has likewise been exploited for the development of small molecules promoting HSC expansion. One of such compounds, the purine derivative StemRegenin1 (SR1) - an

antagonist of AHR - was found to increase CD34⁺ HSC expansion and increase the retention of their engraftment potential (Boitano et al, 2010).

1.7.2.1 Defined culture media systems for murine HSCs

One of the major challenges in HSC *ex vivo* expansion has historically been marked by batch-to-batch variation of specific additives in the culture media, mainly bovine serum albumin (BSA), which replaced foetal calf serum (FCS) when the first long-term *in vitro* expansion systems were developed. Moreover, the difficulty in faithfully replicating the BM microenvironment *in vitro*, had halted the field of HSC expansion until the decade of 2010s. In order to overcome the problem of BSA variation, the Yamazaki laboratory tested 15 different commercially available lots of BSA fraction V (BSA-FV), which allowed for the identification of IL-1 α , which in combination with BSA-FV and recombinant serum albumin (RSA) enhanced HSC self-renewal (Ieyasu et al, 2017). Furthermore, high-performance liquid chromatography analysis (HPLC) of BSA-FV proteins identified hemopexin as a factor which, in combination with RSA and IL-1 α , resulted in expanded HSCs with transplantable potential.

However, it wasn't until members of the Yamazaki and Nakauchi groups published one of the most important breakthroughs in HSC expansion so far - the development of a defined culture media for the expansion of clonal and bulk murine HSC cultures, achieving up to 899-fold expansion yield after 28 days (Wilkinson et al, 2019; Wilkinson et al, 2020). In this study, TPO concentrations were titrated against concentrations of SCF in cultures starting with 50 HSCs per culture well. The concentrations resulting in the highest chimaerism levels in recipients of expanded HSCs were 100 ng/mL⁻ of TPO and 10 ng/mL⁻ of SCF. Next, following a similar approach to Ieyasu et al (2017), the authors tested several different commercially available polymers which could replace BSA in culture. Polyvinyl alcohol (PVA) addition to culture medium containing 50 HSCs resulted in the highest expansion yield, as well as multilineage potential in serial transplantation assays. Thus, through the combination of high TPO concentrations with low SCF concentrations, and the addition of PVA to the cultures in Ham's F12 nutrient mix, has been established as the most effective culture system for murine HSCs to date.

1.7.2.1.1 Applications of F12-PVA expansion conditions

Despite being highly efficient, the F12-PVA based expansion method is not perfect, as a large percentage of the initial cell input will still differentiate and lose self-renewal potential. Still though, the method has enabled the execution of experiments at a larger scale, and

consequently new questions about HSC function and behaviour that can be pursued using this method (Rubio-Lara et al, 2023; Meaker & Wilkinson, 2024). For instance, members of the Yamazaki group conducted a study in which c-Kit⁺ enriched cells were cultured in the F12-PVA expansion system, which were able to reconstitute recipients in serial transplantation assays, displaying remarkably similar engraftment to freshly isolated HSCs (Ochi et al, 2021). Although it is not possible to establish a single-cell culture from enriched BM cell fractions, this study still presents an alternative to sorting, which reduces experimental costs and can increase efficiency as less time is required to complete sample processing. The optimisation of gene-editing approaches in single HSCs has been further advanced by this novel expansion system, which has been elegantly illustrated by a study from the Kent and Yamazaki groups (Becker et al, 2023). Here, the authors demonstrated that replacement of PVA with Soluplus optimised expansion conditions. This allowed for the expansion and profiling of SCID model-derived HSCs which had undergone CRISPR-Cas9 mediated correction of the *Prkdc*^{scid} mutation, which were able to engraft transplant recipients, whereby evidencing the rescue of the immunodeficient phenotype.

One of the first studies to characterise *ex vivo* expanded HSCs in the F12-PVA based system was published by the Kent laboratory only a few years after the publication of the original method (Che et al, 2022). In this study, the content of phenotypic HSCs (pHSC) - defined as EPCR⁺CD45⁺Lin⁻Sca-1⁺c-Kit⁺ (ELSK) - in expansion cultures provided a reliable metric for the identification of engraftable single-cell derived clones grown in the F12-PVA expansion conditions (>20% of ELSK content for clonal cultures), which stably reconstituted transplant recipients for at least 4 months. This relevance of these findings relies on the fact that it is not necessary to isolate phenotypic HSCs through FACS, which increases experimental efficiency, as analysing ELSK content ahead of transplantation will suffice to determine which clones will repopulate a recipient. In a later report by the Bryder laboratory, *ex vivo* expanded HSCs with transplantable potential were defined as Lin⁻ FceR1a⁻ Sca-1⁺ c-Kit⁺ CD150⁺ CD48^{low} EPCR^{high} or candidate HSCs (Zhang et al, 2024).

1.7.2.1.2 Studying malignant HSC biology in expansion cultures

Malignant cell phenotypes have largely been characterised in mouse models of haematological disorders, as discussed above, and this has certainly advanced and transformed the basic and clinical research in the field. However, the coupling of mouse models with novel *in vitro* methodologies can result in even more impactful knowledge of fundamental aspects of disease evolution.

A high-fidelity CRISPR/Cas9 editing system was applied to study *HbS* gene correction in HSCs isolated from the sickle-cell disease (SCD) Townes mouse model on F12-PVA expansion cultures (Wilkinson et al, 2021). Transplantation of Cas9 gene-edited expanded HSCs resulted in restored of haemoglobin A (HgbA) and rescue of normal erythrocyte production. More recently, a comprehensive profiling of clonal dynamics of *ex vivo* expanded HSCs revealed important differences between wild-type and DNMT3A- and NPM1-mutant cellular states and fates (Singh et al, 2025). By leveraging the LARRY barcoding system and mouse models of inducible leukaemic mutations, the authors profiled single-cell derived clones that had been ‘split’ following a similar approach to two previous studies (Zhang et al, 2023; Tian et al, 2021). This approach elucidated the mutational synergistic effect of DNMT3A and NPM1 at the clonal level, which increased HSC fitness in comparison to wild-type or single-mutant HSCs profiled at the 30-day culture endpoint. Therefore, these studies demonstrate that the F12-PVA based system offers a robust method to study different aspects of healthy and malignant HSC clonal dynamics.

However, dissecting the mechanisms by which a malignant clone - carrying either a single or multiple mutations - outcompetes healthy clones in haematological cancers is still a major experimental challenge, as the phenomenon of cell competition has classically been modelled *in vivo*, despite its significant implications on leukaemic disease (Morata et al, 2021; Neerven & Vermuelen, 2023). In the next subsection, the concept of cell competition will be introduced, along with some of the main experimental approaches to model competition at the clonal level.

1.7.2.1.3 The concept of *cell competition*

Competition has categorically been the driving force of natural selection in Darwinian theory, since elimination of the least fit members of species preserves only those which are fittest, and are able to respond effectively to the different stimuli or stresses in their environment (Christiansen & Loeschke, 1990). Hamilton’s perspective on kinship and social interactions between members of different species provides interesting insights into tissue development and evolution: cells which are genetically related will act as ‘neighbours’, therefore promoting tissue formation and, consequently, the development of the organism as a whole (Hamilton, 1964). Cells which are genetically ‘distant’ will need to be removed from a specific organ unit if they will not contribute to its development, thereby being *outcompeted* and eliminated (Johnston, 2014).

Cell competition was originally described in *Drosophila melanogaster*, as an essential mechanism for the correct formation of the fly’s wing and leg disc structures, which was

impaired by heterozygous mutations in ribosomal genes (Bryant & Schneiderman, 1969; Garcia-Bellido & Merriam, 1971). Interestingly, in genetic mosaics, wild-type cells actively removed mutant cells by a form of programmed cell death termed apoptosis, implying that they *outcompeted* their genetically impaired counterparts - the mutants were called *Minutes* due to their smaller body sizes compared to normally developed individuals (Morata & Ripoll, 1975). After these findings, the field of developmental biology advanced rapidly, but the concept of cell competition had been forgotten, until the end of the 1990s, when it rebirthed after the discovery that somatic mutations could influence competition. In a study led by Johnston, loss of the drosophila *Myc* (*dMyc*) gene impaired wing formation in *Drosophila*, as *Myc*-deficient cells were being eliminated by wild-type cells in genetic mosaics, which was one of the first forms of cell competition assays reported (Johnston et al, 1999). Moreover, mutants of *chico*, a size regulator gene, showed growth impairment but not enhanced apoptosis, while still being outcompeted by wild-type cells which proliferated at a normal rate (Bohni et al, 1999). Following from this study, Moreno and coworkers hypothesized that cell competition relies on the sensing of slowly proliferating cells, which are eliminated by apoptosis due to the overexpression of the *brk* gene, which is characteristic of *Minute* heterozygous cells (Moreno et al, 2002).

Since *Myc* had been implicated in a vast number of human solid tumours (Dang, 1999), its role was further profiled on cell competition in genetic mosaics of *Drosophila* carrying between two and four copies of the *dMyc* gene (Moreno & Basler, 2004). This study identified *super-competitors* for the first time - mutant clones which annihilated wild-type clones in a spatial-temporal manner after 72 hrs of *dMyc* induction. Almost a decade later, characterisation of cell competition in functional genetic mosaics of mouse embryos, revealed the presence of super-competitors induced by the overexpression of *Myc*, which was also confirmed by *in vitro* assays of embryonic stem cells.

1.7.2.1.4 Cell competition in haematopoietic stem cells

Cell competition has been previously reported in the haematopoietic system as a mechanism for controlling the size of the HSC pool, likely mediated by cell-extrinsic factors, such as CXCL12, normally regulated by MPPs expressing the CXCR4 receptor (Miao et al, 2022), which goes in line with the principle of organ and tissue size control. Likewise, cell competition in the thymus is an important regulator of thymic precursors, and consequently, T lymphocyte development, primarily mediated by the double-negative (CD4⁻ CD8⁻, DN) fraction of early thymocytes (Ramos et al, 2020; Ramos & Martin, 2021). However, the acquisition of somatic

mutations can lead to altered cell competition, which then results in clonal outgrowth and potentially malignancy.

The principle that haematological disorders, including leukaemia and pre-cancerous diseases such as MDS or MPN, are originated in the stem cell compartment led to the notion that clonal competition must also exist at the HSPC level. The publication of two key studies in the year 2010 led to the discovery of cell competition in HSPCs primarily driven by mutations in the tumour suppressor gene, *p53* (Marusyk et al, 2010; Bondar & Medzhitov, 2010). In the study led by Marusyk, irradiation resulted in a selective advantage of *p53*-deficient HSCs, leading to the clonal expansion and lymphoma development in mice (Masuryk et al, 2010). Next, in the landmark study by Bondar & Medzhitov, cell competition was modelled through the generation of haematopoietic chimaeras by transplanting BM from wild-type and *p53* heterozygous mice (Bondar & Medzhitov, 2010). Prior to transplantation, a fraction of the BM cells was subjected to 1 Gray of irradiation in order to induce stress in the HSPC compartment and then measure the level of competition between irradiated and non-irradiated cells transplanted together. This resulted in higher chimaerism displayed by non-irradiated wild-type cells, which outcompeted their irradiated counterparts, as evidenced by a higher proportion of donor-derived HSPCs in recipient BM, in comparison to a lower contribution by irradiated *p53*^{+/-} cells. Additionally, this mechanism was found to persist for months and to be associated with DNA-damage response, which could be the underlying cause for the acquisition of a senescent phenotype in the irradiated HSPC fraction. The role of *p53* was further investigated in a study by Quijada-Alamo and coworkers, through the generation of CLL cell lines by CRISPR/Cas9, revealing a higher competitive advantage in cells harbouring monoallelic chromosomal deletion at 11q (del(11q)) than in cells with biallelic loss of *ATM* and *p53* (Quijada-Alamo et al, 2021).

1.7.2.1.5 The need for the development of HSC clonal competition models

Thanks to the contribution made by all these important studies in the field of cell competition, it is now known that mutant HSCs can outperform their normal counterparts, or be outperformed by them, depending on the level of induced stress and the mutation they harbour. Loss-of-function in genes such as *TET2* and *DNMT3A* have been shown to increase the self-renewal capacities of HSCs (Challen et al, 2011; Moran-Krusio et al, 2011), promoting the expansion of the stem cell pool, which could also be replicated *in vitro* thanks to the availability of powerful mouse models, particularly the *TET2*^{-/-} mouse model produced by the Rao laboratory (Ko et al, 2011). In a recent report by Yang and colleagues, overexpression of *HOXB4* - a transcription factor involved in the regulation of HSC self-renewal - in haematopoietic progenitors isolated from *TET2* knockout or wild-type mice was reported

(Yang et al, 2025). TET2 knockout HOXB4 over-expressing cells were mixed with TET2 WT HOXB4 over-expressing cells and were treated with several different epigenetic compounds, of which SCG0946 - an inhibitor of the DOT1L methyltransferase - reduced the competitive advantage of TET2 KO HOXB4 progenitor cells, which increased dimethylation of the *Mpl* gene, which encodes the TPO receptor. Likewise, the proportion of *Mpl*⁺ HSCs was increased in TET2 KO BM cells, suggesting that upregulation of TPO signalling might be one of the mechanisms through which TET2 deficient cells acquire a higher competitive advantage. Although not in competition settings, the selective advantages conferred by DNMT3A and TET2 LOF mutations have also been reported in clonal haematopoiesis (Jakobsen et al, 2024).

Nevertheless, there have not been any assays formally measuring the clonal dynamics of cell competition between healthy and malignant HSCs *in vitro*. There are major technical challenges to faithfully replicate this phenomenon *in vivo*, especially at the single-cell level, since the transplantation of two single cells from different genotypes - even if they carry a molecular barcode - might result in loss of one of the cells, as single-cell transplants are error-prone and therefore, are not completely effective (50% of transplant success for mouse ESLAM HSCs) (Kent et al, 2009; Kent et al, 2016). If this is then overcome by transplanting more than one cell from each of the genotypes of interest, it will thereby eliminate the clonal component to the assay and will not allow to characterise cell competition in single-cell derived clones. Notwithstanding, thanks to previous studies in our group (Shepherd et al, 2018; Oedekoven et al, 2021; Che et al, 2022), it is possible to establish single-cell cultures isolated from wild-type and mutant mouse models, in both short-term (Oedekoven et al, 2021) and long-term expansion (Che et al, 2022). Given that TET2 deficiency increases HSC expansion in the BM, resulting in an imbalance in haematopoietic production, this implies that healthy clones are very likely being outcompeted by their malignant counterparts, which is clinically relevant to understand the underlying clonal dynamics of blood cancers. This question will be addressed in Chapter 4 of this doctoral thesis work.

1.8 Thesis aims

The overarching goal of this doctoral thesis is to investigate different aspects of HSC clonal evolution, clonal competition dynamics and the complex interactions between HSCs and their microenvironment in health and disease.

The central aim of Chapter 3 is to understand the implications of anatomical location on fundamental biological properties of HSCs derived from the mouse femur, tibia, ilium and spine at the single-cell level. In the first instance, it seeks to retrospectively trace the life histories of HSCs, using somatic mutations as barcodes, thereby inferring clonal relatedness, and how this influences the seeding of their future microenvironmental site of residence. Likewise, it dissects the transcriptomic profiles of such HSCs, to discover molecular cues which might help distinguish HSCs from one anatomical site to the other, and to make informed inferences about their functionality. Lastly, it seeks to compare the functional performance of HSCs across the different bone marrow locations, in order to determine the impact of geography on self-renewal and multilineage engraftment potential.

The main objective of Chapter 4 is to leverage on somatic mutations which are implicated in various forms of pre-leukaemic disorders to explore the dynamics of clonal competition. As the current state-of-the-art in cell competition models largely relies on *in vivo* assays, work in this chapter is initially focused on the development and validation of a split HSC culture system, to be then adapted for the systematic study of wild-type and TET2 HSC competition at the clonal level. It also seeks to profile the competitive advantage of clones, or lack thereof, and the mechanism(s) underlying such behaviour.

The primary aim of Chapter 5 is to consolidate the effect of somatic mutations on the development and evolution of pre-leukaemic disease - myeloproliferative neoplasms - and the role of the dysregulated microenvironment. Work described in this chapter begins with the implication of a pro-inflammatory niche cytokine - IP-10 - in patients and mouse models of MPNs, particularly in the presence of concomitant mutations in JAK2 and TET2. The chapter then focuses on the generation of a triple-mutant mouse model, carrying JAK2 V617F knock-in, TET2 KO and IP-10 KO mutations. Lastly, through this approach, work in this chapter seeks to unravel the intricate interplay of cell-intrinsic and cell-extrinsic factors on MPN disease biology, at a level of detail which is only possible by leveraging sophisticated *in vivo* models.

Chapter 2 - Materials and Methods

2.1 Mice

Wild-type C57BL/6J/Ly5.2 (CD45.2), C57BL/6J/Ly5.1 *Pep Boy* (CD45.1), and *c-Kit*-defective C57BL/6J-^{W41/W41}Ly5.1 (CD45.1) mice were bred in-house at the Biological Services Facility at the University of York. TET2 loss-of-function (TET2^{-/-}) mice were obtained via Prof. Anajana Rao (La Jolla, US) (Ko et al, 2010). JAK2^{V617F/V617F} knock-in mice (Li et al, 2011) were obtained from Prof. Anthony Green (Cambridge, UK). JAK2^{V617F/V617F} IP-10 knockout (referred as JAK/IP10 hereafter) were obtained by crossing JAK2^{V617F/V617F} with IP-10 (IP-10^{-/-}) knockout mice (B6.129S4-*Cxcl10*^{tm1Adl/J}), purchased from the Jackson Laboratory, US). JAK2^{V617F}/TET2^{-/-}/IP10^{-/-} (JAK/TET/IP10 hereafter) mice were obtained by crossing TET2^{-/-} mice with JAK2^{V617F/+}/IP10^{-/-} mice. Congenic hybrid mice expressing the CD45.1 and CD45.2 epitopes (Ly5.1/Ly5.2) were obtained from Prof. Ian Hitchcock (University of York).

All mice were bred and co-housed in microisolator cages at the animal facility of the Department of Biology at the University of York, co-housing a maximum of 5 animals of the same sex per cage. Animals were kept on 12-hour light/12-hour darkness cycles, at constant temperature between 20-24 °C and 45-65% humidity. Mice were supplied with sterile food pellets, water *ad libitum*, bedding and enrichment. All animal procedures were conducted under the regulations of the Animal Scientific Procedure Act (1986) and the UK Home Office through an Animal Project License (PEAD116C1) owned by Prof. David Kent.

2.1.1 Genotyping by Polymerase Chain Reaction (PCR)

For genotyping of the different MPN mouse models, ear-punch biopsies were obtained from recently weaned mice at the Biological Service Facility, University of York. Samples were immediately processed, otherwise stored at -20°C for later processing. The PCRBio Rapid Extract PCR kit (PCR Biosystems) was used to extract genomic DNA from ear biopsies. A DNA extraction master mix containing 10µL of the Rapid Extract PCR kit buffer A, 5µL of buffer B, and 35µL of PCR-grade ddH₂O per sample, was prepared in individual 1.5-mL PCR clean Eppendorf tubes and each ear biopsy was placed inside. The extraction reaction was incubated for 30 min at 75°C on a heat block, to promote cellular lysis, as well as degradation of nucleases and proteins. Once this incubation period was completed, the samples were further incubated at 95°C for 15 min, and then pulse-centrifuged to recover the condensed liquid from the lid of the tube. DNA was immediately used for PCR or stored at -20°C for later processing.

Primer sequences for JAK2, TET2 and IP-10 used for PCR reactions can be found in Table 2.7. Each PCR reaction master mix contained 12.5µL of KAPA2G fast PCR kit (Roche), 1.5µL of each primer corresponding to the gene of interest (Table 2.1), and 7µL of PCR-grade ddH₂O (24µL). Of note, PCR reactions for each of the three genes were prepared and analysed separately. To each reaction mixture was added 1uL of genomic DNA extracted from ear biopsies, prepared in 0.2-mL PCR tubes (8-strip) (Starlab). The PCR programmes corresponding to each gene are shown in Table 2.2. PCR reactions were run on a single-module (ThermoFisher) or multi-module (BioRad) thermal cyclers. PCR products were analysed on 1% agarose gels, using 1:10000 GelRed dye (Sigma Aldrich) to visualise the products at the end of the electrophoresis run.

Table 2.1 - Primer sequences for genotyping of JAK2, TET2 and IP-10

Primer	Sequence (5' → 3')
JAK2 Forward	5' –GTA CGC TGT CCA CCC AGA TT– 3'
Reverse-1 JAK2	5' –TTC ACA TCT TGT TCC TGT TGC– 3'
Reverse-2 (WT) JAK2	5' –TGC CTC CAT TTC TGT CAT TG– 3'
Wild-type forward TET2	5' – AGC TGA TGG AAA ATG CAA GC – 3'
Wild-type reverse TET2	5' – TCT CAG AGC AAA GAG GAC TGC – 3'
Mutant-forward TET2	5' – GCC ACT TTA GAA GCC TAT TGG A – 3'
IP10 Wild-type	5' - TGC CAC GAT GAA AAA GAA TG - 3'
IP10 Forward	5' - GGG ACA GAA TGG GTT TGT GT - 3'
IP10 Mutant	5' - GCC AGA GGC CAC TTG TGT AG - 3'

Table 2.2 - PCR programmes for genotyping of JAK2, TET2 or IP-10

Gene	Stage	Cycles	Temp	Time	Cycle Type
JAK2	1	1	94°C	1-2 mins	Initial denaturation/e nzyme activation
	2	35	94°C 57°C 72°C	15 sec 15 sec 10 sec	Denaturation Annealing Extension
	3	1	72°C 4°C	5min ∞	Final extension Hold
TET2	1	1	94°C	1-2 mins	Initial denaturation/e nzyme activation
	2	35	94°C 57°C 72°C	15 sec 15 sec 15 sec	Denaturation Annealing Extension
	3	1	72°C 4°C	10 sec ∞	Final extension Hold
IP-10	1	1	94°C	2 mins	Initial denaturation/e nzyme activation
	2	7	94°C 65°C 68°C	20 sec 15 sec 30 sec	Denaturation Annealing Extension
	3	20	94°C 60°C 72°C	15 sec 15 sec 30 sec	

	4	1	72°C 1°C	3 min ∞	Final extension Hold

2.2 Isolation of Haematopoietic Stem Cells

2.2.1 Tissue preparation

Bone Marrow was harvested from tibias, femurs, ilia, sternum and spine from mice between 6 and 30 weeks of age from wild-type and mutant mice, bones were processed individually (Results Chapter 3) or in bulk (Results Chapters 4 and 5) by crushing or flushing (the latter not applicable to sternum and spine) the bone marrow into PBS, 1X supplemented with 2% of Foetal Bovine Serum (FBS) (Gibco Life Sciences). Of note, experiments corresponding to Chapter 3 required mice to age until 29 weeks to allow for the accumulation of genomic mutations, which would subsequently be used for the phylogenetic analysis (See Chapter 3). For experiments corresponding to Chapter 4, mice were used during young adult age (9-12 weeks). In Chapter 5, tissue collection was performed in mice aged 16 weeks old, where the disease monitoring period ended.

The preparation was pipetted through a 70-um filter to obtain a single-cell suspension into a 50-mL Falcon tube placed on ice. Red blood cells were lysed by treatment with Ammonium Chloride (NH₄Cl, StemCell Technologies) on ice. In most instances, enrichment of HSC/HSPC cells was performed by immunomagnetic depletion of lineage-positive bone marrow cells using the HSPC isolation cocktail (StemCell Technologies), and the EasySep RapidSpheres 50001 (StemCell Technologies). This step was not performed for isolation of HSCs from individual bones due to the limited number of bone marrow cells (Results Chapter 3).

The resulting cell pellets were stained with the haematopoietic stem cell antibody panel at the concentrations shown on Table 2.3, and incubated in the dark for 30 min on ice. Antibody excess was removed by topping up cell suspension tubes with PBS/2% FBS and centrifuged at 300 x g for 5 min. Supernatant was discarded and cells were resuspended into 7AAD at a 1:1000 dilution in PBS/2% FBS and filtered once again through a 40-µM cell strainer before proceeding to sorting.

Table 2.3 - ESLAM marker panel for isolation of haematopoietic stem cells

Antibody	Fluorochrome	Clone	Concentration	Volume per sample (µL)	Supplier	Cat. No.	Lot No.
CD45	FITC	30-F11	1:100	0.5	Biolegend	103108	B330230
CD48	APC	HM48-1	1:100	0.5	Biolegend	103412	B341106
Sca-1	BV421	D7	1:200	0.5	Biolegend	108128	
EPCR	PE	eBio1560 (1560)	1:100	0.5	Invitrogen	12-2012-82	2600245
CD150	PE-Cy7	TC15-12F12.2	1:100	0.5	Biolegend	115914	B353358

2.2.2 Fluorescence-Activated Cell Sorting

The gating strategy for the isolation of HSCs is shown in Figure 2.1 (ESLAM gating strategy), which is as follows: Cells -> FSC-H/FSC-A Singlets -> Live (7AAD negative) -> CD48⁻ CD150⁺ (SLAM) -> CD45⁺ EPCR⁺ (ESLAM) (Chapter 3). In some instances, ESLAM-Sca-1^{high} stem cells were isolated for single-cell and bulk sorts (Chapter 4). Due to reduced cell numbers obtained when bones were processed individually (Chapter 5), Sca-1 expression data was collected, however, this marker was not included in the sort gating strategy. Through the ESLAM strategy, around 43% of total HSCs from a mouse (as defined by their serially transplantable potential) can be purified (Kent et al, 2009). For the work involving the F12-PVA based expansion system, the ELSAM Sca-1^{high} strategy was followed, since it allows for the isolation of 60% of the serially-repopulating HSCs from a mouse (Wilson et al, 2015). HSCs were sorted using a Moflo Astrios (Beckman Coulter) or an S8 sorter (Beckton Dickinson). For single-cell assays, one ESLAM or ESLAM-Sca-1^{high} cell was sorted into wells of U- or flat-bottom 96-well plates containing supplemented 50-100 µL of StemSpan- or 200µL of F12-based supportive media (refer to Table for recipe), using the the sorter's single-cell deposition mode. For bulk cell assays, between 20-10000 ESLAM or LSK cells were sorted in purity mode onto well plates containing media or into 1.5-mL Eppendorf tubes either containing media or PBS, 1X. Flow sorting rate was finely regulated to achieve maximum sorting efficiency and minimise the number of abortions.

The gating strategies for the isolation of B cells (B220/CD45R⁺ CD19⁺), T cells (CD3ε⁺), granulocytes (Ly6G⁺ CD11b⁺) and monocytes (CD11b⁺) is shown in Figure X, all populations gated from Cells -> FSC-H/FSC-A Single cells -> Live (7AAD negative) (Results Chapter 3).

A minimum of 5×10^3 and a maximum of 4×10^5 cells from each population were collected into 1.5 mL DNA lobind tubes (Eppendorf) containing 300 μ L of sterile PBS, 1X. Immediately after the sort was completed, samples were centrifuged at 500 x g for 5 min at room temperature. Supernatant was carefully aspirated by pipetting, avoiding to disturb the cell pellet, and discarded. Samples were immediately stored at -20C.

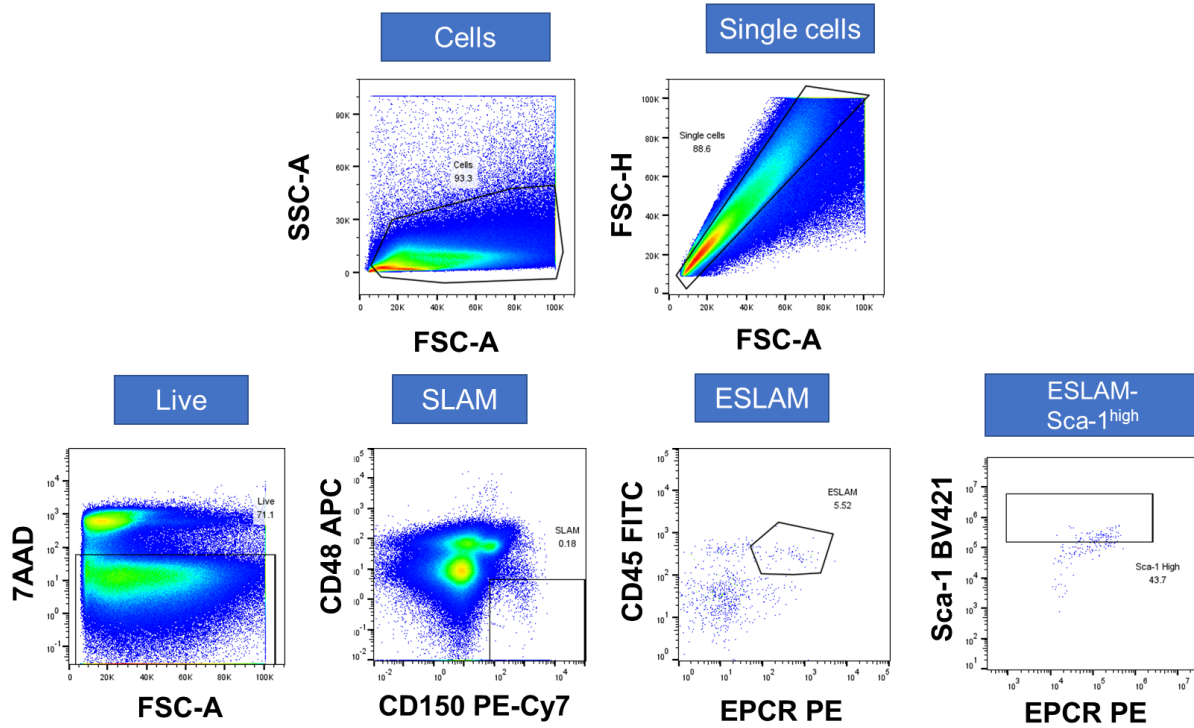


Figure 2.1. ESLAM Isolation strategy. Cells were gated from total events, followed by single cells, and then live cells (7AAD⁻). SLAM cells were gated from Live, to finally gate and purify ESLAM HSCs. The ESLAM-Sca-1^{high} gate was only used for single-cell sorts and corresponding experiments described in Chapter 4.

2.2.2.1 Index-sorting and data analysis

Index-sort data enables the retrospective examination of the phenotype of sorted events in single-cell experiments (Osborne, 2011), which has been useful in resolving clonal heterogeneity of HSCs and optimisation of purification strategies (Schulte et al, 2015), as well as the definition of molecular signatures by coupling transcriptomic data with expression of cell surface markers (Wilson et al, 2015).

To record index-sort data in single-HSC experiments, the index-sort function was enabled in the Summit or FACS Chorus software of the Moflo Astrios or BD S8, respectively. Index-sort data was extracted from the software and exported in CSV format, cell sort files were exported in FCS format. The expression levels of cell surface markers - expressed as median

fluorescence intensity units, MFI - (CD150, CD48, EPCR, CD45) was retrospectively analysed using GraphPad v.10.1.2 and R Studio v 2024-12-01.

2.2.2.2 Isolation of blood cell populations

Male wild-type (CD45.2) mice were divided into two cohorts - the first consisting of 10 and the second consisting of 5 animals - and were serially bled starting at 9 and ending at 27 weeks of age, every 6 weeks, resulting in a total of 4 timepoints collected (9, 15, 21 and 27 weeks). A maximum of 140 μ L of peripheral blood were withdrawn from each mouse. Blood samples were depleted of red blood cells by Ammonium Chloride (StemCell Technologies) treatment (1 mL/sample) for 5 min on ice, followed by a round of thorough vortexing, and further undisturbed incubation on ice for 5 min. Sample tubes were topped up to 3 mL final volume with PBS/2% FBS, and centrifuged at 300 x g for 5 min at room temperature. Pellets were stained with the Lineage antibody master mix at the concentrations and volumes described on Table 2.4. Samples were stained in the dark for 30 min on ice. Antibody excess was washed off by topping up samples with PBS/2% FBS and centrifuged as described above. Prior to sorting, samples were resuspended in a final volume of 400 μ L in 7AAD (1:1000) in PBS/2% FBS and filtered through a 40-um cell strainer into polystyrene FACS tubes (Falcon).

Table 2.4 - Lineage marker panel for isolation of blood cell populations

Antibody	Fluorochrome	Clone	Concentration	Volume per sample (μ L)	Supplier	Cat. No.	Lot No.
Ly6G	BV421	1A8	1:500	0.1	Biolegend	127628	B356918
CD11b	BV785	M1/70	1:500	0.1	Biolegend	101243	B373879
CD3ϵ	PE	145-2C11	1:500	0.1	Biolegend	100308	B346465
B220/CD5R	PE-Cy7	RA3-6B2	1:500	0.1	Biolegend	103221	B357252
CD19	PE-Cy7	eBio 1D3	1:500	0.1	eBioscience	25-0193-82	2450685

2.3 Single-HSC *in vitro* assays

2.3.1 StemSpan Liquid Culture System

Single HSCs were cultured in Stem Span media into 100 μ L in U-bottom 96-well plates between 10 and 14 days. StemSpan (StemCell Technologies) media contained 10% FBS

(unless otherwise stated) (Gibco), 1% Penicillin/Streptomycin cocktail (Sigma Aldrich), 1% L-Glutamine (Gibco), 0.2% β -mercaptoethanol (Gibco), 20 ng/mL IL-11 (Bio-Techne) and 300 ng/mL SCF (StemCell Technologies, Peprotech) (See Table 2.5). For whole-genome sequencing libraries (Results Chapter 3), cloning efficiency (the proportion of sorted wells with growing colonies) was visually assessed on days 2 and 10 after sorting. For single-cell division kinetics assays, cell counts were performed from day 1 to day 4 post-sort (24-96 hours post-sort).

For WGS of single-cell derived clones, culture was ended at day 14 and cells were harvested as follows: plates were observed under the microscope to analyse the quality and viability of the clones prior to harvest to ensure a successful library preparation downstream of this process. Colonies were individually harvested in 100 μ L of sterile PBS, 1X into skirted 96-well polypropylene PCR plates under sterile conditions (Eppendorf) and placed on ice. Plates were centrifuged at 500 x g for 5 minutes at room temperature. After centrifugation, media and PBS were removed by gently flicking the plate into a waste receptacle, and then placed onto a tissue paper bed to remove excess liquid. Plates were covered with 8-cap strips and immediately stored at -80C until the moment of DNA extraction.

Table 2.5 Stem Span media for single-HSC assays

Reagent	Concentration	Volume (μ L)	Final Concentration	Supplier	Cat. No.
Stem Span SFEM	–	900	1X	Stem Cell Technologies	09650
Fetal Bovine Serum	–	100	10%	Gibco	10270-106
Penicillin/Streptomycin	100X	10	1%	Sigma Aldrich	P4333-100mL
L-Glutamine	200 mM	10	1%	Thermo Fisher	25030081
β -mercaptoethanol (β -ME)	50 mM	2	0.2%	Gibco	31350-010
Stem Cell Factor (SCF)	50 μ g/mL	6	300 ng/mL	Peprotech	AF-250-03
Interleukin 11 (IL-11)	10 μ g/mL	2	20 ng/mL	BioTechne	418-ML-025

2.3.2 DNA extraction

DNA from individual colonies and blood cell populations (of numbers larger than 5000 sorted cells) was extracted using the Qiagen DNAeasy kit (Qiagen). Frozen cell pellets (stored in plates or tubes) were thawed and allowed to reach room temperature prior to conducting the technique. Cell pellets were resuspended into 200 μ L of PBS, 1X and transferred into 1.5-mL Lo-Bind Eppendorf tubes. Contents were mixed well, and 20 μ L of Proteinase K solution were added to each sample, followed by 200 μ L of cell lysis buffer. Samples were vortexed gently but thoroughly and incubated at 56°C for 10 minutes on a thermal incubator block. 200 μ L of ethanol was added and samples were vortexed again. The mixture was then transferred to DNA spin columns placed onto a 2-mL round bottom collection tube. Columns were centrifuged at 6000 x g for 1 minute. Flow-through was discarded and the column was placed onto a new collection tube. 500 μ L of the kit's wash buffer 1 were pipetted into each column, samples were centrifuged at 6000 x g for 1 minute, and flow-through was discarded. After placing columns onto new collection tubes, 500 μ L of the kit's wash buffer 2 was added to each of the samples and were centrifuged at 20,000 x g for 3 minutes. The flow-through was discarded and columns were placed onto new, 1.5-mL DNA Lo-Bind tubes to perform DNA elution by carefully pipetting 40 μ L of elution buffer directly into the centre of the spin column membrane. Samples were incubated at room temperature for 1 minute and then centrifuged at 6000 x g for 1 minute. Samples were stored at -20°C.

DNA extraction from single-cell derived colonies and blood cell populations (of number lower than 5000 sorted cells) was performed using the Arcturus Picopure kit (Thermo Fisher). A proteinase K solution was prepared in Arcturus DNA extraction buffer. 50 μ L of solution was added to the low-cell number pellet samples directly into the original sample tubes, mixing well by gently pipetting, and incubating at 60°C for 3 hours on a thermal block. Samples were immediately stored at -20°C.

To quantify the DNA, double-stranded DNA High-sensitivity (dsHS) Qubit quantification reagent was diluted in a 1:200 ratio in Qubit quantification buffer. DNA samples were added at a 1:200 concentration and were read on the Qubit Fluorometer v. 2.0. A maximum of 20 ng of DNA was loaded into wells of a 96-well skirted qPCR plate (Eppendorf) diluted in DNA extraction buffer or topped up with sterile nuclease-free ddH₂O and stored at -20°C. Samples were shipped on dry ice to the Wellcome Sanger Institute (Cambridge) for quality control, library preparation and sequencing.

2.3.3 DNA libraries construction for Whole Genome Sequencing

Libraries were prepared by the low-input DNA sequencing pipeline (Ellis et al, 2021). The NEBNext Ultra II FS reaction buffer and enzyme (New England Biolabs) were used to prepare the fragmentation mix, and it was then added to a pre-chilled 96-well PCR plate (reaction plate). DNA samples were transferred to the reaction plate, which was incubated in a thermal cycler, performing the first step at 37°C for 12 min and the second step for 30 min at 65°C. Ligation mix was prepared using the NEB Next Ultra kit (New England Biolabs), and was added to all samples. PCR amplification was performed using the Kapa HiFi HotStart ready mix and library amplification was done in a thermal cycler. DNA libraries were pooled and transferred in equimolar amounts into 1.5-mL tubes, and quality was assessed on an Agilent Bioanalyser. For WGS, paired-end short sequencing reads (150 base pairs) were generated on an XTEN Illumina machine, resulting in ~ 15X coverage per sample. The Burrows Wheeler Alignment-MEM (BWA-MEM) algorithm was used to align sequences to the mouse reference genome (GRCm39, NCBI).

2.3.4 Construction of phylogenetic trees

Phylogenetic analysis of single-cell derived haematopoietic stem cell clones was performed as previously described (Lee-Six et al, 2018; Spencer-Chapman et al, 2021; Machado et al, 2022; Mitchell et al, 2022; Coorens & Spencer-Chapman, 2024). In brief, genome-wide single-base somatic substitutions were called from aligned sequences using the CaVEMan algorithm, against an unmatched reference murine genome (Jones et al, 2018). All variant sites across all clones were genotyped for each mouse individually. Germline mutations, false positives and artefactual mutations were filtered out. Variant allele frequencies (VAF) were calculated for each mutation in order to determine the clonality of the sample, as VAFs represent the proportion of sequencing reads that report a particular mutation (Lee-Six et al, 2018). Shared mutations (those which are present in more than one sample) are highly confident as they result from mutational processes *in vivo*. These had a VAF binomial distribution mean of ~0.5, thereby indicating that the colony derived from a single cell. These mutations were therefore included in the analysis. Conversely, mutations with lower VAFs (~0.25-0.33), were considered to be private (present in only one colony), as in principle, a lower proportion of DNA molecules carry such mutations, likely resulting from *in vitro* or late *in vivo* (after embryogenesis) processes. These mutations were excluded from the analysis. A binary genotyping matrix was used to reconstruct the phylogenies of stem cells from each mouse separately, in which samples were classified as 'present' (≥ 2 variant reads supporting the mutation), 'absent' (no variant reads available for the mutation) 'unknown' (samples which did

not fall into any of the two previous categories) for all variants. The genotyping matrix was then converted into a *fasta* format file for input into the MPBoot algorithm, which uses a maximum parsimony approach to reconstruct phylogenies (Hoang et al, 2018). Phylogenies were initially generated from a raw mutation count with no branch length adjustment, and were then linearised, i.e., ultrametrically or chronologically ordered, as described previously (Chapman et al, 2021). Samples were classified by tissue of origin (termed *source* in the analysis pipeline) - femur, tibia, ilium or spine. Analysis of Molecular Variance (AMOVA) was performed on the linearised tree to statistically determine sample clustering by tissue of origin (Excoffier et al, 1992).

2.3.5 Single-cell RNA sequencing

Single-cell RNA-seq of HSCs was performed as previously described (Picelli et al, 2014). In brief, single LT-HSCs (ESLAM) cells were sorted into 96-well qPCR plates containing Superase-In RNase inhibitor (20 units/ μ L, Thermo Fisher), molecular biology-grade Triton X-100 (1%, Sigma Aldrich) and Nuclease-free ddH₂O (Sigma Aldrich). Immediately after sorting, plates were placed on ice, covered with standard optical qPCR plate film and vortexed gently but thoroughly for 15 seconds. Plates were centrifuged at 2000 rpm for 1 minute at 4°C, placed on dry ice and stored at -80°C. For reverse transcription, annealing mixture (ERCC Spike-in mixture, ThermoFisher;) were added to each individual well. Plates were incubated at 72°C for 3 min, and centrifuged for 15 seconds at 700 x g, to then add reverse transcription (RT) mix (containing Maxima H Minus reverse transcriptase, RNA inhibitor, 5X Maxima RT buffer, TSO, PEG 8000 and ddH₂O) to each well. Following reverse transcription, pre-amplification of cDNA products was performed using the Terra PCR Direct polymerase and buffer (Takara Bio) in 19 PCR cycles, which is the standard recommended for haematopoietic cells (Bagnoli et al, 2017). For purification of PCR products, Ampure XP beads (Beckman Coulter) were used. Quantification of cDNA and quality control of samples was performed before library preparation, for which the Tagment DNA buffer and the Amplicon Tagment Mix were used (Illumina). Libraries were pooled at equimolar ratios, and cleanup was done using the Ampure XP beads. Samples were sent to Novogene or Azenta Life Sciences and sequenced on the Illumina NovaSeq 6000 or NovaSeq X.

2.3.5.1 Analysis of scRNA-seq data

Data were retrieved from Novogene or Azenta; raw *fastq* files were renamed according to the tissue of origin and location of the well within the plate using a bespoke algorithm in Python. The *Snakemake* script was run for quality control of samples and read trimming (Molder et al,

2021). Trimmed ready data were aligned by the Spliced Alignment to a Reference (STAR) method (Dobin et al, 2012). Aligned data were fed into the Seurat pipeline for analysis in R, along with the dplyr and patchwork packages (Hao et al, 2023). The criteria for quality control were defined as follows: 1) mapped reads between $> 1 \times 10^5$ to $< 3 \times 10^7$, including nuclear genes, mitochondrial genes and ERCC spike-in variants; 2) minimum of 20% reads mapping to known genes; 3) percentage of mitochondrial DNA $> 5\%$, ensuring a minimum of 5% reads mapping to genes outside of the mitochondrial genome; and 4) percentage of ribosomal RNA $> 0.8\%$ for all samples. Cells which did not meet the criteria were filtered out of the dataset and excluded from further analysis. The number of libraries prepared per anatomical location was as follows: 28 from the femur, 24 from the tibia, 38 from the ilium, and 48 from the spine, equating to 138 libraries in total. All samples from the ilium and spine successfully passed the quality control, along with 25 from the femur and 23 from the tibia, resulting in a total of 133 libraries sequenced.

scRNA-seq data was normalised using the global-scale LogNormalise method in Seurat. Principal component analysis was performed on the filtered cell subset to determine the dimensionality of the data; an elbow plot was generated to select the number of components that were contributing to the heterogeneity of the data. The non-linear dimensionality reduction Uniform Manifold Approximation and Projection (UMAP) method, along with the FindNeighbors function, was applied to cells for visualisation and clustering according to the tissue of origin. The FindAllMarkers function was executed to identify differentially expressed genes or *features* across samples from the different tissues. The EnhancedVolcano function (Blighe et al, 2018) was used for visual representation of differentially expressed features, and the VlnPlot (violin plot) function of Seurat was used to represent individual gene expression in each bone.

2.4 F12-PVA based expansion system (28-day assay)

Expansion cultures were initiated with single (1 cell/well) or bulk (25 or 50 cells/well) LT-HSCs (ESLAM) sorted cells into 96-well fibronectin-coated plates, containing 200 μL of F12 media (Table 2.6). Prior to sorting, the inner 60 wells of a 96-well flat-bottom plate were coated with 10 $\mu\text{g}/\text{cm}^2$ (equivalent to 6.4 $\mu\text{g}/\text{mL}$) of fibronectin solution (Sigma Aldrich); plates were incubated overnight at 4°C with fibronectin solution, which was rinsed with 3x washes of sterile PBS, 1X. After cell sorting, plates were incubated at 37°C , $5\% \text{CO}_2$ and 90% relative humidity for 5-7 days, and from that point onwards media was replaced with freshly prepared media (with freshly added cytokines) three times per week. At the end of the culture (either 14 or 28

days), cells were processed for several distinct downstream applications (transplantations) or analysis (proteomics, transcriptomics or flow cytometry).

Table 2.6 - F12 media for HSC expansion

Reagent	Concentration	Volume (μL)	Final concentration	Supplier	Cat. No.	Lot No.
Ham's F12 Mix	—	958	1X	Gibco	11765054	N/A
Penicillin/Streptomycin/L-glutamine	100X	10	1%	Thermo Fisher	10378-016	N/A
Insulin/Transferrin/Selenium/Ethanolamine	100X	10	1X	Gibco	51500-056	N/A
Hepes buffer	1 M	10	1 mM	Gibco	15630-049	N/A
Polyvinyl-alcohol (PVA)	100 mg/mL	10	10 mg/mL	Sigma Aldrich	P8136-250G	SCLB0875
SCF	100 ug/mL	0.1	10 ng/mL	Peprotech	AF-250-03	N/A
Thrombopoietin (TPO)	100 ug/mL	1	100 ng/mL	Peprotech	AF-315-14-1MG	N/A

2.4.1 Split expansion cultures

Single-cell cultures were grown for two weeks as detailed above, with the same frequency of media changes. At day 14 of culture, cloning efficiency was assessed, i.e., the number of clones formed per sorted well. On average, cloning efficiency was of 75% (45 of 60 wells contained visibly forming single-HSC derived clones). Furthermore, purity of the cultures was also verified by visual assessment whereby clones containing large amounts of dead or differentiated cells (which appear to be larger in size) were excluded. Therefore, an average of 6 of 60 (10%) colonies would be excluded due to the presence of death or terminally differentiated cells. This then left a total of 39 wells per every 60 wells sorted which could be split and transferred into new culture plates. Thereon, clones were divided into four equal fractions (50 μL of total volume) and each fraction was transferred to a new culture plate, creating four split clones from each of the original selected clones. Cultures were grown for further 14 days and were used for downstream applications or analysis.

2.4.1.1 Wild-type versus TET2 Clonal Competition Assays

For cell competition assays, single ESLAM cells from wild-type (C57BL/6J/Ly5.1, *Pep Boy*) and TET2 (C57BL/6J) mice were sorted and cultured separately, following a similar workflow graphpadas for split culture assays. Additionally, for this particular assay, the CD45 congenic system was incorporated to be able to discriminate between wild-type clones (expressing CD45.1) and TET2 clones (expressing CD45.2) when they were set in competition. On day 14 of culture, clones were visually assessed as described above. An equivalent number of clones from each genotype were selected, split four ways and distributed across a 3 x 3 arrangement which we termed a *Competition Matrix*. Each competition matrix consisted of 9 unique competition wells, where split, individual clones from wild-type (50%) and TET2 (50%) competed against one another, alongside 3 untouched wild-type and TET2 control clones for a total of 15 wells (Figure 4.3, Chapter 4). Thus, each competition plate contained four competition matrices, along with 12 control wells of wild-type and 12 controls of TET2 clones. In every experiment, at least seven competition plates were prepared and cultured for a total of 28 days (14 days post-splitting/mixing). When the culture ended, clones were processed for downstream applications or analysis.

2.4.1.2 Phenotypic analysis of 28-day expanded clones

For phenotypic profiling, clones were analysed by flow cytometry on the last day of culture, or in some instances, on day 14 of culture. Clones were harvested by pipetting up and down gently but thoroughly several times, in order to collect a homogenous sample for analysis. According to different experimental purposes (which will be discussed in the Results section), either all of the clone contents or a fraction of the contents (20%-50%) were transferred to a polypropylene V-bottom plate, after which wells were topped up to a final volume of 200 μ L with PBS, 1X. Plates were then centrifuged at 4°C for 5 min at 300 x g. Supernatant was discarded and antibody master mix (Tables 2.7 and 2.8) was added to all wells, and were incubated for 30 min in the dark on ice. Samples were rinsed one time with PBS, 1X and centrifuged as above. Samples were resuspended in 7AAD (1:1000) prepared in PBS, 1X, and were then analysed on the Cytotflex LX375 or LX355, collecting 50-70% of sample volume, mixing and backflushing in between samples. Data were analysed on Flowjo v 10.10 (BD Biosciences), and plotted on GraphPad Prism v 10.1.2 (GraphPad Software).

Table 2.7 - ELSK marker panel for 10- and 28-day culture analysis

Antibody	Fluorochrome	Clone	Concentration	Volume per sample (μL)	Supplier	Cat. No.	Lot No.
CD45	FITC	30-F11	1:200	0.25	Biolegend	103108	B330230
CD11b	APC	M1/70	1:300	0.2	Biolegend	101212	B408151
CD117 (c-Kit)	APC-Cy7	2B8	1:200	0.25	Biolegend	105286	B405703
Sca-1	BV421	D7	1:200	0.25	Biolegend	108129	B419089
EPCR	PE	eBio1560 (1560)	1:200	0.25	Invitrogen	12-2012-82	2600245
Gr-1	PE-Cy7	RB6-8C5	1:300	0.2	BioLegend	108416	B379114

Table 2.8 - ELSK marker panel for competition culture analysis

Antibody	Fluorochrome	Clone	Concentration	Volume per sample (μL)	Supplier	Cat. No.	Lot No.
CD45.1	PE-Cy7	A20	1:200	0.25	Biolegend	110705	B352777
CD45.2	AF700	104	1:200	0.25	Biolegend	109821	B375109
CD11b	BV605	M1/70	1:300	0.2	Biolegend	101212	B408151
CD117 (c-Kit)	APC-Cy7	2B8	1:200	0.25	Biolegend	105286	B405703
Sca-1	BV421	D7	1:200	0.25	Biolegend	108129	B419089
EPCR	PE	eBio1560 (1560)	1:200	0.25	Invitrogen	12-2012-82	2600245
Gr-1	FITC	RB6-8C5	1:300	0.2	BioLegend	108405	108406

2.4.1.3 poly(A) bulk RNA sequencing of expanded clones

For transcriptomic analysis of clones in competition assays, clones identified as competitors by flow cytometry were selected. Analysis was performed on the *control* clones corresponding to each of the selected competitors. Thus, 50% of clone contents were collected for RNA extraction, which were transferred to 1.5 mL DNA Lo-Bind tubes, topped up with PBS, 1X and centrifuged for 5 min at 5000 x g. The supernatant was carefully removed and 50 μL of the Arcturus Picopure extraction buffer (Applied Biosystems) were added to each sample. Samples were incubated at 42°C for 30 min on a dry block heater, and then centrifuged for 2

min at 3000 x g, supernatant was recovered and stored at -80°C until RNA isolation was to be performed.

For RNA isolation, the purification columns from the ArcTurus kit were pre-conditioned by adding 250 µL of conditioning buffer, incubated for 5 min at room temperature, and centrifuged for 1 min at 16000 x g. To each sample, 100 µL of molecular grade Ethanol (70%) were added, and samples were transferred to the pre-conditioned columns. Tubes were centrifuged for 2 min at 100 x g to promote RNA binding to the column, immediately followed by a second centrifugation step at 16000 x g for 30 seconds to discard the flowthrough. Columns were rinsed once with the wash buffer provided with the kit. DNase treatment was performed for all samples to ensure sample quality and purity: DNase I, prepared in RNase-free buffer (Qiagen), was added to each sample, and samples were incubated for 15 min at room temperature. Samples were rinsed once with wash buffer 1, and twice with wash buffer 2 provided with the kit. The purification column was placed onto 0.5-mL tubes (which were at the same time securely placed inside 2-mL tubes to avoid breakage of the column or collection tubes due to centrifugation). Elution buffer was added to each column, pipetting 11 µL into the centre of the column without directly touching the column. Samples were incubated at room temperature for 1 min, centrifuged for 1 min at 100 x g to allow distribution of the elution buffer, and then at 16000 x g for 1 min to finally elute the RNA.

For library preparation, the quality of RNA samples was assessed on the Bioanalyzer 2100 (Agilent Technologies) by the Genomics Lab from the Technology Facility, University of York. Samples with poor quality or lower-than-threshold concentration were discarded and removed from the workflow. Libraries were prepared using the NEBnext Multiplex Oligos library prep kit and Unique Dual Index Primer Pairs for Illumina next generation sequencing (NGS) (New England Biolabs). Then, pooling of libraries was done at equimolar ratios, and were sent to Novogene or Azenta Life Sciences for paired-end 150 base sequencing on an Illumina NovaSeq 6000 or NovaSeq X.

Data were retrieved from Novogene or Azenta, and aligned to the mouse reference genome (GRCm39) by STAR method. Quality control of data was done by the CutAdapt trimming method v 5 (Martin, 2011). Gene counts were obtained through the Python Library HTSeq (High-Throughput sequencing), which counts the number of aligned reads overlapping with each gene's exons (Anders et al, 2015). Differential gene expression (DGE) analysis was performed on RStudio by the DESeq 2 method (Love et al, 2014), and principal component analysis (PCA) was performed. Estimation of log-fold change in gene expression was done by empirical bayesian data shrinkage. Volcano plots of differentially expressed genes were

produced using the `ggplot2` and `AnnotationDbi` packages (CRAN repository). Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway enrichment analysis was performed using the `clusterprofiler` package, and pathways were directly visualised on the KEGG website (<https://www.genome.jp/kegg/pathway.html>) using the `pathview` package. Representative heatmaps of differentially expressed genes from ordered DESeq2 results were produced using the `pheatmap` package; single gene expression whisker plots were produced using the `ggplot2` and `ggpubr` packages.

2.4.2 Data-Independent Acquisition Mass Spectrometry (DIA-MS)

Supernatants were collected from competition cultures on days 26 and 28 of the experiments, and transferred to polystyrene V-bottom plates each time media replacements were done. Supernatants were centrifuged for 5 min at 500 x g at 4°C, then carefully collected and transferred once more to polypropylene V-bottom plates. Plates were stored at -80°C until mass spectrometry analysis was performed. Supernatants from two timepoints were pooled and transferred to 2-mL protein Lo-Bind tubes (Eppendorf). Ice-cold acetone was added to each sample at a 4:1 ratio. Samples were thoroughly vortexed and incubated at -20°C for 1 hour, and were then centrifuged at 15000 x g at room temperature. Supernatants were discarded, and tubes were incubated at room temperature for 30 min with lids off to allow the acetone to completely evaporate. Samples were dissolved in 125 µL of Ammonium Bicarbonate (AmBic) (Sigma Aldrich), followed by 6 µL of 0.1 M of dithiothreitol (DTT) (Sigma Aldrich), and incubated at 56°C for 30 min on a dry block heater. Samples were removed from the heater and 6 µL of 0.2 M alkylating agent iodoacetamide (IAA) (Sigma Aldrich) were added, and samples were incubated for 30 min in the dark at room temperature. For protein digestion, Trypsin was added, and samples were incubated overnight at 37°C. The next day, digestion was quenched by addition of 10% trifluoroacetic acid (TFA) (Sigma Aldrich), and samples were dried in a SpeedVac Vacuum Concentrator (ThermoFisher Scientific) for approximately 4 hours. Sample loading into EvoTips was performed following the protocol by EvoSep: 1-propanol were added to a 96-well plate, and the plate was set aside. Mass spectrometry grade acetonitrile with 0.1% formic acid were added to EvoTips and centrifuged for 60 sec at 700 x g. Evotips were then soaked into the 1-propanol on the 96-well plate for 1 min (tips would have acquired a pale white colour at the end). For tip equilibration, 0.1% formic acid (prepared in mass spectrometry grade water - were added, and tips were centrifuged once again as above. EvoTips were placed onto an EvoSep tip tray, and 20 µL of each sample were loaded into each corresponding tip, and were centrifuged again as above. Tips were rinsed with 0.1% formic acid, and a final volume of 100 µL of formic acid were added to the tips for sample preservation, centrifuging at 700 x g for 10 sec. Samples were then introduced on a 15-cm

performance column with elution, using a pre-set 30 SPD standard method with a 44-min gradient to ensure correct measurement of the proteome, on an EvoSep One UPLC. Data were acquired using the DIA-MS method on a Bruker timsTOF high-throughput mass spectrometer, between 400-1200 mass-to-charge (m/z) ratio, using 25 Th DIA windows for each run (Li et al, 2021 DIA emerging proteomics technology).

2.4.2.1 DIA-MS Data analysis

Acquired data were searched in the Spectronaut software v 19 (Biognosys) against the Swiss Prot Mouse subset of UniProt. The search was conducted at 1% false discovery rate (FDR). Results were filtered by a required protein group q -value of <0.01 and a minimum of two peptides using the Konstanz Information Miner (KNIME) software. An average of 5500 protein groups were reported across all samples. Normalisation of data was performed on R Studio using the trimmed means of M -values method (Robinson & Oshlack, 2010). Z -scores were calculated and data were $\log_2$ -transformed, and representative volcano plots were made for significant differences in protein intensities using the `gplot` and `EnhancedVolcano` packages.

2.5 Transplantation assays

2.5.1 Single-cell transplantations

Single LT-HSCs (ESLAM) were isolated from adult (10-12 weeks of age) male C57BL/6J mice (CD45.2) by FACS into 96-well U-bottom plates, as described above. Sorting efficiency of single HSCs was confirmed by observing the presence of one and one cell only in each well under the microscope. Wells with one and one cell only were selected for transplantation. For syringe loading, 400 μL of sterile PBS were uptaken into a 0.5-mL insulin syringe (29-30 gauge), to then load the single cell by ejecting approximately 200 μL of PBS into the well and then aspirating all of the liquid in the well several times, ensuring that the single HSC would be correctly loaded. *c-kit* defective W41 (Ly5.1) recipients received a single dose of 360 cGy of irradiation shortly before transplantations. Mice were placed inside a heatbox at 37°C to promote vasodilation, which aids tail vein injections. A total volume of 500 μL of suspension containing the single-cell was injected into each of the mice for transplantation experiments. Mice were kept under support care for the first four weeks of the experiment to promote their recovery, welfare and wellbeing.

2.5.2 Transplantations from expanded clones

In vivo functional assays were done for split or competition experiments from *ex vivo* expanded clones. From split cultures, 50% of total clone contents were collected, and from competition cultures, 30% of the clone contents were collected and prepared. Cells were transferred to 1.5-mL lo bind tubes (Eppendorf), topped up with PBS, 1X and centrifuged for 5.5 min at 300 x g at 4°C. Supernatant was carefully removed and samples were resuspended into 300 µL of sterile PBS, 1X for injections. For split cultures, w41 recipients were sub-lethally irradiated and reconstituted immediately after with the samples. For competition assays, CD45.1 or CD45.2 BL6 recipients were lethally irradiated, and reconstituted with the expanded clone samples along with 2.5×10^5 whole bone marrow cells from hybrid CD45.1/CD45.2 donors, as a palliative method to restore haematopoiesis after lethal irradiation. Monitoring of chimaerism was done from week 4 to week 24 (with 4-week intervals), and peripheral blood samples were analysed by flow cytometry, using the lineage marker panel presented in Table 2.9. Bone marrow and spleen were collected from recipients at the end of the transplant experiment and analysed by flow cytometry to look for chimaerism levels in both these tissues using the antibody panel described in Table 2.10. Data were analysed using Flowjo v 10.1.2 (BD Biosciences).

Table 2.9 - Lineage panel marker for analysis of blood, spleen and bone marrow from transplant recipients (chapters 3 and 4)

Antibody	Fluorochrome	Clone	Concentration	Volume per sample (µL)	Supplier	Cat. No.	Lot No.
CD45.1	PE-Cy7	A20	1:250	0.2	Biolegend	110730	B435520
CD45.2	AF700	104	1:250	0.2	Biolegend	109822	B375109
CD3ε	PE	145-2C11	1:250	0.2	Biolegend	100308	B346465
CD11b	BV605	M1/70	1:250	0.2	Biolegend	101216	B370143
CD45R/B220	APC	RA3-6B2	1:250	0.2	Biolegend	103221	B357252
Ly6G	BV421	RB6-8C5	1:250	0.2	Biolegend	108416	B356918

Table 2.10 HSC/HSPC panel for analysis of spleen and bone marrow from transplant recipients (chapter 3 and 4)

Antibody	Fluorochrome	Clone	Concentration	Volume per sample (μL)	Supplier	Cat. No.	Lot No.
CD45.1	FITC	A20	1:200	0.25	Biolegend	110730	B352777
CD45.2	AF700	104	1:200	0.25	Biolegend	109822	B375109
EPCR	PE	eBio1560 (1560)	1:200	0.25	Invitrogen	12-2012-82	2600245
CD150	PE-Cy7	TC15-12F12.2	1:200	0.25	Biolegend	115914	B353358
CD48	APC	HM48-1	1:200	0.25	Biolegend	103412	B341106
CD117 (c-Kit)	APC-Cy7	2B8	1:200	0.25	Biolegend	105826	B405703
Sca-1	BV421	D7	1:200	0.25	Biolegend	108133	B369997
Linage cocktail	Streptavidin BV605	—	1:200	0.25	Biolegend	405229	B366423

2.5.3 Limiting-dilution assays (LDA)

To determine HSC frequency in femur, tibia, ilium and spine, limiting dilution assays were performed as follows: Bone marrow was harvested from each individual bone, prepared and enriched for stem and progenitor cells as described above. The total number ESLAM cells required for each of the bone/ HSC groups (femur, tibia, ilium or spine/ 5 or 20 HSCs) were sorted in bulks into StemSpan media without cytokines in 1.5 mL Lo-bind tubes. Cells were injected into sub-lethally irradiated w41 recipients, in a maximum volume of 400 μL. Chimaerism was monitored from 4 to 16 weeks post-transplant (in 4-week intervals), and peripheral blood samples were analysed as described above. At the end of the experiment, bone marrow and spleens from each recipient were processed and analysed by flow cytometry. Data were analysed using Flowjo and plotted on GraphPad Prism. Determination of HSC frequencies in each of the bones was performed using the web-supported tool at the Extreme Limited Dilution Assays (ELDA) website (<https://bioinf.wehi.edu.au/software/elda/>, Walter and Eliza Hall Institute) (Hu & Smyth, 2009). Data were tested for inequality in

frequencies across all groups, and pairwise tests were performed for all groups, including data from single-cell transplantation experiments.

2.6 Disease monitoring in MPN mouse models

Cohorts of Wild-type, JAK2, TET2 and JAK/TET/IP10 mutant mice were bled routinely every 4 weeks starting at 8 weeks of age through to 16 weeks (and on some occasions, up to 24 weeks of age) to monitor disease evolution. Mice were placed inside a heatbox for 10 min to stimulate vasodilation before conducting the procedures. Using 25-gauge butterfly needles (BBraun), 20-50 μ L of peripheral blood were collected from the tail vein into EDTA-coated tubes (Greiner). Blood was analysed on the VetABC Blood Counter (Scil Vet) and blood count values were plotted using GraphPad Prism. In some instances, particularly for mice carrying a JAK2^{V617F/V617F} mutation, blood samples were diluted 1:1 in deionised water to obtain detectable haematocrit values. Total white blood cell count (WBC) proportion values were used to normalise high haematocrits.

2.6.1 Flow cytometry of stem/progenitor and mature cell populations

Bone marrow, spleen and blood samples (for serum isolation) were collected from mice at the endpoint of the disease monitoring study cohorts.

2.6.1.1 Spleen processing

Spleens were extracted from mice after death confirmation. Spleens were weighed and collected into 1 mL of PBS/2% FBS and kept on ice. To create a spleen single-cell suspension, a 70- μ M canister filter was placed onto a 50-mL Falcon tube placed on ice. Spleens were mashed through the filter into the 3 mL of PBS/2% FBS using the plunger of a 2-mL syringe. 7 mL of PBS/2% FBS was added to rinse the filter and plunger, to achieve a final suspension volume of 10 mL. 70% of this cell suspension was reserved for staining with the HSC/HSPC panel (Table 2.11) and 30% for the Lineages panel (Table 2.12).

2.6.1.2 Bone Marrow

Femurs, tibias and ilium bones were collected into 1.5 mL of PBS/2% FBS in 2-mL Eppendorf tubes. Bone marrow was flushed with 3 mL of PBS/2% FBS into 50-mL Falcon tubes placed on ice. As with spleen samples, 70% of sample volume was reserved for HSC/HSPC panel staining and 30% for Lineage panel staining.

Table 2.11 - Lineage panel marker for analysis of spleen and bone marrow at end of disease monitoring (chapter 5)

Antibody	Fluorochrome	Clone	Concentration	Volume per sample (μL)	Supplier	Cat. No.	Lot No.
CD71	FITC	R17217	1:250	0.2	Biolegend	113806	B365140
TER119	PE-Cy7	TER-119	1:250	0.2	Biolegend	116221	B410265
CD3ε	PE	145-2C11	1:250	0.2	Biolegend	100308	B346465
CD11b	BV605	M1/70	1:250	0.2	Biolegend	101216	B370143
CD45R/B220	APC	RA3-6B2	1:250	0.2	Biolegend	103221	B357252
Ly6G	BV421	RB6-8C5	1:250	0.2	Biolegend	108416	B356918

Table 2.12 - HSC/HSPC panel for analysis of spleen and bone marrow at end of disease monitoring (chapter 5)

Antibody	Fluorochrome	Clone	Concentration	Volume per sample (μL)	Supplier	Cat. No.	Lot No.
CD45	FITC	30-F11	1:200	0.25	Biolegend	103108	B330230
EPCR	PE	eBio1560 (1560)	1:200	0.25	Invitrogen	12-2012-82	2600245
CD150	PE-Cy7	TC15-12F12.2	1:200	0.25	Biolegend	115914	B353358
CD48	APC	HM48-1	1:200	0.25	Biolegend	103412	B341106
CD117 (c-Kit)	APC-Cy7	2B8	1:200	0.25	Biolegend	105826	B405703
Sca-1	BV421	D7	1:200	0.25	Biolegend	108133	B369997
Linage cocktail	Streptavidin BV605	—	1:200	0.25	Biolegend	405229	B366423

2.6.1.3 Red blood cell lysis of bone marrow and spleen

For depletion of red blood cells of samples to be stained with the HSC/HSPC panel only, 3 mL of PBS/2% FBS were added without disturbing the cell pellet, followed by 5 mL of ice-cold ammonium chloride (0.5% final concentration). Samples were incubated on ice for 5 min,

vortexed briefly but thoroughly, and incubated for further 5 min. Samples were centrifuged at 300 x g for 5 min at room temperature and supernatant was discarded.

2.6.1.4 Antibody staining of bone marrow and spleen

For antibody staining, 50 µL of each respective master mix (Table 2.11 and 2.12) was added to each of the bone marrow and spleen samples according to the sample groups - HSC/HSPC or Lineages panel staining. Samples were stained in 96-well round-bottom plates for staining, and were incubated for 30 min on ice in the dark. To each well, 100 µL of PBS/2% FBS were added, and plates were centrifuged for 5 min at 300 x g at room temperature. Plates were flicked onto the sink to remove the supernatant. HSC/HSPC panel-stained samples had a secondary staining step, and so were further incubated and centrifuged as described above. When all staining rounds were completed, each sample was resuspended into final volumes of 400 µL (for bone marrow) and 600 µL (for spleen) of 7AAD (1:1000). Data were acquired on the BD LSR Fortessa X20 flow cytometer; data analysis was performed on Flowjo v.11 (BD); plotting was done using GraphPad Prism.

2.6.2 Blood and serum isolation

Mice were euthanised by CO₂ induction and cervical dislocation as secondary method of culling. Blood was collected by heart snip with a disposable Pasteur pipette and transferred to 1.5 mL Eppendorf tubes, which were placed on ice. Sample tubes were then incubated for 30 min at room temperature to allow samples to clot, and were next centrifuged for 10 min at 2000 x g at 4°C. The upper layer of the centrifugation product contained the serum, which was carefully aspirated with a 200-µL pipette and immediately stored at -20°C. The clotted blood fraction was discarded.

2.6.2.1 Serum cytokine profiling by Multiplexed assays

The Merck-Millipore 32X and 10X mouse serum cytokine kits were used to analyse the presence of inflammatory molecules in the mouse models that had been serially bled as part of the disease monitoring cohort studies. Serum samples were thawed and brought up to room temperature; then centrifuged at 200 x g for 3 minutes to pellet all cellular debris and leave a clear serum supernatant at the top. A 1:2 dilution of each sample was prepared in the kit's Assay Buffer in 0.5 mL Eppendorf tubes, to a final volume of 60 µL. Mouse protein quality controls were prepared by reconstituting each quality control vial in 250 µL of deionised water, followed by brief vortexing, and incubating the vials at room temperature for 10 min. Was buffer

for the immunoassay was prepared by diluting 60 mL of the 10X stock in 540 mL of deionised water; the serum matrix buffer was prepared in 2 mL of the assay buffer included with the kit, and incubated for 10 minutes at room temperature for full reconstitution.

The concentrated Mouse Cytokine standard was reconstituted in 200 μ L of assay buffer; subsequent serial dilutions were prepared from the concentrated standard, each containing a total volume of 250 μ L (six standards in total). The pre-coated immunoassay plate was washed with shaking before the addition of standards, quality controls and samples (25 μ L of each reagent or sample), which were allowed to reach room temperature prior to loading them the plate. Pre-mix Magnetic beads were added to each well of the plate, followed by an incubation step of 5 min, letting the plate rest on a 96-well strong plate magnet (Merck). Plate was flicked into a waste receptacle and excess liquid was removed by carefully tapping the plate on a tissue paper bed. The serum cytokine marker ready-made mix was pipetted into each well of the plate to incubate samples with the antibody cocktail overnight in the dark at 4C with shaking (500 rpm). The next day, Streptavidin-coated beads were added to each well and incubated for 1 hr at room temperature in the dark with shaking (500 rpm). Wells were rinsed with wash buffer twice before resuspending each of the samples into 150 μ L of Luminex Sheath Fluid (Thermo Fisher Scientific).

Samples were immediately analysed afterwards: events were acquired on the Luminex 200 (ThermoFisher Scientific) instrument with xPONENT version 3.1 software (ThermoFisher Scientific) (verification and calibration of the instrument were performed prior to the run according to the manufacturer's recommendations and instructions). A total of 50 events per beads were acquired in 100 μ L of sample; reporter gain was set to default (for a low photomultiplier, PMT), with time of acquisition limited to 60 seconds per sample. The customized gain settings were set for a 32- or 10-plex experiment according to the manufacturer's instructions.

After acquisition was complete, data were retrieved from the instrument in Excel spreadsheet format. Data analysis and dimensionality reduction were performed on R Studio v 2024-12-01 (Posit), using the ggplot2, corrr, ggcorrplot, FactoMineR and ggfortify packages (CRAN Repository). Plots for individual cytokine expression grouped by genotype were produced on GraphPad Prism v10.1.2 (GraphPad Software).

Chapter 3 - Transcriptional, Functional and Phylogenetic Characterisation of Mouse Haematopoietic Stem Cells

Introduction to the chapter

Whether and how the anatomical location of HSCs influences their function and output is a question that remains largely unexplored in the field, with only a handful of recent published works exploring this question for the first time (Wu et al, 2024; Koh et al, 2024; Niizuma, 2025). These studies have focused on distinct aspects concerning HSC biology; e.g., response to specific types of stress, or long-term reconstitution or functional decline of HSC populations with age, although none have undertaken a thorough study on the features that might distinguish HSCs that reside in one anatomical location from another. To date, there are no reports concerning the molecular and functional properties of HSCs localised to specific niches. In addition, most of the phylogenetic studies of the blood forming system have been performed on the CD34⁺ fraction of the human BM (Lee-Six et al, 2018; Mitchell et al, 2022; Spencer-Chapman et al, 2021), however, due to the lack of reliable immunophenotypic markers for human HSCs, this fraction is likely to contain many more committed progenitors compared to primitive HSCs. In this chapter, I present an extensive and comprehensive characterisation of highly purified mouse ESLAM HSCs at the single-cell level to explore potential differences in gene expression (single-cell RNAseq) and function (single-cell transplantations), while also reconstructing the life histories of HSCs (phylogenetics) isolated from four anatomical sites: femur, tibia, ilium (hip bone) and spine. These four bones have historically been the choice anatomical sites for researchers to conduct the majority of experiments in the HSC field, given their accessibility, streamlined tissue processing protocols and BM cellularity (Kent et al, 2007; Kent et al, 2016; LoCelso & Scadden, 2007; Challen et al, 2009; Ema et al, 2006). Historically, our research group has relied on the use of these four bones, to conduct experimental work that has led to the publication of several projects over the years (Oedekoven, 2021; Shepherd et al, 2018; Che et al, 2022; Talarmain et al, 2022; Belmonte et al, 2024).

For the work described in this present chapter, highly purified HSCs were isolated from femur, tibia, ilium and spine from two male C57BL/6J (wild-type) mice, which were processed individually on two separate dates (Figure 3.1). This was done to improve experimental efficiency due to the particularly large size of the experiment, involving 312 single-cell derived colonies, from which genomic DNA was extracted on an individual sample basis; in addition to 291 single-cell transplant recipients, on which functional assessments were performed in

order to validate findings from the phylogenetic and transcriptomic analyses. Hence, this is the largest, broadest clonal profiling of murine niche-localised HSCs to date, in order to answer three main scientific questions:

- 1) How related are HSCs and their progeny HSC from different anatomical regions?
- 2) How do molecular profiles of niche-localised HSCs compare and does this inform about their functional performance?
- 3) What are the functional differences between HSCs derived from the four selected bones?

The chapter begins with a brief immunophenotypic profiling of femur, tibia, ilium and spine BM, together with index-sorting data, in order to create a framework for the following experimental stages. This is followed by a highly detailed study of HSC inter-relatedness using acquired somatic mutations as an endogenous barcode to determine whether HSCs that reside in different anatomical locations have distinct origins developmentally or are preferentially retained in specific stem cell niches. Next, it continues with molecular profiling of single HSCs to assess their similarity and differences according to bone origin, and then proceeds to test functional differences on serial transplantation assays, with a large set of single-cell transplants, ending with limiting dilution assays (LDA) for functional data validation. In addition, a comparison of the molecular profiles between paired groups of femur/tibia and spine/ilium HSCs to further understand the fundamental functional differences observed throughout the transplantation assays.

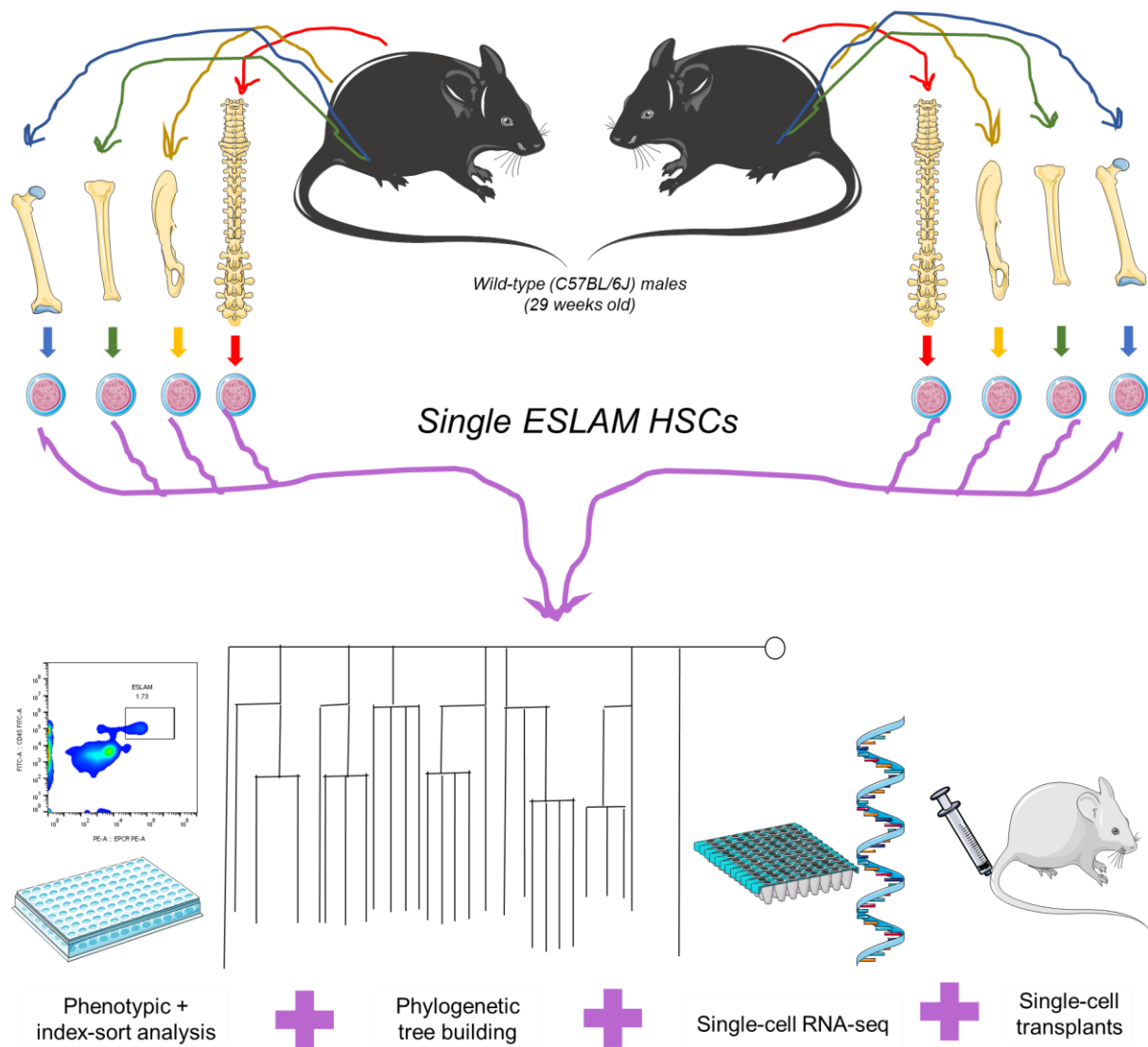
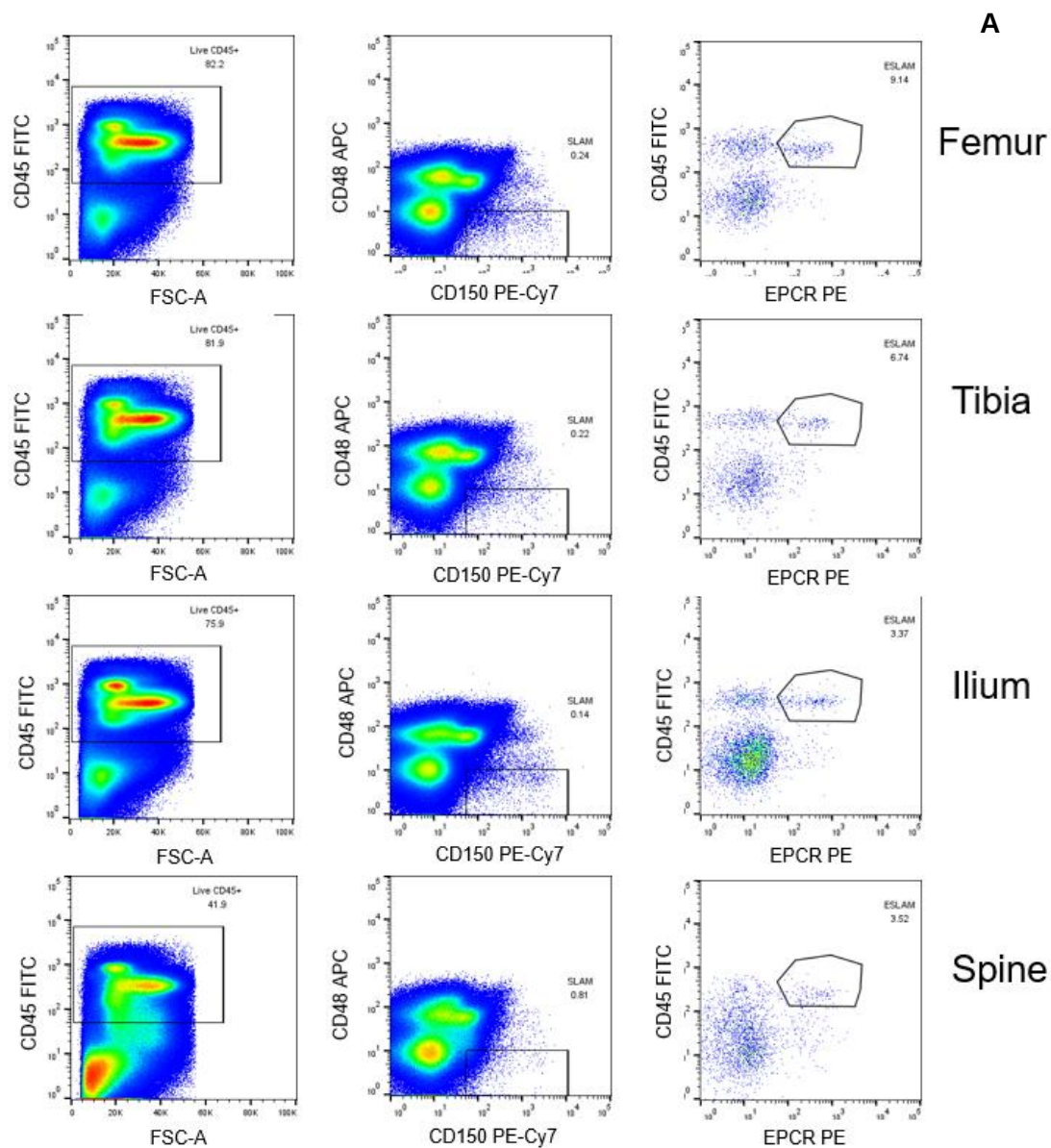


Figure 3.1 Experimental design for the characterisation of niche-localised single HSCs. Two male C57BL/6J wild-type mice (Ly5.2) were aged until 29 weeks (6.7 months) and bone marrow was harvested from each mouse on separate dates due to the complexity and size of the experiment. Given that mutations accumulate linearly with age, both in mice and humans, mice were allowed to age until 29 weeks in order to obtain the highest possible number of somatic substitutions acquired in the genome, thereby enabling the reconstruction of phylogenetic trees from WGS data. Single HSCs were purified from four different geographical locations: femur (blue arrow), tibia (green arrow), ilium (yellow arrow), and spine (red arrow). Index-sort data was collected from single-cell sorts to compare HSC marker expression across bones. Single-HSC derived clones were profiled by WGS to reconstruct a phylogenetic tree from each mouse. Molecular profiles were analysed by scRNA-seq and functional performance was assessed by single-cell transplantations.

3.1 Immunophenotypic analysis of bone marrow did not reveal changes in ESLAM frequencies across bones

Prior to the forthcoming experimental work, I decided to investigate the content of ESLAM cells in the BM of the femur, tibia, ilium and spine through flow cytometry analysis. By following a conventional gating strategy for immunophenotypic profiling, the proportion of live CD45⁺ cells was reduced by almost 50% in the spine than for the rest of the bones (Fig 3.2 A). However, when following a conventional sort gating strategy, ESLAM frequencies were comparable across the four tissues. Still, it was noted that CD45 expression was slightly lower in the ESLAM gate from the spine, so I moved to investigate whether this would also be reflected at the single-cell level.



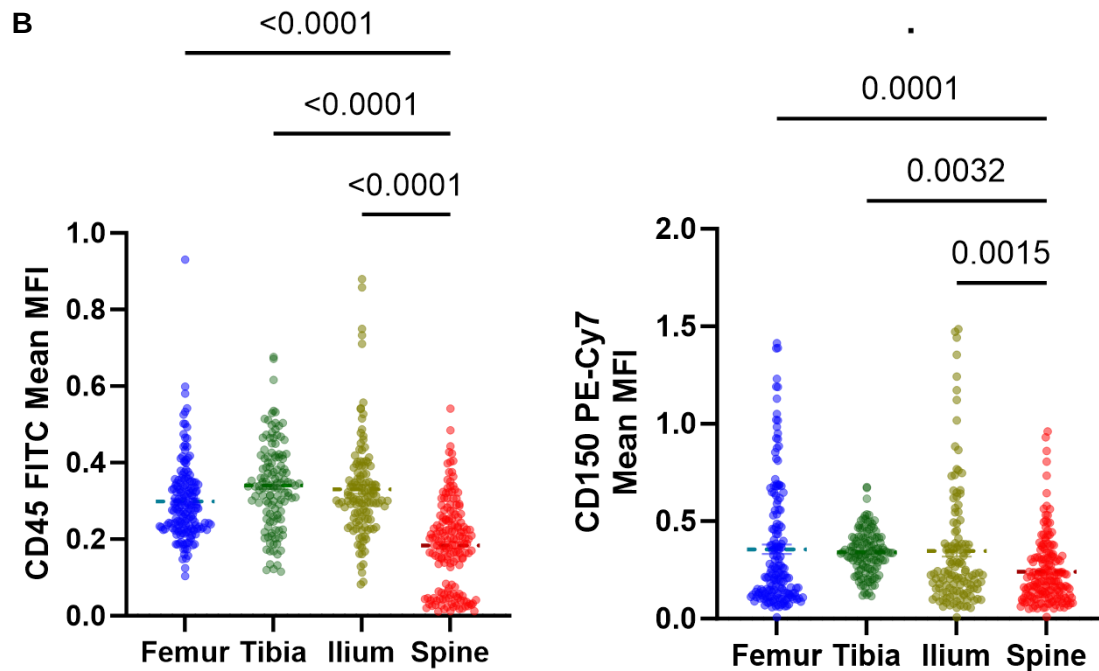


Figure 3.2 Analysis of ESLAM frequencies and cell surface markers in femur, tibia, ilium and spine bone marrow. A) Phenotypic profiling of bone marrow from different geographical locations. Live CD45⁺ frequency was nearly two-fold lower in the spine bone marrow, than for the rest of the bones, which resulted in lower ESLAM frequency calculated from CD45⁺ cells. All three other bones showed comparable frequencies of Live CD45⁺ and ESLAM cells. Bone marrow from three mice was analysed, one representative example is shown. B) Index-sort data analysis of HSC surface markers in single cells. The expression of CD45 and CD150 (SLAM) was significantly elevated in femur, tibia and ilium single HSCs in relation to spine HSCs (femur, n= 168 HSCs; tibia, n = 128 HSCs; ilium, n = 126 HSCs; spine, n=168 HSCs). Ordinary one-way ANOVA was applied to the data to calculate statistical significance between HSC groups.

3.1.1 CD45 and CD150 expression was significantly reduced in individual ESLAM HSCs in the spine in comparison to other anatomical regions

To gain a more precise insight into the expression of the ESLAM immunophenotypic markers in individual cells from each bone, I analysed the index-sort data (Chapter 2, section 2.2.2.1) obtained from the two main experimental dates (Figure 3.2B). While the expression of CD48, Sca-1 and EPCR did not vary significantly across bones, CD45 and CD150 expression was significantly higher in the femur, tibia and ilium compared to the spine, thereby confirming the findings from the immunophenotypic analysis (Figure 3.2A). The most prominent differences were observed in the expression of CD45, which explained the reduction of ESLAM proportion in the spine observed in the initial immunophenotypic analysis (calculated from viable CD45⁺).

These findings provided the initial piece of evidence that, immunophenotypically, BM cells from these four anatomical regions were not identical. At the same time, it indicated that CD45 and CD150 expression was higher in individual HSCs from femur, tibia and ilium than in cells from the spine. What these potential differences relied upon, and how closely related HSCs were according to niche location were questions to be addressed in the next experimental phase.

3.2 Construction of phylogenetic tree of murine HSCs from naturally occurring mutations

To reconstruct the life histories of mouse HSCs, and to determine the origins of clones in context of their geographical location, I followed a systematic approach, in which two male mice were selected from two cohorts of littermate C57BL/6J (Ly5.2/Ly5.2) mice. Mice were male for greater mutation detection sensitivity. Every 6 weeks - starting at 9 weeks of age -, cells of the granulo-myeloid fraction (GM, Ly6G⁺CD11b⁺), CD11b⁺ monocytes, B (B220⁺CD19⁺) T (CD3ε⁺) cells from peripheral blood collected from these mice were profiled by FACS. At the end of the pre-sampling period two mice were selected for whole genome sequencing analysis; selection was done based on the numbers of cells from each population isolated across all timepoints - mice which showed the most consistent, as well as higher numbers of cells, were selected for the experiment.

Both mice were allowed to age up to 29 weeks (6.7 months, roughly equivalent to a human aged 30-40) (Flurkey et al, 2007), since this would yield a higher number of mutations acquired per cell, thus increasing resolution of genomic data. BM was harvested from one femur, one tibia, one ilium and from the spine (which was shared across the other experimental downstream applications which will later be described in this chapter). Single ESLAM cells were isolated by FACS and index-sorted, and subsequently expanded in the liquid StemSpan culture system with SCF (300 ng/mL) and IL-11 (20 ng/uL) added as growth factors and FBS to promote colony growth. Single-cell derived colonies from all plates were incubated and left undisturbed. Seven days post-sort, plates were examined under the microscope to verify cloning efficiency, i.e., that HSCs were dividing and forming colonies in culture. The femur, tibia and ilium plates were cultured for a total of 14 days. In the plate containing single HSCs isolated from the spine, cell sorting efficiency was lower due to technical difficulties with the sorting instrument, for which the cells required a slightly extended period of culture (+3 days), in order to capture a sufficient number of clones to proceed with the WGS approach.

The total number of colonies individually harvested and processed for library preparation was 312: 82 HSC-derived colonies from the femur, 83 from the tibia, 94 from the ilium and 53 from the spine. After DNA quantification and quality control, the number of libraries which were successfully prepared and submitted for whole genome sequencing was 290: 81 colonies from the femur, 83 from the tibia, 92 from the ilium and 37 from the spine (Table 3.1). WGS data obtained for all of these colonies were put through the computational analysis pipeline for mutation calling and phylogenetic tree building.

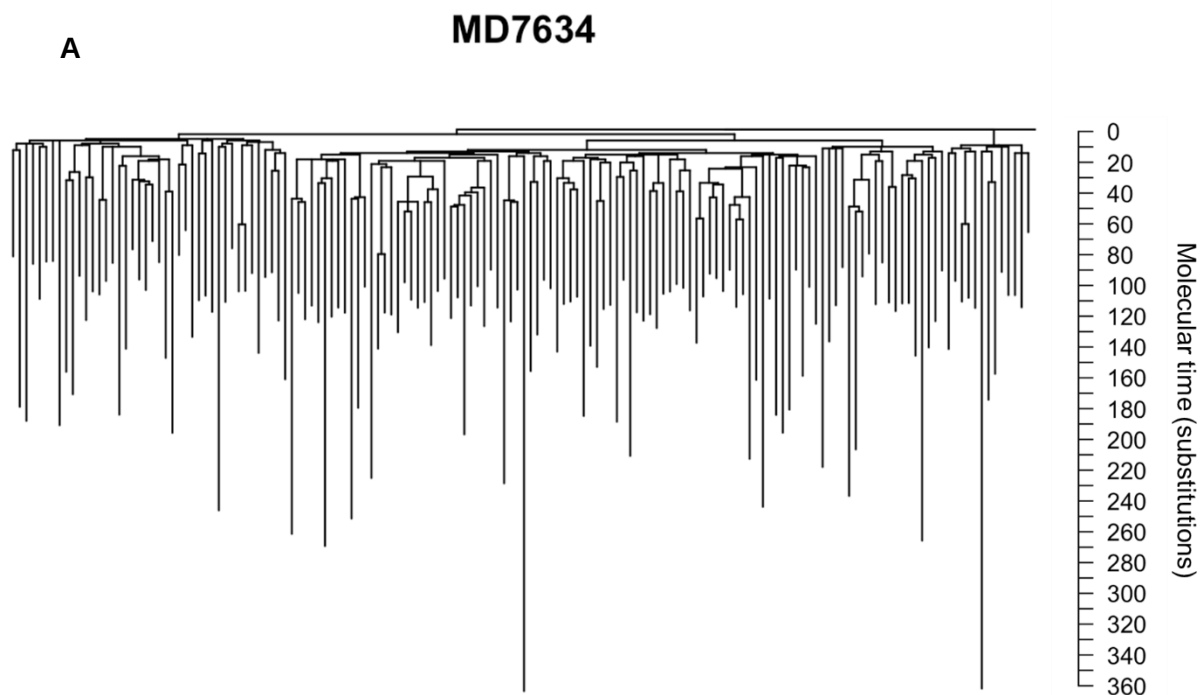
Table 3.1. Summary of single-cell derived colony samples profiled by WGS.

No.	Date	York mouse ID	Sanger mouse ID	TISSUE_PHENOTYPE	n
1	05 June 2023	S341/22_2	MD7634	Femur	40
2	05 June 2023	S341/22_2	MD7634	ilium	42
3	05 June 2023	S341/22_2	MD7634	Spine	35
4	05 June 2023	S341/22_2	MD7634	Tibia	37
5	12 June 2023	S348/22_2	MD7635	Femur	40
6	12 June 2023	S348/22_2	MD7635	ilium	48
7	12 June 2023	S348/22_2	MD7635	Spine	2
8	12 June 2023	S348/22_2	MD7635	Tibia	46

3.2.1 Somatic mutation calling identified over 24,000 mutations between the two experimental animals

In the mutation discovery phase of phylogenetic analysis, 12,573 somatic mutations were identified in the first mouse, and 11,504 in the second mouse, equating to 24,077 mutations called between the two mice (Figure 3.3A). Variant allele frequency (VAF) analysis showed that the majority of these mutations occurred in more than one colony (VAF ~ 0.5), and were therefore shared and highly likely to have been acquired in a single cell *in vivo* (Figure 3.3B). Since VAF frequency of autosomal mutations follows the principle of binomial distribution, in which each mutation is present in only one of the autosomes, the VAF mean of shared or clonal mutations will be 0.5 (Lee-Six et al, 2018). VAFs lower than 0.5 are likely to be artifactual, as a result of mutational processes *in vitro*, and therefore present in one colony only, which is why they are termed *private* or *sub-clonal* mutations.

Further analysis revealed low VAF densities for 3893 mutations from the first mouse and 4755 from the second mouse were lower than 0.5 (VAF = 0.26 and 0.28, respectively), implying that these mutations might be artifactual. Shared mutations, as a result of natural mutation acquisition with age (Alexandrov et al, 2020), produce a signature similar to the SBS1 mutational profile, a clock-like mutational signature characterised by the prevalence of C>T substitutions at CpG nucleotides, and a significant correlation of number of acquired mutations with age, which is what was observed on both experimental animals (Welch et al, 2013; Alexandrov et al, 2015). However, due to the low VAF distribution means of a significant portion of the called mutations, we applied a binomial mixture model to the data to determine how many of the private mutations were in fact artifactual (occurred in only one colony). Our estimates showed that 1545 and 1940 of the private mutations in the first and second mouse, respectively, exhibited a mutational pattern in accordance with the SBS1 signature. Hence, the binomial mixture model allowed to categorize approximately 40% of the private mutations as likely clonal, which were confidently included in the following analysis stages, whereas sub-clonal mutations were discarded as they are uninformative for the construction of phylogenetic relationships between cells. After filtering, the estimated number of somatic substitutions (SBS) acquired per cell at the end of the mouse lifespan (6.7 months) was of approximately 65.22 SBS, in agreement with previous reports (Cagan et al, 2022; Kapadia et al, 2025).



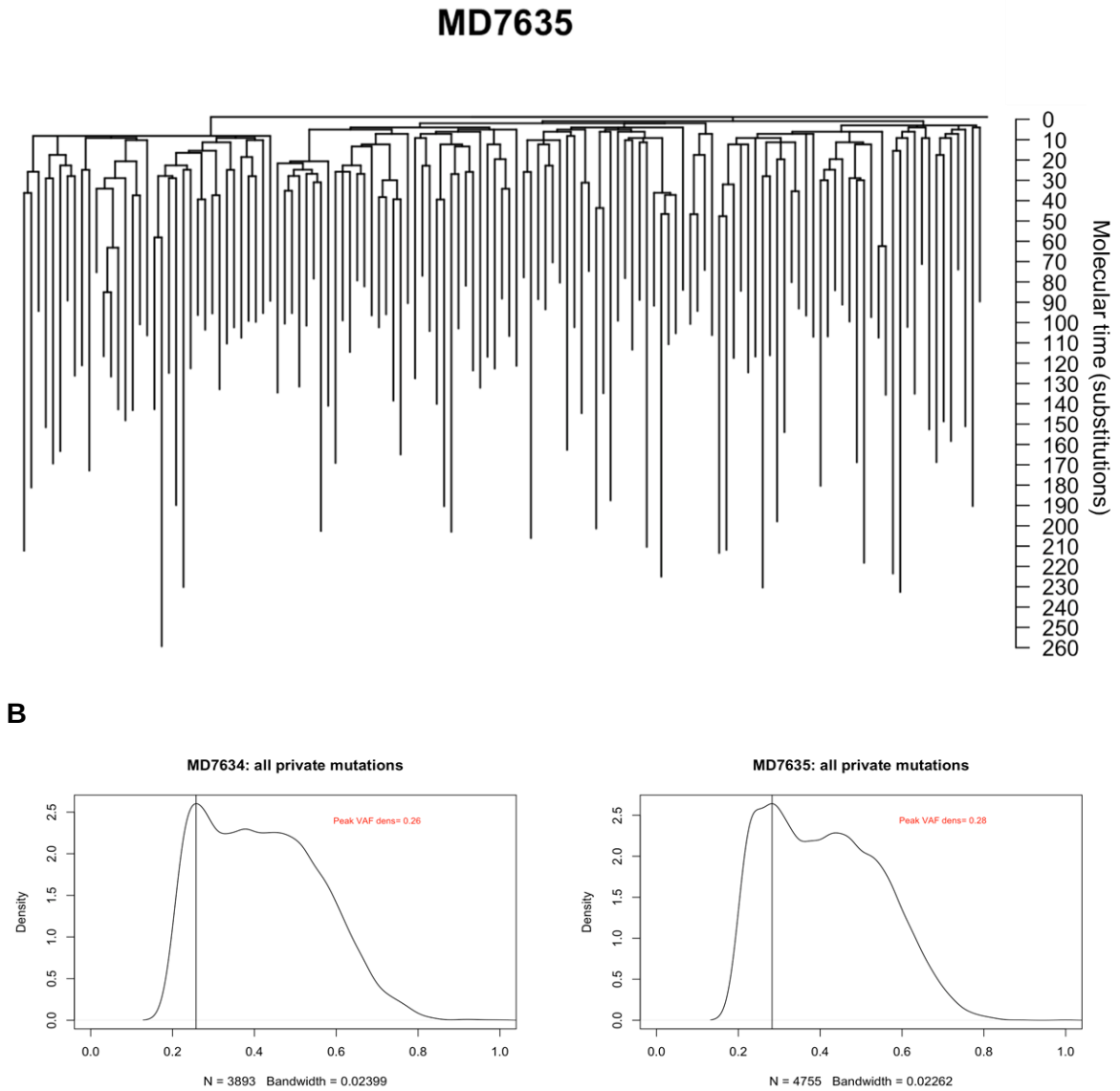


Figure 3.3. Phylogenetic analysis of single-HSC derived clones. A) Phylogenetic trees corresponding to the first (top) and second (bottom) experimental animal, which were aged until 29 weeks (6.7 months). Molecular time is calculated by the number of substitutions acquired per cell. As mutational burden increases with age, somatic mutations can be used as endogenous barcodes to infer the clonal relatedness of HSCs. B) VAF profiles of private (not shared) mutations detected in the first (left) and second (right) experimental animal. VAF cutoff was set at >0.5 since this confidently identifies mutations which were acquired *in vivo* and therefore present in more than one colony. Therefore, mutations with less than 0.5 VAF were removed from the analysis, as they probably resulted from mutational processes occurred *in vitro*.

3.2.2 Ultrametric ordering of phylogenies showed that HSCs cluster by tissue of origin, and reside with the same tissue throughout life

Once variant calling and filtering were completed, the phylogenies of single-cell derived HSC clones were reconstructed from the clonal mutations (found in more than one of the profiled

colonies) identified in each mouse. The trees were ordered in a chronological manner, i.e., all tip nodes were set at the same distance from the ancestral node (Page & Holmes, 1991), so that branch lengths could be extended to the maximum number of acquired mutations, which were expressed as mutations (substitutions) of molecular time, thus creating 'ultrametric' trees (Figure 3.4). This ordering showed that mutation burden was fairly uniform for both mice, which was expected since both mice were of the same strain and age at the moment of tissue harvest; in addition, genomic DNA from all samples from each mouse was extracted and processed at the same time, in order to maintain consistency across experiments. Nevertheless, the number of substitutions found in the tree of the first mouse was higher (360 substitutions) (Figure 3.4A) than in the second mouse (260 substitutions) (Figure 3.4B). This could be attributed to a larger number of colonies from the first mouse which were profiled, in comparison to the second mouse (fewer colonies due to technical issues with sorting efficiency, as explained above). Despite this, the observed average mutation burden was of approximately 130 substitutions in molecular time, i.e., the mutation acquisition rate across the individual's lifetime until the moment of sampling, after which there were no more bifurcations or *coalescences* (branching points) observed, and consequently, new *lines-of-descent* were not formed any more. Therefore, branch lengths were adjusted to a maximum of 130 mutations of molecular time across both trees, for a more precise interpretation of the phylogenetic data.

Since our main interest lay in examining relationships between HSCs from different anatomical locations, sample clustering by tissue of origin analysis was applied to the ultrametric trees with adjusted branch lengths. Remarkably, from early in life - even before 10 substitutions in molecular time - HSCs descended from common ancestors, which then coalesced and formed clades which showed preference for being restricted to single BM locations, i.e., separate clusters were formed for femur, for tibia, for ilium and for spine. HSCs and their progeny HSCs within these clades continued to reside within the same anatomical region throughout the mouse's lifetime. Since our analysis was restricted to HSC progeny, it is likely that the non-HSC progeny could be randomly distributed across the tree, i.e., committed progenitors and cells from the different blood lineages might not preferentially cluster by bone of origin, although future experiments should address this query.

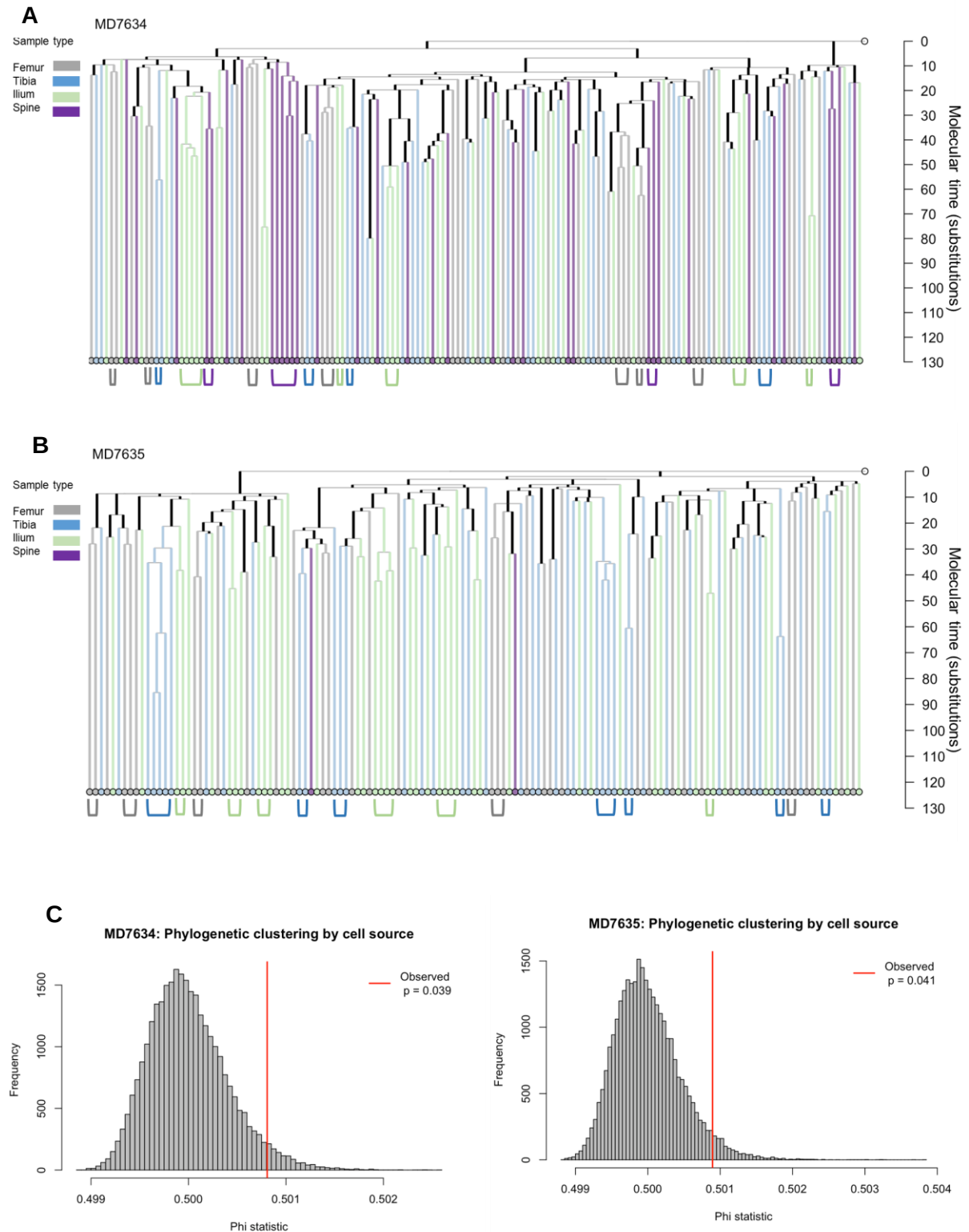


Figure 3.4 Clustering of HSCs by tissue of source. Ultrametric trees corresponding to the first (A) and second (B) mouse, showing clustering by bone marrow location of origin. Individual bone clusters are indicated by brackets at the bottom of each tree, using different colours to distinguish clusters from different bones. In each cluster, coalescences gave rise to lines-of-descent which did not bifurcate any further, and remained within their tissue of origin, forming tissue-specific HSC clades. C) AMOVA analysis of tissue clustering by source in the first (left) and second (right) phylogenetic tree. Phi statistic denotes association between HSC location of origin and position on the phylogenetic tree.

3.2.2.1 Embedding of HSCs in tissue of origin identified by phylogenetic analysis was consistent across both experimental animals and bones

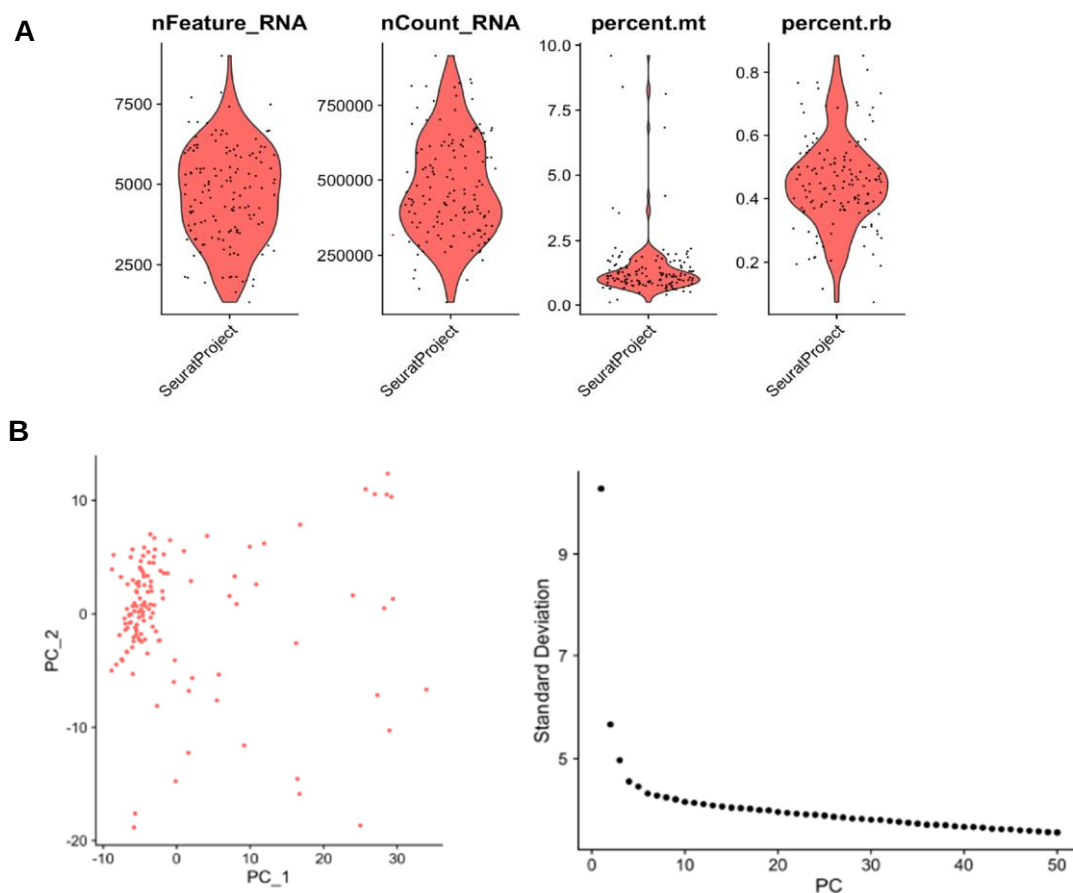
Furthermore, HSC clustering was not more prevalent in one tissue than in the others - HSCs from all four bones formed clusters regardless of tissue source, and these were consistently observed across the entirety of both trees. In order to confirm the former and to discard tissue-specific clades as a result of chance, Analysis of Molecular Variance (AMOVA) was applied to the data from the ultrametric tree to statistically calculate the probability of clustering by tissue source (Figure 3.4C). AMOVA results for both trees ($p = 0.039$ and $p = 0.041$ for the first and second tree, respectively) confirmed that HSC clustering was indeed determined by anatomical location and not as a result of random chance. The formation of tissue-specific clades of HSC progeny suggested that HSC migration between bones is highly uncommon following their initial seeding in a particular bone, despite sharing a common ancestor in embryogenesis, as none of the lines-of-descent were observed to change location within the tree later in life.

3.3 Molecular profiling of single HSCs from individual bones

The next phase of the experiment consisted on exploring the potential transcriptomic differences in HSCs from different bones, which would also shed light on some of the functional aspects of HSC self-renewal capacity, which will be discussed later. Thus, for molecular profiling, single ESLAM cells from each of the four BM locations (femur, tibia, ilium and spine) were index-sorted into 96-well PCR plates and profiled using single cell RNA-sequencing via the SMARTseqII protocol (Picelli et al, 2014). We profiled 25 HSCs from the femur, 23 from the tibia, 37 from the ilium and 48 from the spine. Thus, a total 141 libraries were prepared, of which all passed the quality control stage and were sequenced using the NGS Illumina technology (see Methods).

Initially, a first-pass quality control analysis was performed on all the samples to discard potential artefacts in the data. After applying quality control cut-offs as described in the methods sections, 133 of 141 samples (94%) met the criteria: 48 samples from the spine, 23 from the tibia, 25 from the femur and 37 from the ilium (Figure 3.5A). Individual HSC isolations were performed on two separate dates and a batch-correction analysis was conducted to determine whether samples collected on different dates were distinct from one another. This analysis confirmed that no batch effect was present and library preparation and sequencing of samples was done at the same time for both batches.

Next, a principal component analysis (PCA) was performed on the data, in order to select the principal components which contributed the most to data variability, and would be subsequently selected for the differential expression analysis stage (Figure 3.5B). As a result, the first five principal components were selected for differential expression analysis. Likewise, as part of the first pass analysis, cells were profiled for cell cycle phases (Figure 3.5C). Results showed no clustering of samples according to cell cycle phase. However, the analysis did confirm that most of the cells were in phase G_0/G_1 of the cycle (resting, quiescent phase), although this was not affected by bone of origin. Notably, from the PCA plot it was inferred that principal component 1 (PC1) was driving a group of cells to the right handside region of the plot, which could be attributed to differences in cell cycle scorings which would need further exploration, and more importantly, potentially distinct molecular profiles exhibited by HSCs from the four different anatomical locations.



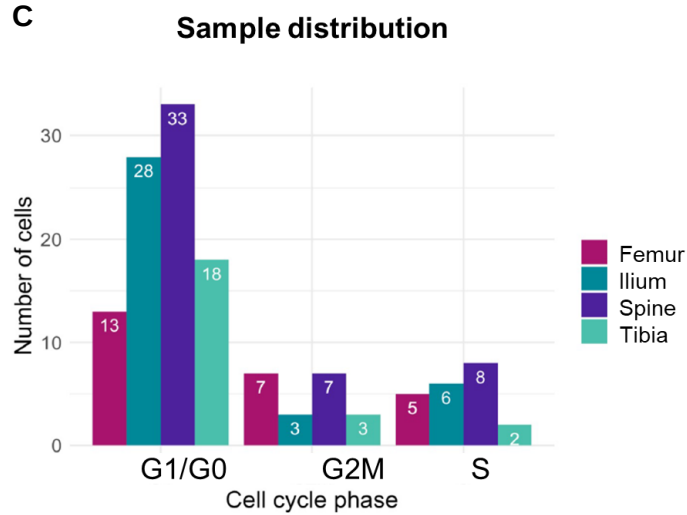


Figure 3.5. Quality control and first-pass analysis of single-cell RNA sequencing data using the Seurat pipeline. A) Samples were examined for mitochondrial contamination, as well as a minimum number of transcript reads to select samples above the set thresholds (see Methods). B) Principal component analysis plot produced with DimPlot from the Seurat pipeline (left). Standard deviation analysis showing that the majority of data heterogeneity was captured within the first five components, which were then selected for further analysis. C) Cell cycle analysis of HSCs from different bones. Plot displays the number of cells from each anatomical site distributed across the phases of the cell cycle. Most of the cells were found in the G1/G0 phase of the cell cycle, while the remaining cells were uniformly distributed across the G2M and S phases. However, the presence of a visible cluster being driven by PC1 on the plot shown in (B) suggested that cell cycle phase could also contribute to differences in the data.

3.3.1 Non-linear dimensional reduction analysis identified two visible clusters comprised of HSCs from all four bone marrow locations

Following from the data quality assessment and selection of principal components for differential gene expression analysis, we proceeded to perform uniform manifold approximation and projection (UMAP) analysis on HSCs from all four bones. Interestingly, two visibly distinct clusters were observed on the UMAP plots: one larger cluster on the upper left region of the plot, and one smaller cluster in the lower right corner of the UMAP (Figure 3.6A). Both these clusters were present on both experimental dates, and each of them were comprised of HSCs from all four anatomical locations, therefore indicating that there was a consistency in the data obtained from two independent experimental days (Figure 3.6B).

Furthermore, cell cycle analysis revealed that the larger cluster was enriched for cells found in the G1 phase, while it was notable that cells in the smaller cluster were mostly found in the G2M and S phases of the cell cycle (Figure 3.6C). Together, these results were suggestive that these two HSC clusters were likely to be molecularly distinct, which led to the next phase of data analysis, in which we compared gene expression profiles between these two clusters, which we termed HSC1 (large) and HSC2 (small).

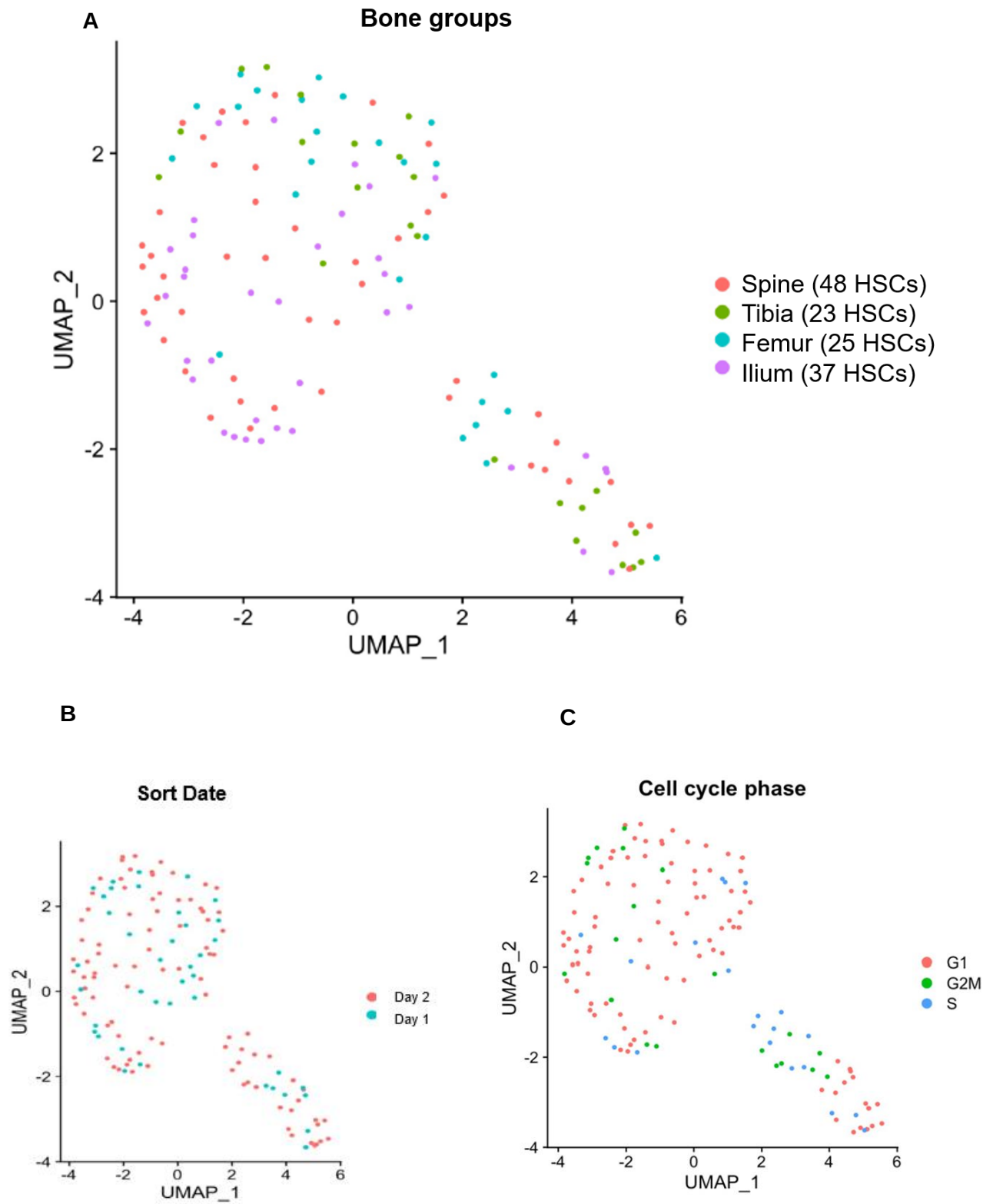


Figure 3.6. Dimensionality reduction analysis of scRNA-seq of HSCs from different bones. A) Unified Manifold Approximation (UMAP) plot of scRNA-seq by bone of origin. The table to the right shows the number of cells profiled from each bone. Two visibly distinct clusters were identified from the analysis: a larger cluster towards the upper left region and a smaller cluster on the lower right-hand side region of the plot. B) UMAP showing distribution of samples across the two experimental dates in each cluster. C) UMAP of cell cycle scoring for cells in the small and large clusters, irrespective of bone of origin. While both clusters were present on both experimental dates, it was noted that the larger cluster was more enriched for cells in the G1/G0 phase of the cycle, while the smaller cluster contained cells found in the G2M and S phases.

3.3.1.1 Cells contained within the HSC1 cluster exhibited HSC gene expression patterns, including genes such as *Procr*, *Kit* and *Gata2*

Given the identification of two cell clusters on the UMAP plot resulting from the DEG across single HSCs derived from all bones, we decided to explore the potential differentially expressed genes in HSCs from the HSC1 cluster versus HSC2. Datapoints corresponding to each individual cell on the UMAP plot were selected by applying the CellSelector command function of the Seurat analysis pipeline to infer their gene expression patterns (Butler et al, 2018) (Figure 3.7). Out of the 133 cells analysed, 34 were found in the HSC2 cluster, and 99 cells comprised the HSC1 cluster (Figure 3.7A). A total of 9694 genes were identified using the Seurat FindMarkers function, resulting in 347 differentially expressed genes between the two clusters ($Q < 0.05$, $\log_2FC > 1$) (Figure 3.7B). Interestingly, even though only 18 of these genes were differentially expressed in cells from the HSC1 cluster, these were genes characteristically associated with HSC expression patterns, including *Procr* (EPCR) ($p = 1.26e-08$), *Kit* (CD117 or c-Kit) ($p = 4.86e-09$), *Gata2* (GATA-binding protein 2) ($p = 8.2e-07$), and *Mpl* (TPO receptor) (full list found in Table 3.2). In addition, the *Adgrg1* gene ($p = 2.4e-09$) - encoding for the adhesion G-protein coupled receptor 1 - was likewise found to be upregulated in this cluster, which is of particular interest since it is involved in the positive regulation of the RhoA (Rho GTPase family member A) signalling (Lala & Hall, 2022), a crucial pathway for multilineage potential in HSCs (Zhou et al, 2013). Contrastingly, even though the most numerous, DE genes in HSCs from the HSC2 cluster included markers of mature cell lineages such as CD52 ($p = 1.86e-20$), expressed on the surface of circulating lymphocytes and monocytes (Picaaluga et al, 2007); and CD33 (Siglec3) ($p = 7.84e-15$), found in early committed myeloid progenitors (Nguyen et al, 2006). However, two members of the cell division cycle-related family - *Cdca3* and *Cdca8* - were found among the HSC2 DE genes, which both regulate proliferation via mitotic activation through different protein complexes (Cui et al, 2021; Uchida et al, 2012). From our previous analyses, we inferred that the HSC2 cluster was more enriched for cells found in the G2M and S phases, therefore agreeing with the cell cycle associated genes identified in such cluster.

These results were suggestive of a potential gene signature characterising HSCs from different bones distributed across these two clusters. Considering these data, we asked our next question - is it possible that HSCs in the small cluster would exhibit upregulation of genes associated with better HSC functional performance in vivo? Would this correlate with the HSC gene expression patterns observed across the different bones?

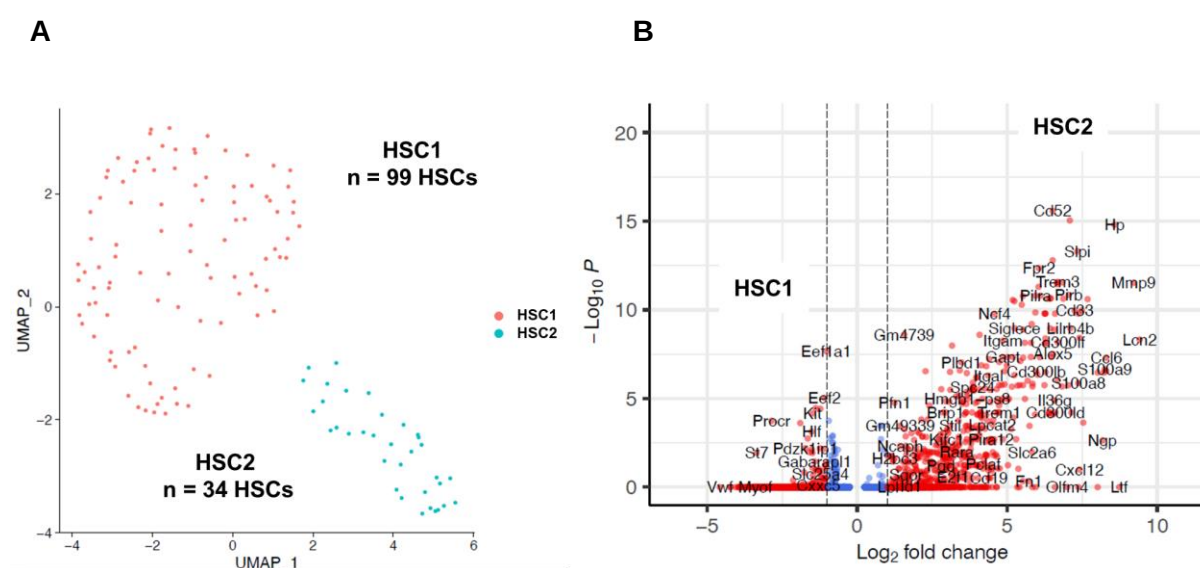


Figure 3.7. Differential gene expression analysis between HSC1 and HSC2 clusters. A) UMAP plot produced using CellSelector from the Seurat pipeline. Of the 133 HSCs analysed, 99 were found in the large cluster, and 34 in the small cluster. B) Enhanced volcano plot showing DE genes in the small cluster (right) over the large cluster (left). Notably, even though only 18 genes were differentially expressed in the large cluster, they included HSC-associated genes, such as *Procr*, *Kit*, *Gata2* and *Mpl*. Likewise, within this cluster, the high expression of *Adgrg1*, a gene implicated in positive regulation of RhoA signal transduction, was indicative that cells in the large cluster could potentially correlate with functional HSC gene expression signatures (described in next section). A total of 9694 genes were identified, 347 differentially expressed between the two clusters ($Q < 0.05$, $\log_2FC > 1$).

Table 3.2. DE genes in HSC2 cluster over HSC2 cluster (FindMarkers)						
Marker	Q	avg_log2FC	p_val	pct.1	pct.2	gene
St7	0.01158	-3.34801	8.01E-07	0.294	0.737	St7
Procr	0.000182	-2.85337	1.26E-08	0.382	0.838	Procr
Tbxas1	0.000243	-1.89575	1.68E-08	0.588	0.939	Tbxas1
Pdzk1ip1	0.006248	-1.7244	4.32E-07	0.618	0.889	Pdzk1ip1
Saraf	0.001784	-1.64458	1.23E-07	0.706	0.949	Saraf
Gimap6	0.0106	-1.58765	7.33E-07	0.676	0.909	Gimap6
Hlf	0.000742	-1.51684	5.13E-08	0.676	0.98	Hlf

Gm19590	0.007434	-1.50983	5.14E-07	0.559	0.96	Gm19590
Kit	7.03E-05	-1.491	4.86E-09	0.912	1	Kit
Gata2	0.011867	-1.48712	8.20E-07	0.706	0.98	Gata2
Gabarapl1	0.036076	-1.41093	2.49E-06	0.735	0.97	Gabarapl1
Adgrg1	3.48E-05	-1.38162	2.40E-09	0.971	1	Adgrg1
H2-Q7	0.006395	-1.24368	4.42E-07	0.912	1	H2-Q7
Cnbp	3.76E-05	-1.22318	2.60E-09	0.971	1	Cnbp
Tmem176b	0.006992	-1.10146	4.83E-07	0.765	0.98	Tmem176b
Eef2	9.31E-06	-1.0885	6.44E-10	1	1	Eef2
Mpl	0.047169	-1.04006	3.26E-06	0.735	0.99	Mpl
Eef1a1	2.13E-08	-1.03083	1.48E-12	1	1	Eef1a1

3.3.2 Global differential gene expression analysis identified specific gene expression exclusively in HSCs from the femur and tibia

After investigating the molecular profiles of the small and large clusters, differential gene expression analysis was performed on data from all different bones, across the two clusters. We applied the FindAllMarkers function from the Seurat pipeline on all data to compare each bone against every other bone (femur vs tibia, femur vs ilium, femur vs spine, tibia vs ilium, tibia vs spine, ilium vs spine) (Figure 3.8). A total of 1636 genes were identified in the comparison, although only a small number of genes exhibited significantly higher expression levels in femur or tibia when compared against all other bones (Figure 3.8A). No genes were significantly upregulated or exclusively expressed in HSCs from the ilium and spine locations.

In the tibia, the genes with higher expression were: Gm16091 (predicted gene Gm16091) ($p = 6.63\text{e-}08$), *Npc1* (intracellular cholesterol transporter 1) ($p = 5.79\text{e-}07$), *Ctdspl* (CTD small phosphatase) ($p = 1.39\text{e-}06$), *Igsf6* (Immunoglobulin superfamily member 6) ($p = 2.26\text{e-}06$), and *Mirt1* (Myocardial infarction associated transcript 1) ($p = 3.14\text{e-}06$) (Figure 3.8B). In the femur, there were two genes with significantly higher expression: *Zcchc3* (Zinc finger, CCH-C domain containing 3) ($p = 3.05\text{e-}07$) and *Ggact* (γ -glutamylamine cyclotransferase) ($p = 7.96\text{e-}07$) (Figure 3.8B). A summary of DE genes in femur and tibia HSCs can be found in Table 3.3. Considering the findings from the DE analysis between the two clusters, together with the specific gene expression patterns observed in femur- and tibia-derived HSCs, we decided to

perform a scoring for two molecular signatures to determine the functional performance of HSCs *in vivo*.

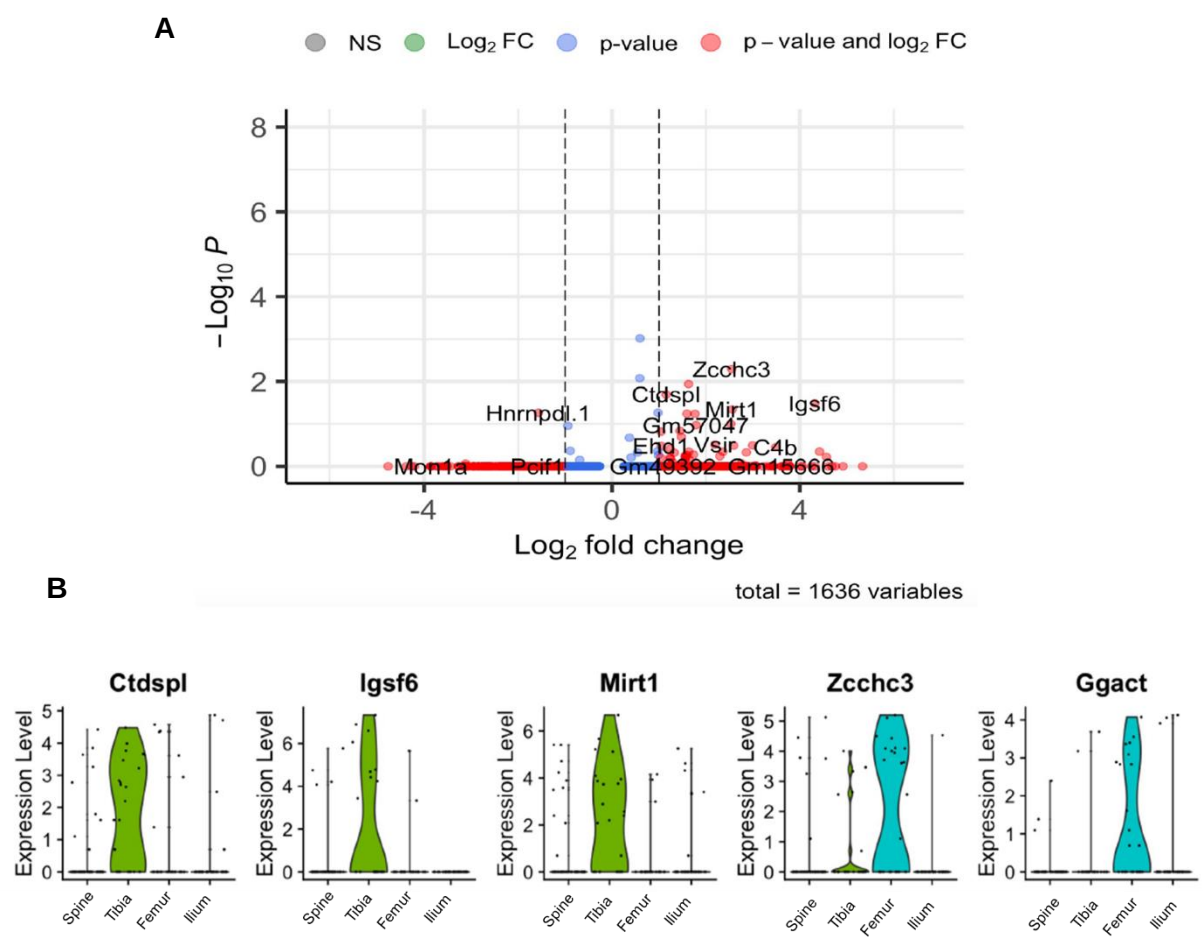


Figure 3.8. Differential gene expression analysis of HSCs from four bone marrow locations. A) Enhanced volcano plot, showing Log₂FC and p adjusted values for 1636 variables analysed through the Seurat pipeline for all four bones. DE genes were only found in femur and tibia HSCs, and none for spine and ilium HSCs. B) Violin plots corresponding to the four significantly differentially expressed genes for tibia (green), and two DE genes in femur (blue) HSCs. Plot shows normalised gene expression levels across HSCs from all different bones. Threshold values: Q < 0.05 and Log₂ FC >1.

Table 3.3 DE genes in femur and tibia (FindAllMarkers)							
marker	Q	avg_log2FC	p_val	pct.1	pct.2	cluster	gene
Gm16091	0.000959	0.592665	6.63E-08	0.304	0.009	tibia	Gm16091
Npc1	0.008375	0.588457	5.79E-07	0.826	0.236	tibia	Npc1

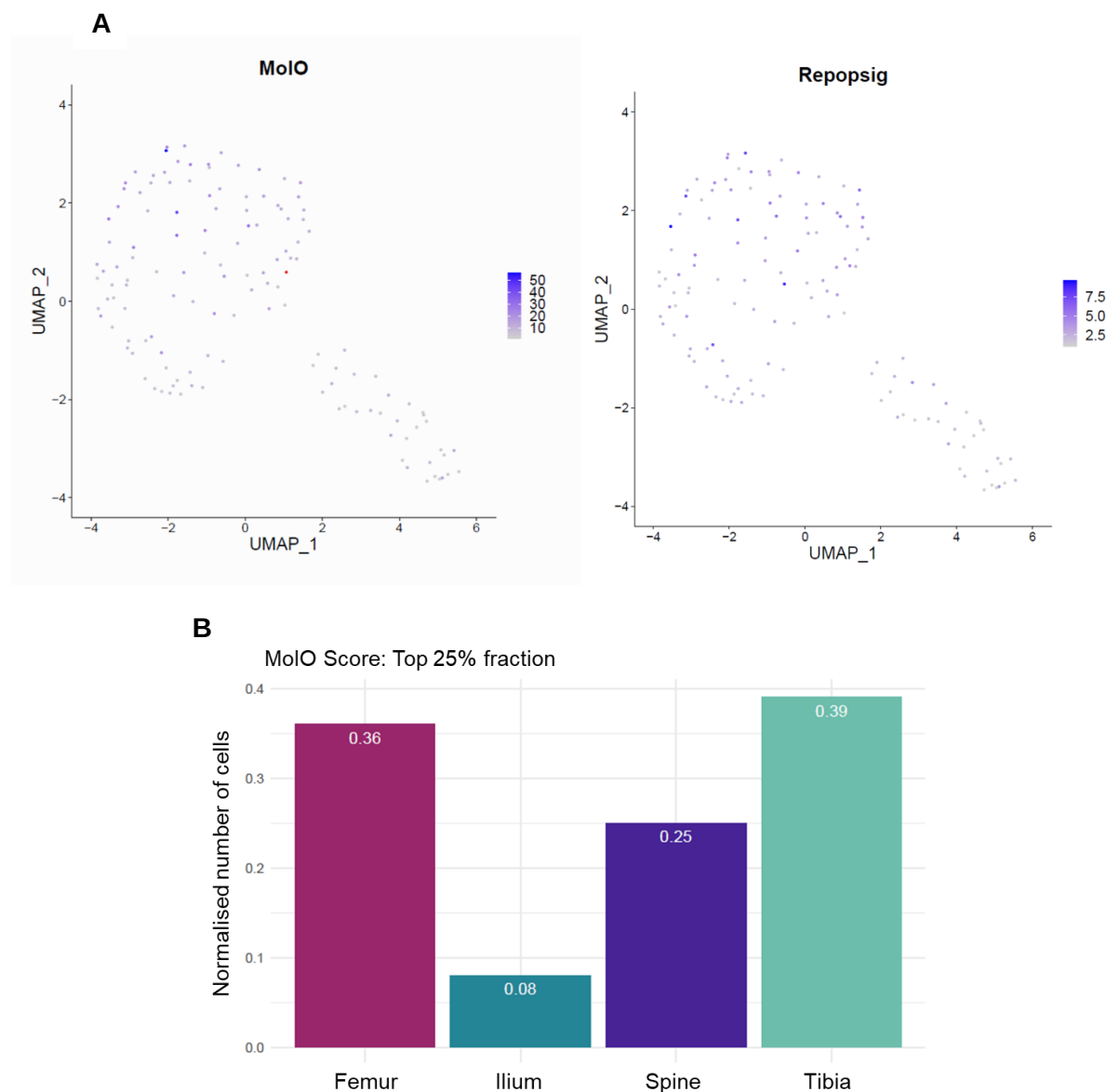
Ctdspl	0.020218	1.149629	1.40E-06	0.696	0.182	tibia	Ctdspl
Igsf6	0.032719	4.314623	2.26E-06	0.391	0.055	tibia	Igsf6
Mirt1	0.045566	2.55932	3.15E-06	0.652	0.173	tibia	Mirt1
Zcchc3	0.005078	2.548468	3.51E-07	0.56	0.12	femur	Zcchc3
Ggact	0.011519	1.62888	7.96E-07	0.48	0.074	femur	Ggact

3.3.3 Femur and tibia HSCs exhibited high scores of two functionally-defined HSC gene signatures, MoIO and Repop, in comparison to ilium and spine

This stage of data analysis focused on analysing the gene expression profiles of HSCs from the four bones in accordance with two functionally-defined molecular signatures for HSCs: the Molecular Overlap Signature (MoIO), which was originally developed to link the transcriptional profile with self-renewal *in vivo* capacity of freshly isolated single HSCs (Wilson, Kent et al, 2015) and the Repopulating Signature (RepopSig), which likewise links the molecular profile of HSCs to their functionality in transplants (Che et al, 2022). The RepopSig was originally developed to profile and determine the self-renewal potential *ex vivo* single-cell expanded clones. Since it shares many of the gene features with the MoIO signature, RepopSig can also inform about functional potential in freshly isolated HSCs. Therefore, we estimated the scores for each of the cells within the four BM groups.

To conduct this analysis, the gene ID lists for each of the signatures were obtained from the supplementary material of the online versions of the corresponding publications, and were integrated to the Seurat analysis pipeline, thus establishing the numerical scores for each of the signatures (0-50 for MoIO and 0-8 for RepopSig). The results from this analysis strongly suggested that the two major cell clusters observed on the UMAP plots had different functional HSC content as the cells in the lower right had both a lower MoIO (Figure 3.9A, left) and lower RepopSig score (Figure 3.9A, right), regardless of the bone they were isolated from. On the other hand, cells in the upper left cluster had the highest MoIO and RepopSig scores, comprising the large HSC cluster. This agrees with our earlier findings, in which cells from the large cluster displayed higher expression of *Procr*, *Kit*, *Mpl* and *Gata2*, resulting in a nice correlation with genes present in both molecules signatures. Furthermore, by normalising total

HSC number across the four bones, we estimated the number of cells found within the top 25% scoring fractions for each signature (Figure 3.8B-C). The analysis demonstrated that femur- and tibia-derived HSCs scored the highest for both MoIO (Figure 3.9B) and Repop (Figure 3.9C) signatures, while spine- and ilium-derived HSCs displayed lower scores, and this was also reflected on a single-cell analysis basis (Figure 3.9D). This demonstrated that MoIO and Repop scores were lower in cells of the HSC2 cluster, irrespective of the anatomical site from which they were harvested. It also indicated, however, since HSCs from the femur and tibia scored the highest for these molecular signatures, and were preferentially located within the large cluster - in addition to displaying a specific gene expression pattern observed in the global DE analysis - they were likely to have a superior performance in functional *in vivo* assays



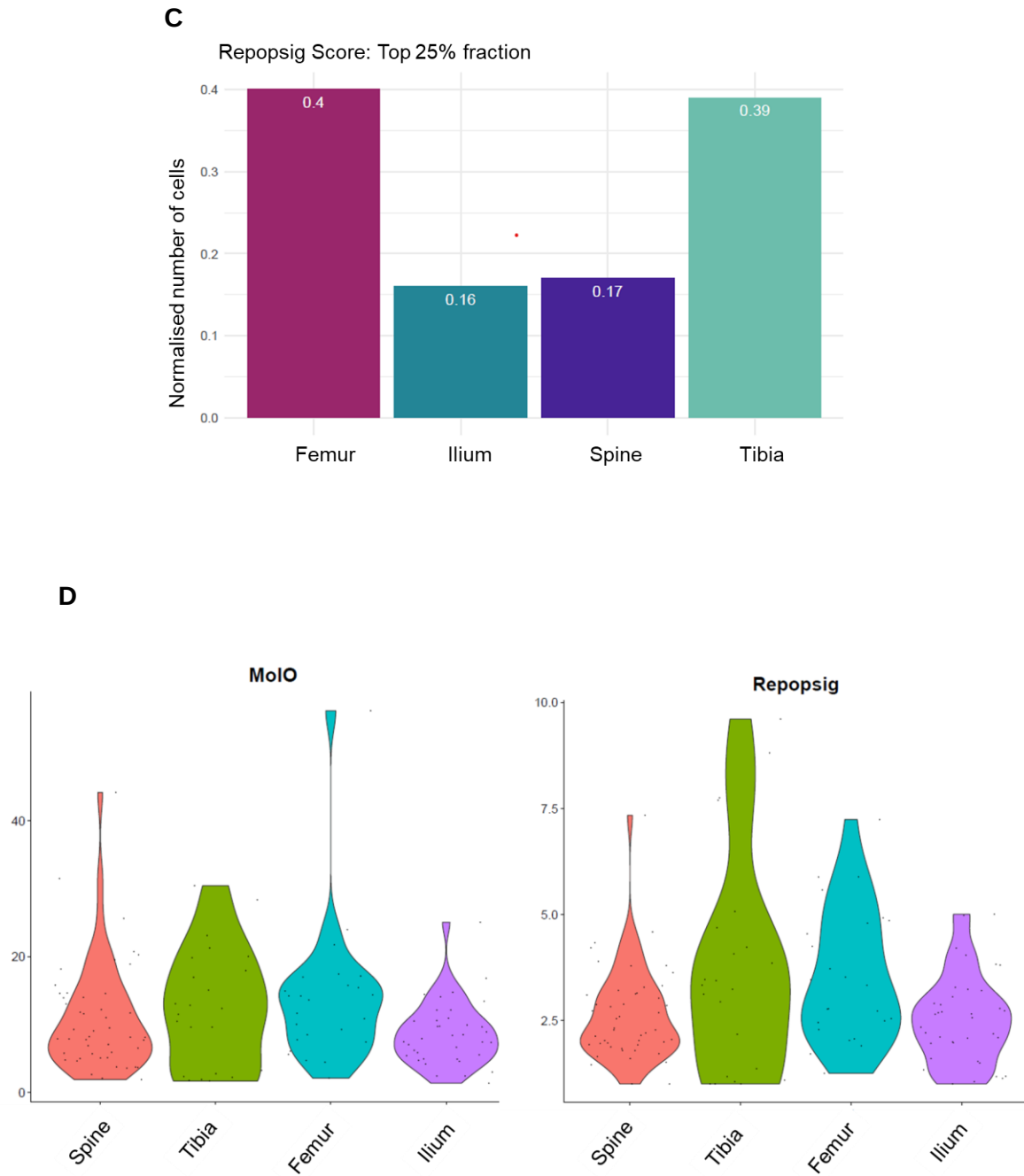


Figure 3.9. MoLO and RepopSig analysis in HSCs from different anatomical regions. A) UMAP feature plots of the MoLO (left) and Repop (right) scores for all samples across the HSC1 and HSC2 clusters. Signature scores were determined by arbitrary units according to previous reports (Wilson et al, 2015; Che et al, 2022), - MoLO = 0-50 units; Repop = 0-8 units - represented by the purple colour scale to the right of each UMAP. B-C) Barplot displaying the normalised number of cells found within the top 25% scoring fraction for the MoLO (B) and Repop (C) signatures. D) Violin plots for the overall score of each signature for single femur-, tibia-, ilium- and spine-derived HSCs. Y axis represents the scores for each signature, set at the same scale as for the UMAP plots.

3.3.4 Long-term reconstitution potential of HSC from different geographical locations

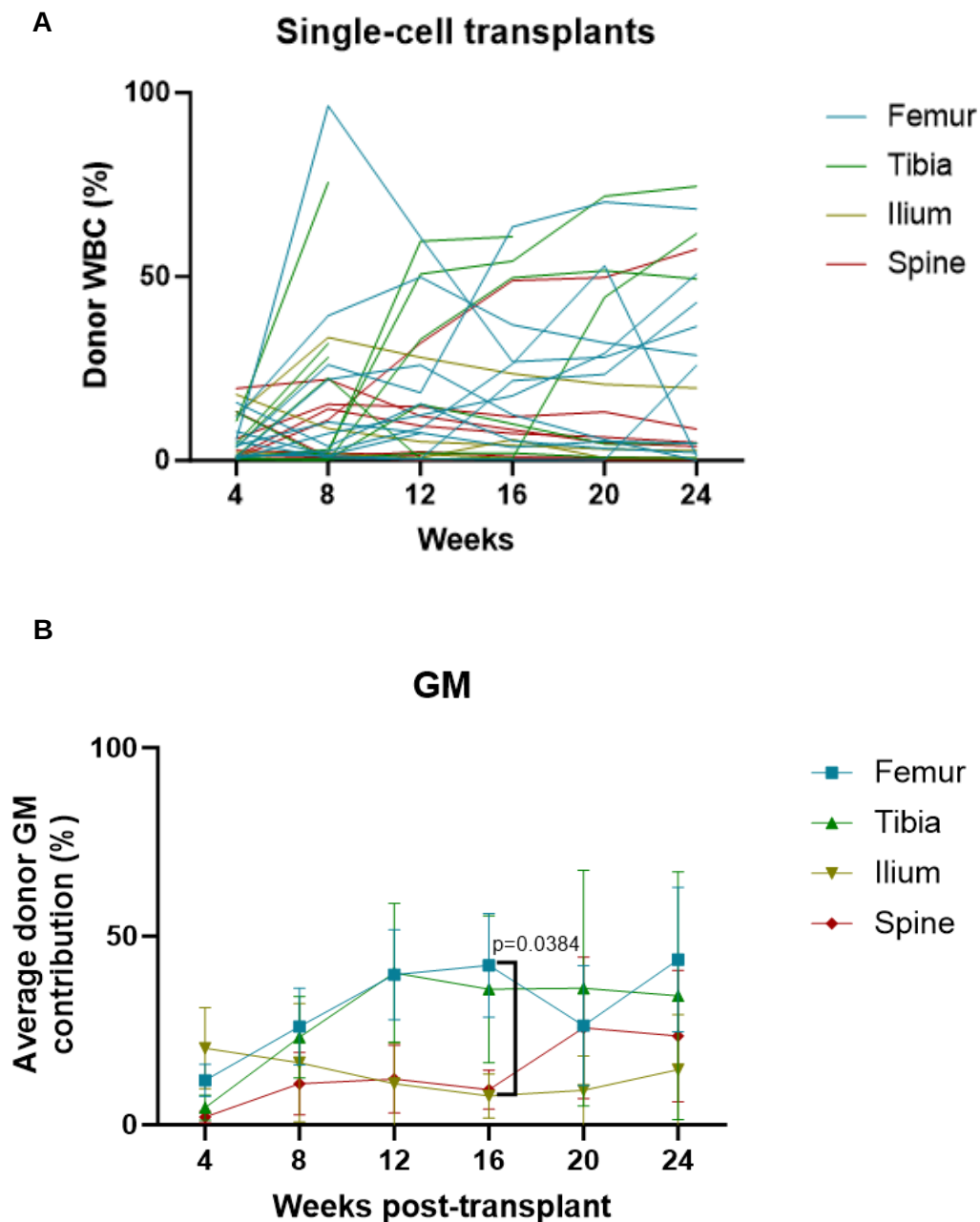
To functionally validate the findings from the scRNA-seq analysis - which suggested a potentially higher reconstitution capacity for femur and tibia HSCs - single HSCs from the four bones characterised in the study were isolated by FACS sorting, and then injected into sub-lethally irradiated congenic Ly5.1(C57BL/6^{w41/w41}) (CD45.1) recipients. The distribution of recipients per BM group was as follows: 76 for femur, 70 for tibia, 74 for ilium and 71 for spine, which injected with a single cell each, equating to a total of 291 injected recipients across two independent experiments. Of these recipients, 21 were sacrificed due to causes unrelated to the transplant prior to the 16-week bleed timepoint, leaving a total of 270 animals post-16 weeks.

Monitoring of chimaerism from the two transplantation experiments revealed differences in the proportion of repopulated recipients of HSCs based on the original BM source. Within the femur group, 10 single HSCs of the 76 recipients (13.15%) showed detectable chimaerism levels of <0.5% at 16 weeks; 6 of 70 from the tibia group (8.6%) recipients were reconstituted, with similar proportions observed in the spine group (8.4%). Lastly, only 4 of 74 of the ilium BM HSCs recipients were repopulated (5.4%). Overall, the number of repopulated recipients across the two experiments was 26 out of 291 injected animals ~ 8.93% in total which is slightly lower than previous reports but still an acceptable frequency of long term functional HSCs. Prior to the transplant endpoint, 2 of the 26 positively engrafted animals were sacrificed due to causes unrelated to the experiment, therefore leaving 24 animals from which BM would be harvested and transplanted into secondary recipients (described later in this chapter)

HSCs from the femur and tibia displayed the strongest overall chimaerism levels, with an average of 22% for the femur and 30% for the tibia of total circulating WBC contribution. Contrastingly, spine-derived HSCs displayed an average chimaerism level of 12%, whereas cells from the ilium achieved an average donor chimaerism of approximately 5%. While HSCs from all groups maintained steady reconstitution levels across experimental timepoints, donor chimaerism levels of mice receiving HSCs from the femur, tibia and spine stably increased from the 4- through to the 24-week timepoint. ilium bone-derived HSCs kept similar chimaerism levels throughout the transplant, with only very mild decreases between 12 and 16 weeks post-transplantation. In spite of no statistically significant differences in donor chimaerism levels for specific BM groups, the average donor contribution to the circulating GM fraction was significantly elevated in the femur HSC recipients than in spine HSC recipients at 16 weeks ($p = 0.0384$) (Figure 3.10B). Overall, long-term multilineage contribution potential was observed to be consistent and steadily increasing throughout time for femur- and tibia-

derived HSCs, while reduced contribution was observed in recipients of HSCs from ilium and spine (Figure 3.10C).

Given that serial monitoring of donor contribution to the overall WBC fraction in primary recipients hinted at the possibility of HSCs from different bones displaying distinct differentiation patterns, we conducted a more thorough analysis to describe the long-term programmes of differentiation in HSCs from individual bones.



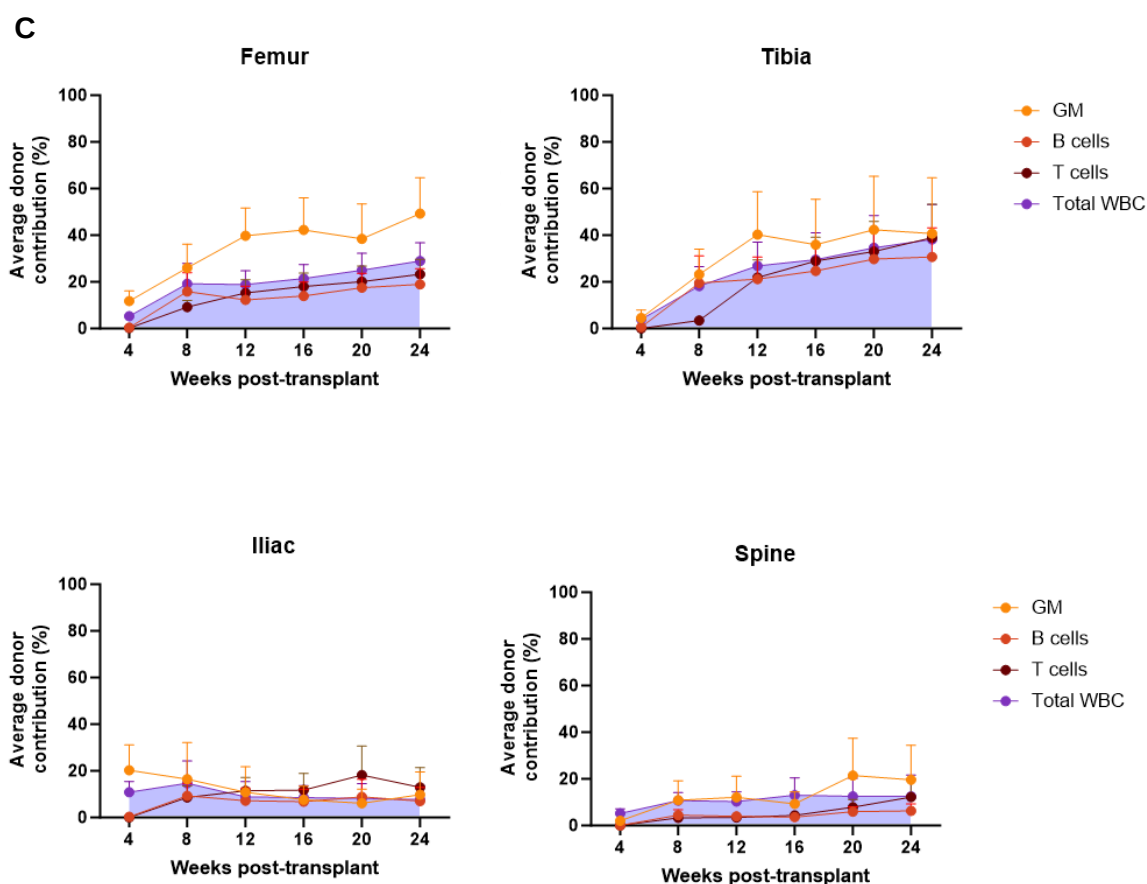


Figure 3.10 Functional assessment of single HSCs obtained from different anatomical locations. A) Repeated measures plot of single-cell transplant data collected from 291 primary recipients from 4 to 24 weeks post-injections. After 16 weeks, 21 recipients were sacrificed due to causes unrelated to the experiment. 26 recipients were positively engrafted at 16 weeks, showing detectable chimaerism in the circulating WBC fraction of <0.5%. Since two of these recipients were sacrificed prior to the 24-week experimental endpoint, 24 animals remained to conduct secondary transplantations. Higher chimaerism levels were observed for femur and tibia repopulating HSCs, while HSCs from ilium and spine were the lowest throughout the transplant. B) Donor contribution to the circulating GM fraction, showing a statistically significant ($p = 0.0384$) increase in donor-derived GM in femur HSC recipients in comparison to spine HSC recipients (one-way ANOVA was applied to calculate statistical significance). C) Multilineage contribution by HSCs from each bone marrow group in PB of primary recipients. Shaded purple area represents contribution to total WBC fraction, with each line representing contribution to each individual lineage (GM, B- and T-lymphocytes).

3.4.1 HSCs from different anatomical regions displayed distinctive haematopoietic differentiation programmes

Considering the results from the index-sort data analysis, as well as the recently obtained single-cell transplant data, we reasoned that - in the same way that HSCs from individual bones exhibit variable HSC marker expression levels, as well as different long-term multilineage contribution capabilities - they were equally likely to display unique

haematopoietic differentiation patterns according to bone of origin. To achieve this, I undertook a similar data analysis approach to Dykstra et al (2007). In this study, multilineage engraftment potential of single LT-reconstituting cells (LT-RCs) was profiled through serial transplantation assays, in which four distinct haematopoietic differentiation programmes were described (Figure 3.11A). Based on the relative contribution to the GM lineage in reference to the B- and T-cell lineage for at least 16 weeks or more after transplantation, as well as their ability to serially reconstitute recipients, LT-RCs were classified into four clusters, namely:

- α cells, which display higher contribution to the GM lineage relative to B and T cell production, with a high donor derived GM: (B+T) ratio ($>2:1$). α cells likewise exhibit extensive long-term self-renewal potential in serial transplantation (secondary and tertiary recipients), in which they also display a higher contribution to the GM lineage.
- β cells yield an equal contribution to the myeloid and lymphoid fractions, with a balanced donor-derived GM: (B+T) ratio. Similar to α cells, β LT-RCs are also able to serially repopulate recipients, generating cells from the β , γ or δ subtype.
- γ cells, which make a higher contribution towards the lymphoid lineage, and have therefore a low GM: (B+T) ratio ($<1:4$). Contribution of at least 1% to both the myeloid and lymphoid lineages by γ cells is sustained at 16 weeks but GM contribution decreases gradually after this time. Unlike α and β cells, γ LT-RCs do not repopulate recipients in serial transplantation.
- δ cells contribute mainly to the lymphoid lineage, but stop contributing to the GM (at least 0.5%) fraction after 16 weeks. Their GM: (B+T) ratio is low, and are unable to serially repopulate recipients.

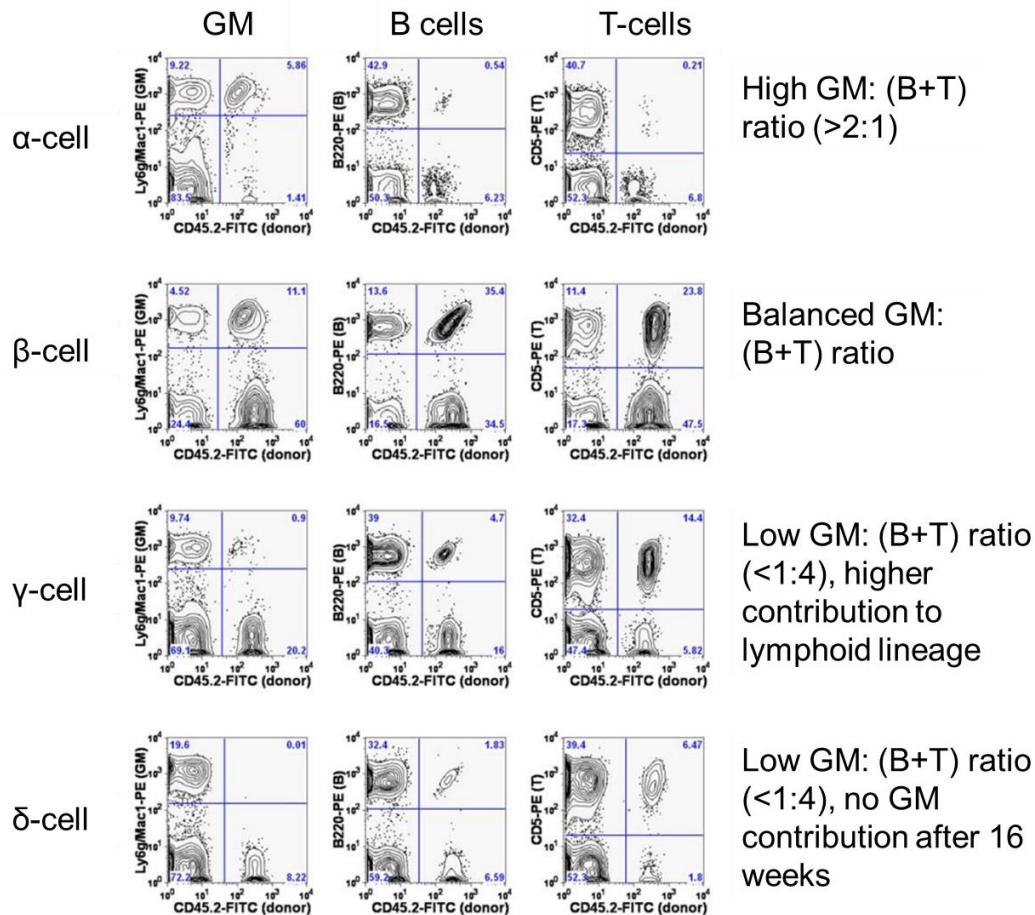


Figure 3.11 Subtypes of LT-RCs defined by their donor-derived GM:(B+T) ratios. α cells have a high ratio (>2:1), contributing mostly to the myeloid fraction, and display durable self-renewal potential. β cells equally contribute to the myeloid and lymphoid lineages, and therefore have a balanced GM:(B+T) ratio. These LT-RCs are equally able to serially repopulate recipients. γ LT-RCs have a low GM:(B+T) ratio, and are not able to reconstitute secondary or tertiary recipients. δ cells preferentially contribute to the lymphoid lineages, with their GM contribution decreasing after 16 weeks. In a similar way to γ cells, δ cells are unable to give rise to donor-derived progeny in serial transplantation. Figure adapted from Dykstra et al (2007).

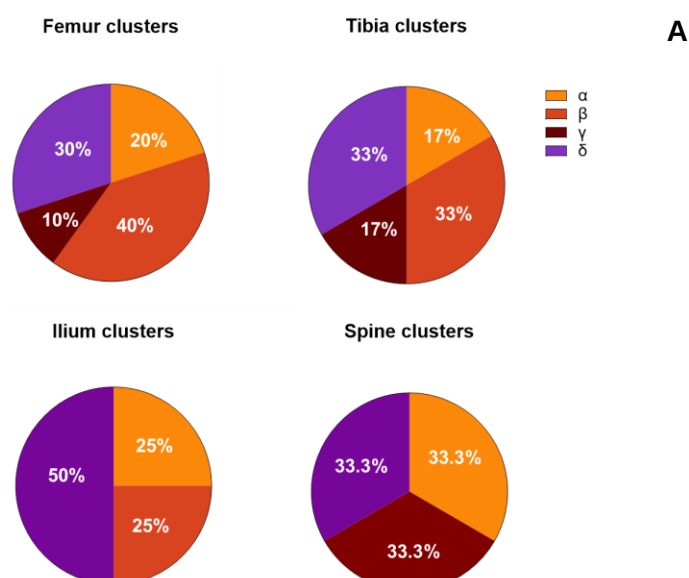
3.4.1.1 HSCs from femur and tibia exhibited α and β LT-RC differentiation patterns, accounting for higher long-term self-renewal potential

The donor-derived GM:(B+T) ratios for HSCs from each anatomical location were calculated (as in Dykstra et al, 2007) from the multilineage contribution data at 16 weeks post-transplantation (Figure 3.12). All four subtypes of long-term reconstituting HSCs were detected in the femur and tibia (Figure 3.12A). The α, β, and δ subtypes were found in the ilium, while δ, γ, and α subtypes were found in the spine. Notably, 60% of repopulating HSCs in the femur belonged to the α and β subtypes, indicating that the majority of HSCs derived from this geographical location displayed a more durable self-renewal potential *in vivo*. In the tibia, and

ilium, 50% of engrafting HSCs were comprised by the durable subtypes of LT-RCs. Nonetheless, in the ilium, the remaining 50% of reconstituting HSCs were of the δ subtype, which accounted for the low contribution to the circulating GM fraction in primary recipients at 16 weeks (3-fold lower than tibia, and nearly five times lower than femur). The spine had an equal distribution of the subtypes identified, each comprising a third of the reconstituting HSCs. Therefore, nearly 67% of reconstituting HSCs derived from the spine were of the δ and γ subtypes, which implied that their long-term self-renewal potential was restricted according to previous data (Dykstra et al, 2007; Benz et al, 2012).

To confirm these findings, data were integrated into a ternary graph, which plots the relative contribution of HSCs to each lineage along each of the vertices of a triangle (Figure 3.11B). Indeed, femur HSCs were preferentially clustered towards the apex of the ternary plot, which corresponds to the highest GM:(B+T) ratio. Conversely, spine- and ilium- derived HSCs clustered towards the higher T:(GM+B) ratio, corresponding to the δ subtype. HSCs from the different bones distributed across the plot, indicating their differing contributions to blood lineages, and as a result, distinctive differentiation patterns.

Overall, these findings showed that the majority of durable self-renewing HSCs are concentrated within the femur and tibia, which accounted for 53.8% of cells of the α/β subtypes identified among bones. In contrast, HSCs from the ilium and spine displayed patterns corresponding to restricted self-renewing LT-reconstituting HSC subtypes. To corroborate these results, I performed secondary transplantations from whole BM suspension from the primary transplant recipients. In the next section, I will present the results from the analysis of the cellular composition and relative donor chimaerism in haematopoietic organs, before transitioning into the serial transplantation assays.



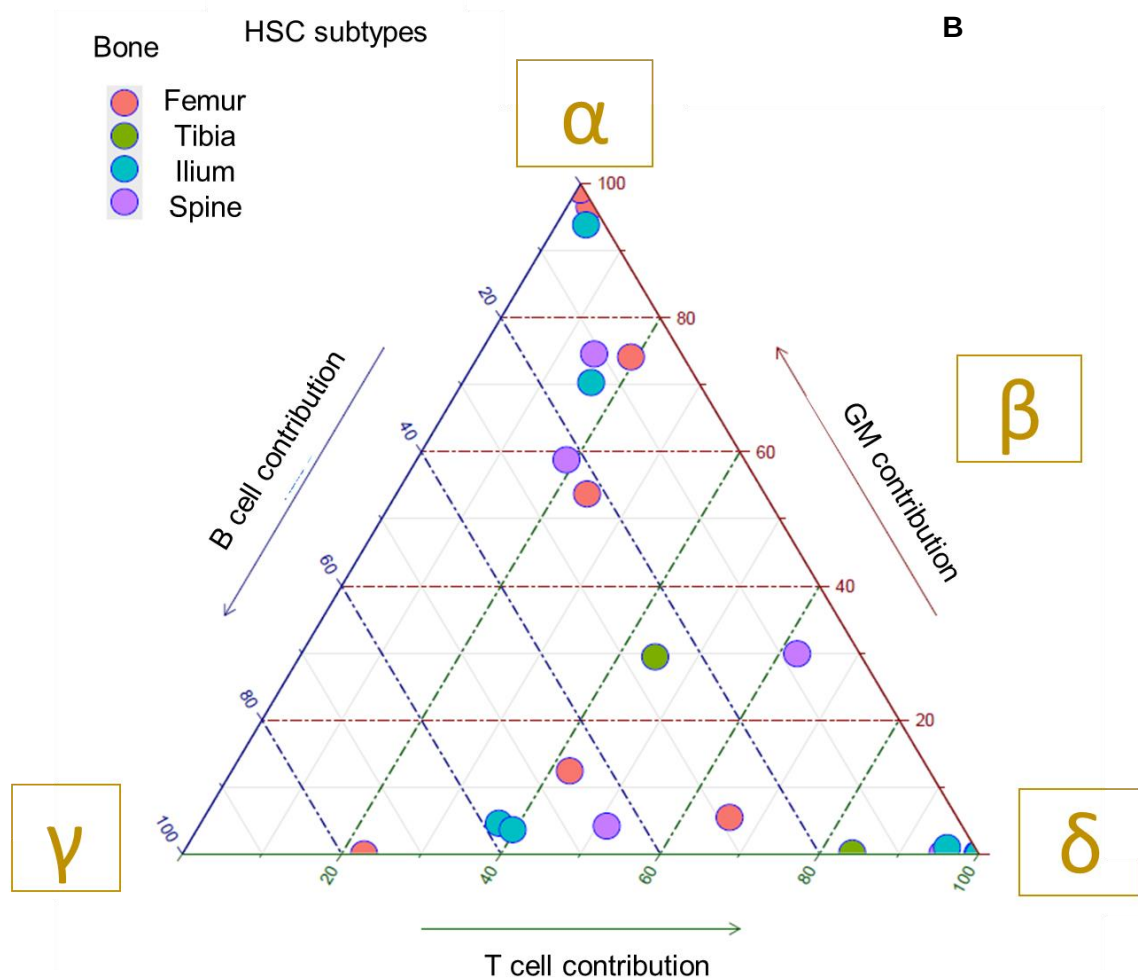


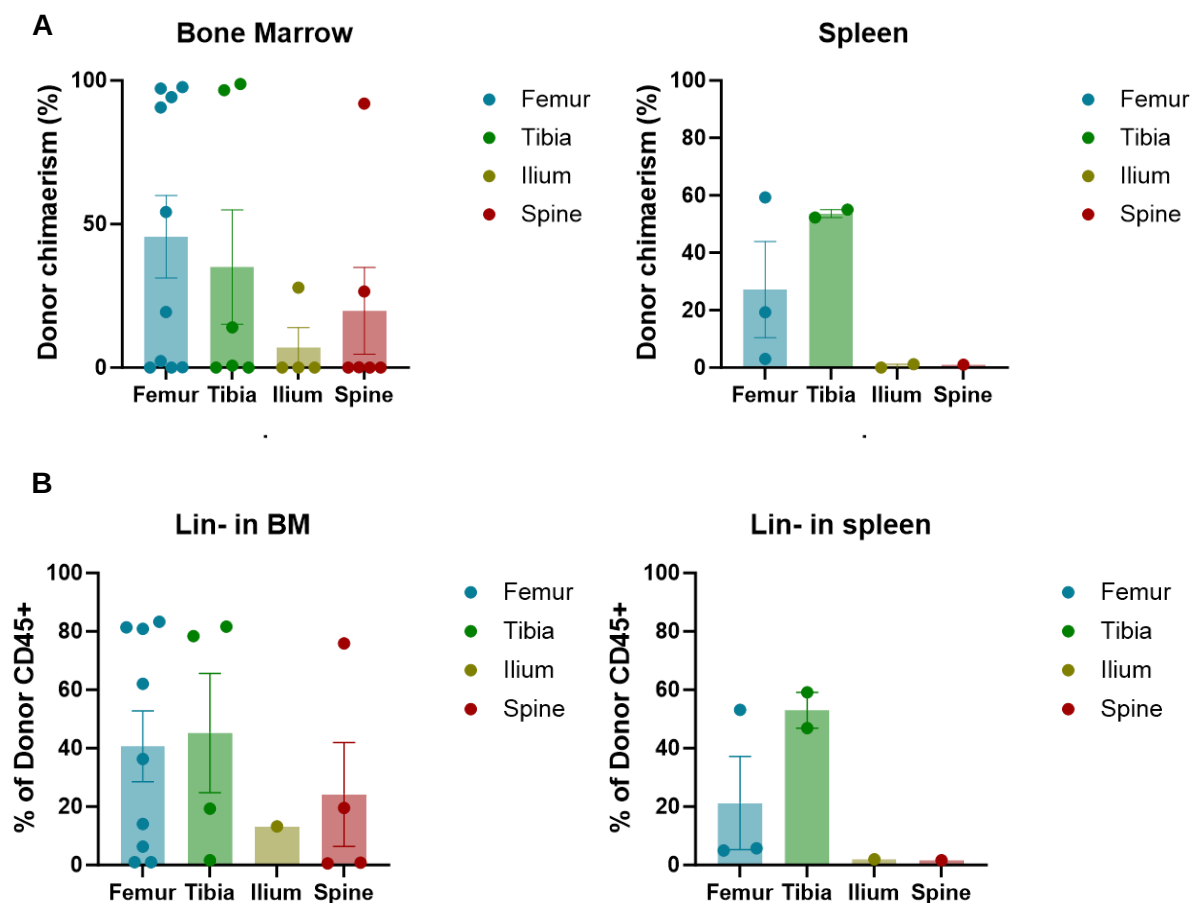
Figure 3.12. Differentiation patterns exhibited by niche-localised HSCs. A) Distribution of LT-RC subtypes in femur, tibia, ilium and spine. The majority of the long-term self-renewing HSCs concentrated in the femur and tibia, whereas cells with restricted potential comprised the largest fraction of LT-RCs in the ilium and spine. B) Ternary plot showing clustering of HSC subtypes by bone of origin. Relative contribution to each lineage (GM, B- and T-cell) runs along each of the vertices of the triangle. The apex of the triangle corresponds to high GM contribution, while the lower left angle corresponds to high B-cell contribution, and the lower right angle corresponds to high T-cell contribution. HSCs which are clustered towards the centre of the plot belong to the β subtype (balanced GM:(B+T) ratio)).

3.4.1.2 Reconstitution of the CD45⁺ cell fraction in the bone marrow and spleen of primary recipients was successfully achieved by femur- and tibia-derived HSCs

After determining the differentiation programmes followed by niche-localised HSCs, and prior to performing serial transplantations, I analysed the cellular composition and contribution of donor HSCs in haematopoietic tissues of 24 positively-engrafted primary transplant recipients (Figure 3.13).

In accordance with their contributions to the circulating WBC fraction, the femur and tibia-derived HSCs made the most notable contribution to the total CD45⁺ fraction in the BM and spleen of primary recipients (Figure 3.13A). Relative donor-derived contribution by spine HSCs was 2-3-fold lower than for femur and tibia HSCs in the BM. In the spleen, neither ilium- nor spine-derived HSCs were able to make any contributions to the CD45⁺ fraction. Contrastingly, tibia HSCs made the most significant contribution to such fraction in the spleen, almost doubling that made by the femur.

Likewise, the donor-derived fraction of the HSC/HSPC compartment in both tissues was higher in femur and tibia HSC recipients than in ilium and spine recipients (Figure 3.13B-D). In the BM, HSCs from the femur and tibia made comparable contributions to the Lin⁻ fraction, while in the spleen, tibia-derived HSC exhibited the highest proportion of donor-derived Lin⁻ among HSCs from the four bones (Figure 3.13B). This pattern was also observed for the LSK (Figure 3.13C) and ESLAM fractions (3.13D) in both BM and spleen, whereby femur and tibia had equivalent contributions, but tibia HSCs were the major contributors in the spleen. Ilium-derived HSCs made a minimal contribution to the ESLAM fraction in the BM, and in the spleen, spine- and femur-derived HSCs displayed comparable donor-derived ESLAM frequencies (Figure 3.13D).



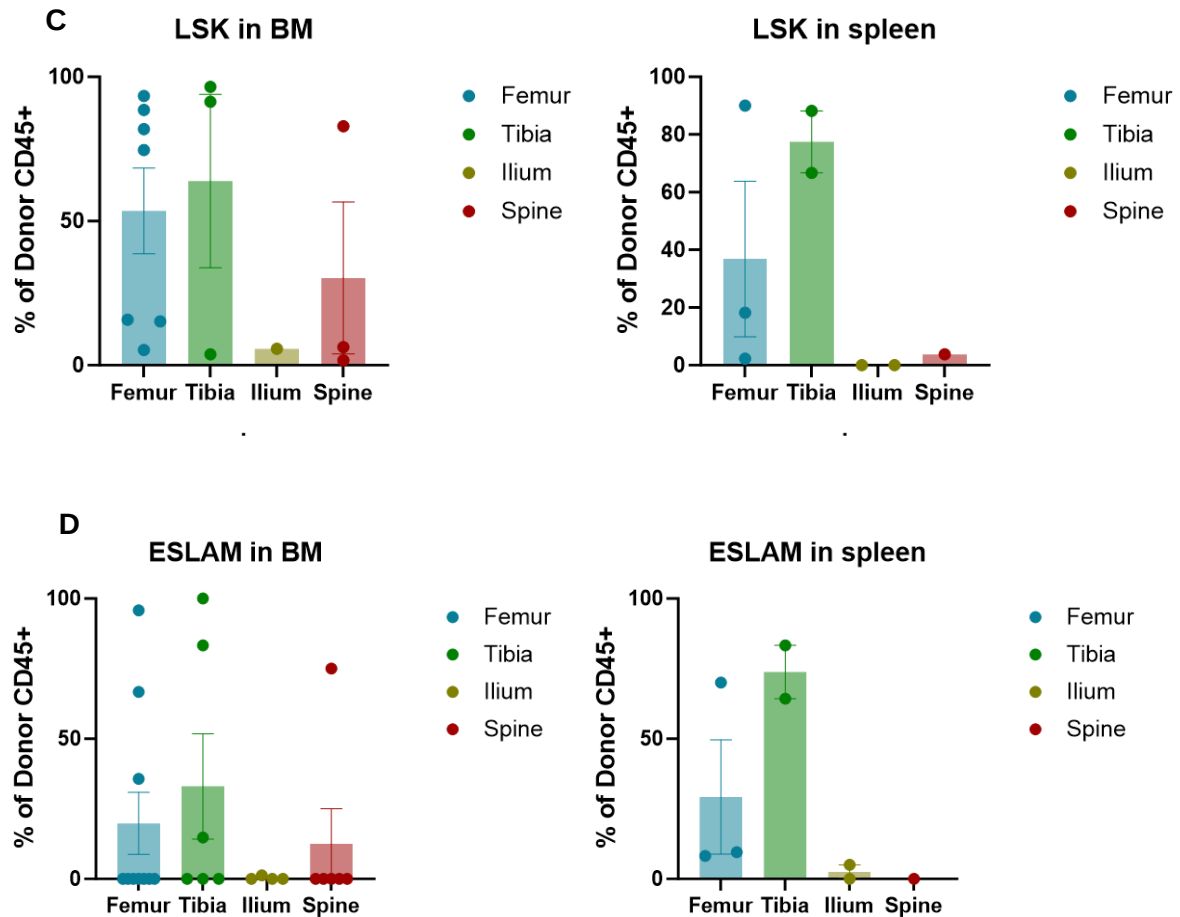


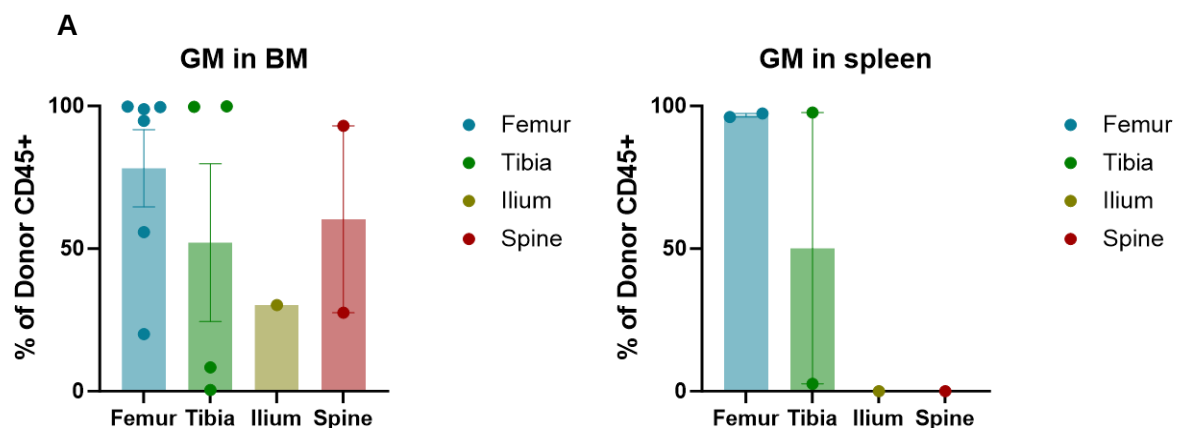
Figure 3.13. Analysis of the donor-derived CD45+ fraction in bone marrow and spleen of primary transplant recipients. A) Relative donor chimaerism in the bone marrow (left) and spleen of recipients (right). While HSCs from all four bones had detectable (>0.5%) progeny in the bone marrow, only femur and tibia-derived HSCs repopulated the spleen of recipients. B-D) Analysis of the HSC/HSPC compartment. B) Donor-derived Lin-frequencies in bone marrow (left) and spleen (right). C) Donor-derived LSK fraction in bone marrow (left) and spleen (right). D) ESLAM donor contribution in the aforementioned haematopoietic tissues. HSCs from tibia made higher contributions to the ESLAM fractions in both tissues, followed by femur HSCs. Spine-derived HSCs were able to reconstitute a detectable proportion of ESLAMs in the bone marrow, but not the spleen. n = 24 recipients in total: Femur, n = 10; tibia, n = 5; ilium, n = 3; spine, n = 6.

3.4.1.3 The highest relative GM contribution levels were exhibited by femur-derived HSCs in the bone marrow and spleen

Analysis of the mature cell lineages in bone marrow and spleen (Figure 3.14) of recipients agreed with the findings from PB chimaerism and donor-derived HSC/HSPC frequencies. The highest donor-derived GM proportion was observed in the bone marrow of femur HSC recipients (Figure 3.14A). Notably, spine HSCs also made a substantial contribution to the GM fraction (60.25% versus 78.15% from femur HSCs), while tibia- and ilium-derived HSCs were

closer together in terms of GM contribution. Strikingly, in the spleen, 100% of the GM fraction was donor-derived in femur HSC recipients, followed by the tibia, which was reduced by almost exactly half (50.15%). Relative contributions to the B-cell compartment in bone marrow remained at comparable levels across bones (Figure 3.14B). As observed for the GM fraction, neither ilium- nor spine-derived HSCs were able to make any contributions to the B-cell fraction in the spleen. The highest donor-derived frequency of B cells was observed in tibia HSC recipients. Finally, analysis of the T-cell compartment showed similar results to the B-cell lineage contributions (Figure 3.14C). Collectively, these data indicate that femur and tibia HSCs display consistent multilineage engraftment in peripheral blood, as well as in haematopoietic tissues, and that ilium and spine make sporadic contributions to either the HSC/HSPC or mature cell compartment in these tissues. This therefore supports the results from the GM:(B+T) ratio calculation for HSCs from the four anatomical regions, in which femur and tibia contained the greater proportions of durable HSC subtypes.

Having concluded the primary transplantation experiment, I then moved on to perform secondary transplantations of bone marrow from recipients of HSCs from different geographical regions.



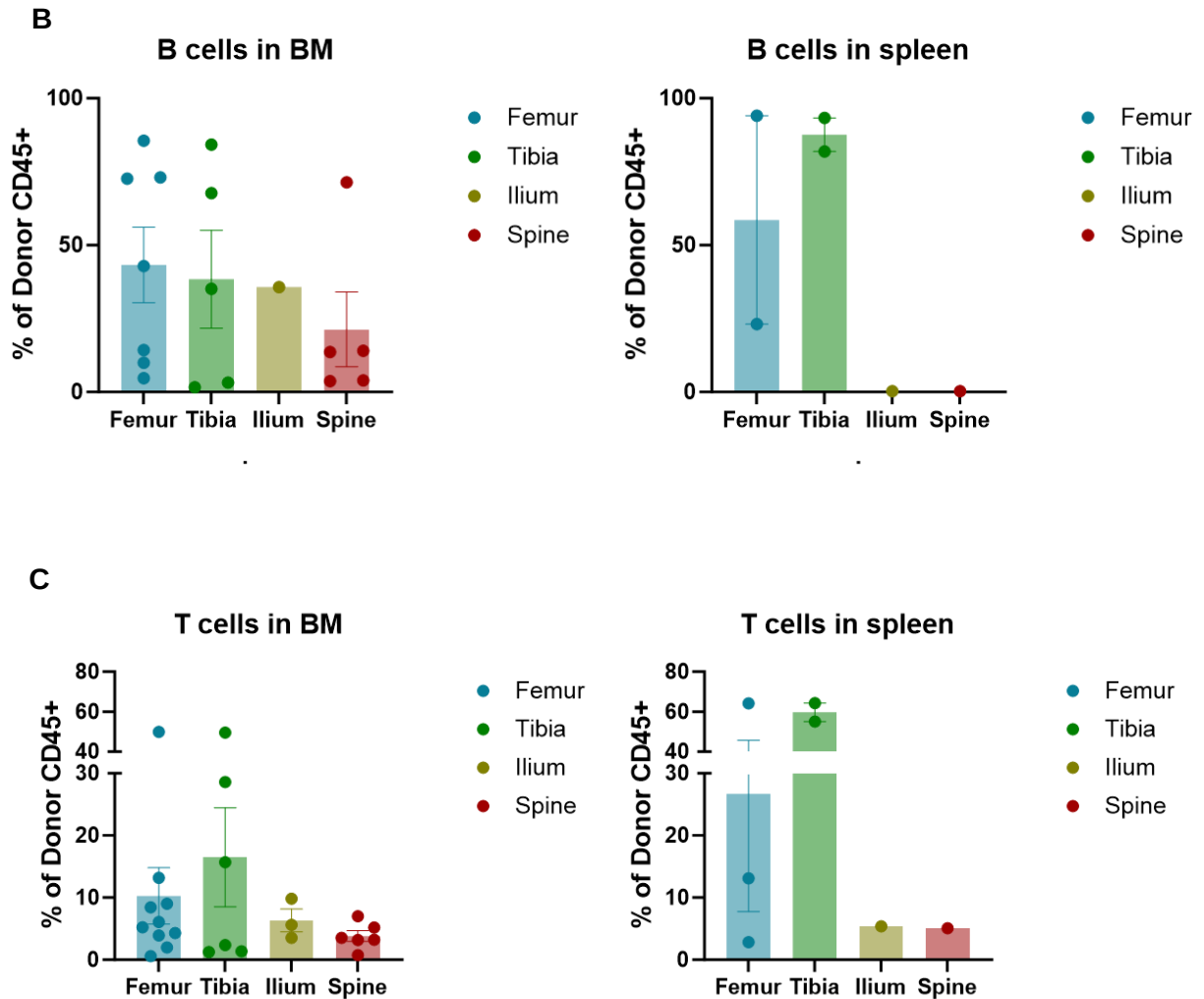


Figure 3.14. Analysis of mature cell lineages in the bone marrow and spleen of primary transplant recipients. A) Donor-derived GM frequencies in bone marrow (left) and spleen (left). HSCs from all four bones made contributions to this fraction in the bone marrow, but not in the spleen, whereby only femur and tibia-derived HSCs were able to reconstitute the GM fraction. In both tissues, the femur made the highest contributions. B) B-cell contribution in the aforementioned tissues. C) T-cell contribution in haematopoietic tissues. The tibia made the greatest contributions to the lymphoid lineages in the spleen, whereas in the bone marrow, HSCs from all anatomical regions made comparably detectable contributions. One-way ANOVA analysis did not detect any statistically significant differences across groups. $n = 24$ recipients in total: Femur, $n = 10$; tibia, $n = 5$; ilium, $n = 3$; spine, $n = 6$.

3.4.2 The largest proportion of serially transplantable HSCs were found in the femur and tibia

The gold-standard method to determine the long-term self-renewal potential of HSCs is serial transplantation, which sets the most stringent threshold for HSC functional activity *in vivo*. Since this was the first time that HSCs from different geographical locations were being

functionally characterised, I decided to conduct a secondary transplantation assay, and in this way determine in which locations do the best functionally performing HSCs reside.

A fraction of the harvested BM from primary recipients was reserved to prepare single-cell suspensions and inject them into secondary recipients (see Methods). A total of 24 BM suspensions (10 from femur, 5 from tibia, 3 from ilium and 6 from spine) were injected into 24 myeloablated animals (Figure 3.15). Of note, all of the primary recipients - which became secondary donors - were stably reconstituted by the donor HSCs for at least 16 weeks (relative chimaerism levels of >0.5%).

Evaluation of peripheral blood chimaerism in secondary recipients (Figure 3.15A) affirmed the findings from the single-cell primary transplant assay - HSCs derived from femur and tibia HSCs were successful in serially repopulating recipients for 16 weeks, displaying the highest levels of relative donor chimaerism (average of 12.47% for femur, and 11.59% for tibia). In contrast, only one sample from the ilium (33.33%) and one from the spine (17%) were able to reconstitute secondary recipients. Furthermore, analysis of chimaerism per individual samples revealed that HSCs with the strongest engraftment corresponded to the α subtype in femur and tibia (2 from femur and 1 from tibia) at 16 weeks post-transplantation. β subtype HSCs, made up the remaining fraction of serially repopulating HSCs (2 from femur, 1 from tibia, 1 from ilium and 1 from spine). In agreement with previous studies (Benz et al, 2012; Dykstra et al, 2007), γ and δ HSCs did not repopulate any of the secondary recipients, irrespective of bone of origin (Figure 3.15A). This also explains why a third of recipients (8 out of 24) were serially repopulated, as all of these were transplanted with α and β -reconstituted primary donors. Moreover, 75% of positively engrafted animals were comprised by recipients of femur and tibia HSCs, while the remaining 25% was equally distributed between ilium and spine recipients.

Subsequent analysis of multilineage contribution in the peripheral blood of secondary recipients reinforced the findings (Figure 3.15B). The total WBC fraction was consistently reconstituted in femur and tibia secondary recipients, maintaining stable relative donor chimaerism levels throughout the experiment (8-16 weeks). This accords with the principle that α and β cells display similar multilineage engraftment in secondary transplantations, as they do in primary transplants (Benz et al, 2012). On the other hand, donor chimaerism in the ilium secondary recipient decreased in a time-dependent manner. Likewise, while contribution to the circulating GM fraction increased towards the 16-week timepoint in the spine recipient, overall WBC contribution was of only 1.07% at 16 weeks (Figure 3.15B). Therefore, not only did femur and tibia contain the greatest proportion of HSCs with durable, serially engraftable potential, but they represented the highest fraction of serially repopulated recipients. More

importantly, α and β cells from ilium and spine were not able to achieve the same level of multilineage engraftment as did those derived from femur and tibia.

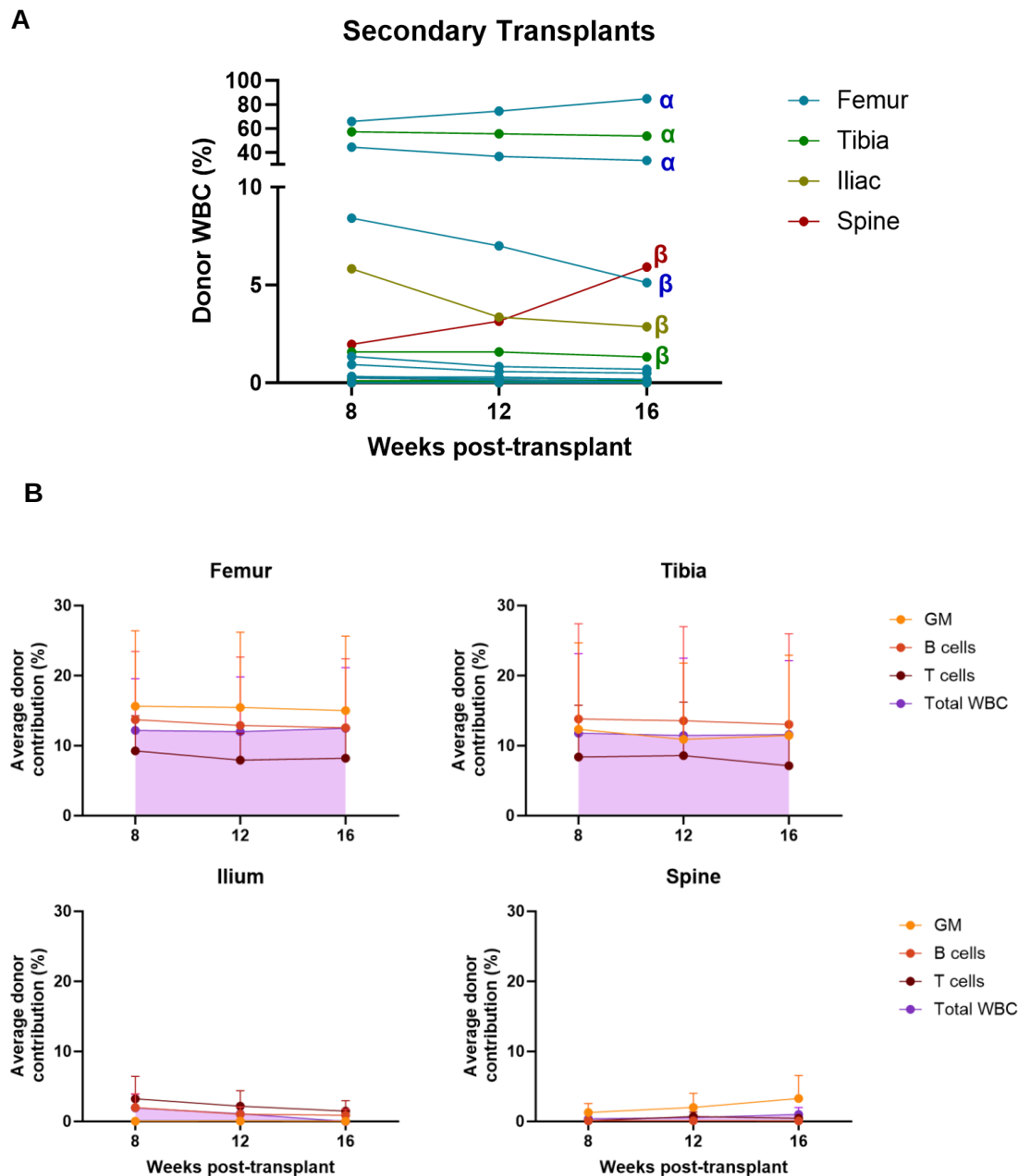
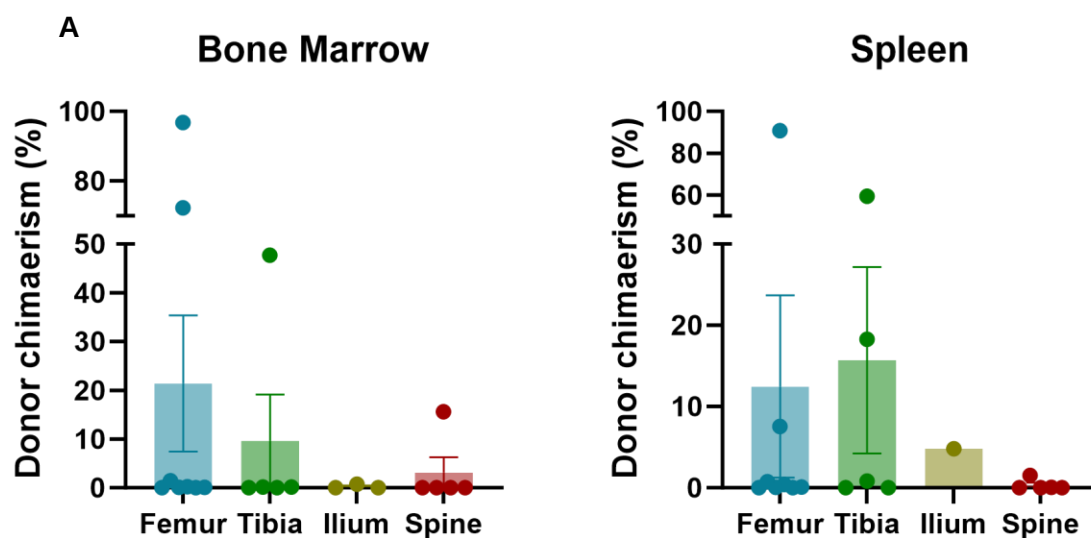


Figure 3.15. Secondary transplantation of whole bone marrow suspensions from femur, tibia, ilium and spine HSC primary recipients. A) Evaluation of donor chimaerism in peripheral blood of secondary recipients. In line with primary single-cell transplant data, 75% of positively engrafted animals were reconstituted with bone marrow suspensions from femur and tibia HSC recipients. These also corresponded to α and β HSCs, which are able to serially repopulate recipients, unlike γ and δ HSCs, as per previous reports (Benz et al, 2012; Dykstra et al, 2007). B) Multilineage contribution profiling in recipients by bone marrow donor group. Donor-derived WBC fraction, as well as individual myeloid and lymphoid lineages were stably and consistently reconstituted by bone marrow of primary femur and tibia HSC recipients. HSCs from ilium and spine, although classified as β HSCs, did not attain similar relative contribution levels.

3.4.2.1 The bone marrow and spleen of were reconstituted in greater proportion in femur and tibia secondary recipients

In order to further profile the long-term self-renewing potential of niche-localised HSCs in serial transplantation, I analysed the BM and spleen of secondary recipients immediately after the 16-week blood collection timepoint (Figure 3.16). BM of femur HSC secondary recipients displayed the greatest relative chimaerism levels (21.38%), being two-fold higher than in tibia (9.6%) and 7 times higher than in spine (3.14%) recipients (Figure 3.16A). In a similar manner to primary transplant recipients, relative donor contribution was higher in the tibia recipients (15.7%), in comparison to femur (12.6%) and ilium (4.79%) recipients. Lastly, analysis of the GM fraction showed a greater contribution in femur recipients (89.25%), while being at comparable levels in tibia recipients (Figure 3.16C). Collectively, these results reinforce the increased functional performance of femur- and tibia-derived HSCs compared to those residing in the ilium and spine, as evidenced by single-cell transplantation data, multilineage reconstitution potential, and the ability to serially repopulate animals, demonstrating their durability and long-term self-renewal potential.

To conclude the functional profiling of HSCs from different BM locations, I conducted limiting dilution assays, in order to determine the frequency of functional HSCs, and to provide greater statistical power to the single-cell transplantation data that I collected. The results from these experiments will be described in the following section.



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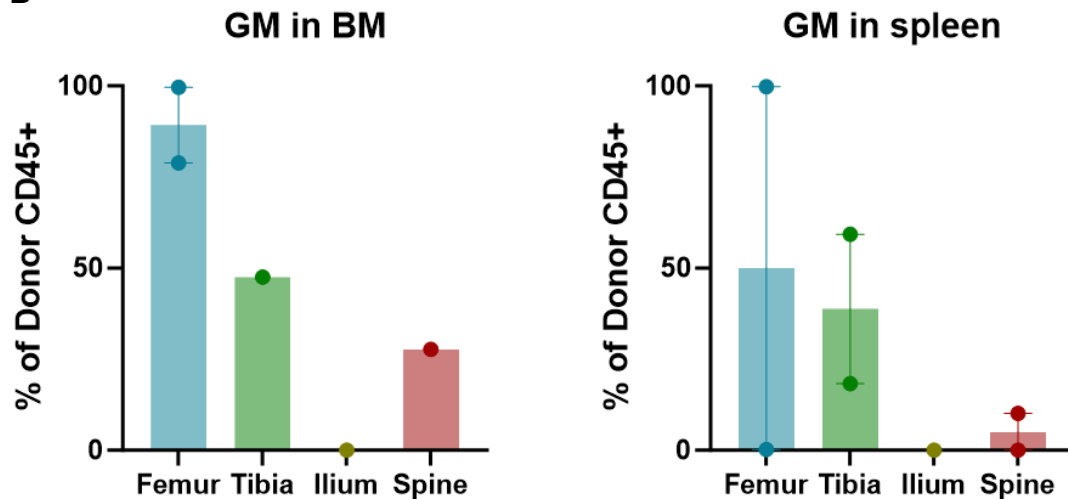


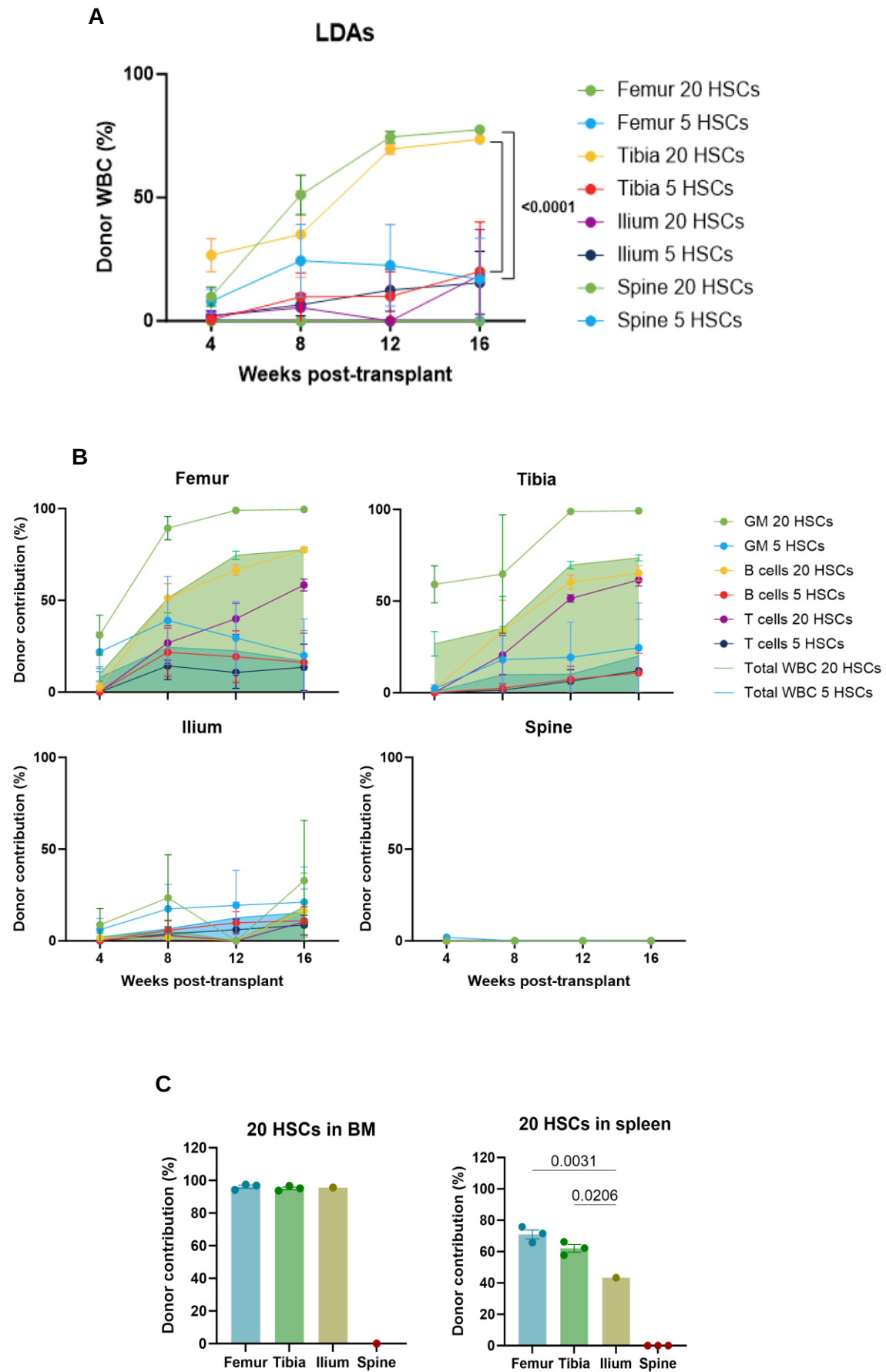
Figure 3.16. Analysis of haematopoietic tissues in secondary transplant recipients. A) Donor contribution to the CD45+ fraction in bone marrow (left) and spleen (right) of recipients. Femur and tibia recipients displayed greater proportion of donor-derived CD45+ cells among each of the recipient groups (recipients of femur, tibia, ilium or spine HSCs), supporting the findings from single-cell transplantation assays. B) Donor-derived GM fraction in bone marrow (left) and spleen (right) of recipients. Femur-derived primary samples made the highest contribution to the GM fraction in the bone marrow, while the highest donor-derived GM fraction was observed in tibia HSC secondary recipients.

3.4.3 Limiting dilution assays affirmed the long-term self-renewal potential of femur and tibia-derived HSCs

Throughout the next section, I will describe how the frequency of long-term reconstituting HSCs was determined through limiting dilution assays (LDAs) (Figure 3.17). The experimental design of these assays consisted mainly in testing two small HSC numbers in myeloablated recipients: 20 and 5 ESLAM HSCs injected per recipient, so that we could then perform an extreme limiting dilution assay (ELDA) calculation of HSC frequency together with the single-cell transplant data. A total of 32 recipients were transplanted, distributed as follows: 5 recipients per bone in the 5-HSC group, and 3 recipients per bone in the 20-HSC group. All recipients were repopulated by donor HSCs, with variable levels of chimaerism, in the femur, tibia and ilium 20- and 5-HSC groups. None of the spine HSC recipients were repopulated, which further supported our earlier observations from the single-cell transplantations, as well as the molecular profiling data, in which spine HSCs displayed lower scores for functional gene expression signatures, namely, MoIO and RepopSig signatures.

Chimaerism levels of both femur and tibia in the 20-HSC group were significantly higher at 16 weeks than for ilium within the same group ($p = 0.0088$ and $p = 0.013$, respectively); contrastingly, no significant differences were observed in the 5-HSC group across bones (Figure 3.17A). When contemplating the contribution of each group to each individual WBC fraction (Figure 3.17B), donor-derived granulo-myeloid cells accounted for 100% of the total found in the blood of femur and tibia 20-HSC recipients at 16 weeks, which progressively and stably increased from the 4-week timepoint, while also significantly contributing to B- and T-lymphocyte production throughout the duration of the experiment (Figure 3.17B). Therefore, femur and tibia-derived HSCs indeed possess a more durable self-renewal potential than do HSCs from the ilium and spine. These data also supported the α and β differentiation patterns inferred from single-cell transplant data of femur and tibia-derived repopulating HSCs, as they also possess higher multilineage reconstitution capacity than ilium and spine.

Analysis of donor chimaerism in the BM and spleen of recipients was performed immediately after the 16-week timepoint. Donor chimaerism in BM of 20-HSC recipients from femur, tibia and ilium was at comparable levels for all BM groups, indicating that, despite differences in multilineage contributions, high doses of HSCs allow for the almost complete reconstitution of the recipient BM. Contrastingly, BM of 5-HSC doses from tibia had no detectable chimaerism. Ilium recipients displayed higher donor-derived BM chimaerism than femur recipients; however, since only 1 ilium recipient in this group was reconstituted, this difference was not statistically significant. In the spleen, a similar trend was observed in femur, tibia and spine 20-HSC recipients, although chimaerism levels were lower than in the bone marrow, but still above 50% for femur and tibia recipients. Intriguingly, tibia recipients still did not exhibit any detectable chimaerism, in contrast to femur and ilium recipients, which indicated that functional HSC frequency in tibia was lower, and they were required to be injected in higher doses in order to repopulate the spleen and marrow of the recipients. Nonetheless, to verify the veracity of this statement, and of all the results obtained from the single-cell and LDA functional assays, I performed an ELDA calculation of HSC frequency in each bone.



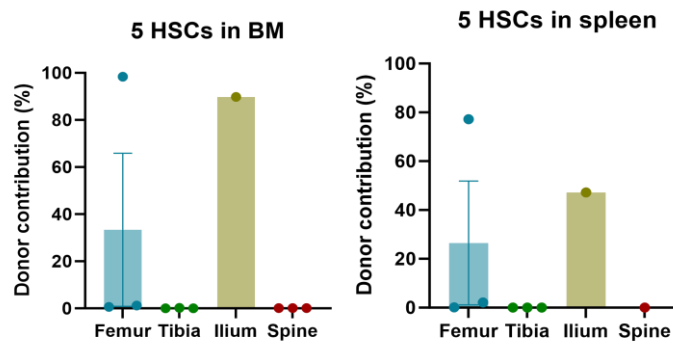


Figure 3.17. Limiting dilution assays of HSCs from different tissues, at two cellular doses: 20 and 5 HSCs per bone. A) Contribution to total circulating WBC in the peripheral blood of recipients. At 16 weeks, relative chimaerism levels were significantly elevated for femur and tibia HSCs in comparison to ilium at the 20-HSC dose. B) Analysis of individual lineage contribution by bone group and HSC dose. In agreement with our single-cell transplant data, animals were successfully engrafted by HSCs from femur and tibia at both doses, with consistent multilineage engraftment from 4 to 16 weeks post-transplant. Relative donor contribution to the myeloid and lymphoid lineages was lower than the overall average for femur and tibia HSCs at both the 5- and 20-HSC doses. None of the spine HSC recipients were reconstituted, at any of the doses tested. C) Analysis of donor chimaerism in the bone marrow and spleen of recipients at the 20- (top) and 5-HSC (bottom) dose. 20 HSCs from femur, tibia and ilium reconstituted the bone marrow at comparable proportions; however, at the 5-HSC dose, only recipients of femur and ilium HSCs showed detectable chimaerism. A similar pattern was observed in the spleen. One-way ANOVA analysis was performed to calculate statistical significance between groups.

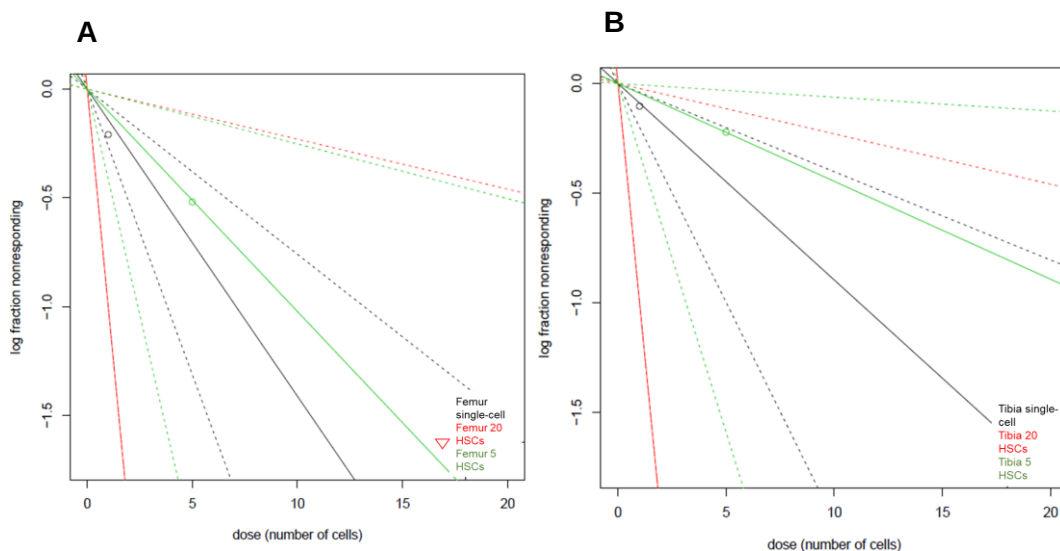
3.4.3.1 ELDA analysis estimated the highest frequency of long-term reconstituting HSCs found in the femur and tibia

Once all functional assays were completed, data were compiled and input into the ELDA software analysis tool (available online at: <https://bioinf.wehi.edu.au/software/elda/>), to estimate the frequency of HSCs in each of the four bone marrow locations profiled throughout this chapter. In total, there were 3 different HSC doses tested: 1, 5, and 20; and four anatomical regions, which equated to 12 experimental groups to analyse (Figure 3.18). First, the analysis was conducted in four groups: femur, tibia, ilium and spine at the three HSC doses tested in each of these groups (Figure 3.18A-D). Frequencies of long-term self-renewing HSCs per 100 cells tested (between single-cell transplants and LDAs) were:

- Femur = 49.7/100 HSCs
- Tibia = 45.8/100 HSCs
- Ilium = 21.4/100 HSCs

- Spine = 3.8/100 HSCs

Indeed, femur and tibia were the anatomical regions in which the largest frequencies of long-term reconstituting HSCs reside, whereas the spine contained the lowest proportion of these cells. To further validate this, a limited dilution model was fit to the data from all different bones at all doses tested. The overall test of differences in stem cell frequencies executed by ELDA (analogous to a one-way ANOVA test) (Hu & Smyth, 2009), demonstrated that HSC frequency was statistically significant across all bone marrow groups ($p = 0.0171$) (Table 3.4). Next, by executing a pairwise comparison between groups, the analysis calculated the highest frequencies of functional HSCs in femur and tibia (20-HSC) group in comparison to the spine ($p = 0.0393$), supporting our previous findings. Albeit no statistically significant differences were calculated when comparing across single-HSC transplant groups, it was clearly evident that the spine contained the least frequency of long-term reconstituting HSCs. Therefore, in accordance with all functional and molecular profiling data, the femur and tibia were the sites where HSCs with durable self-renewal and multilineage contribution potential resided.



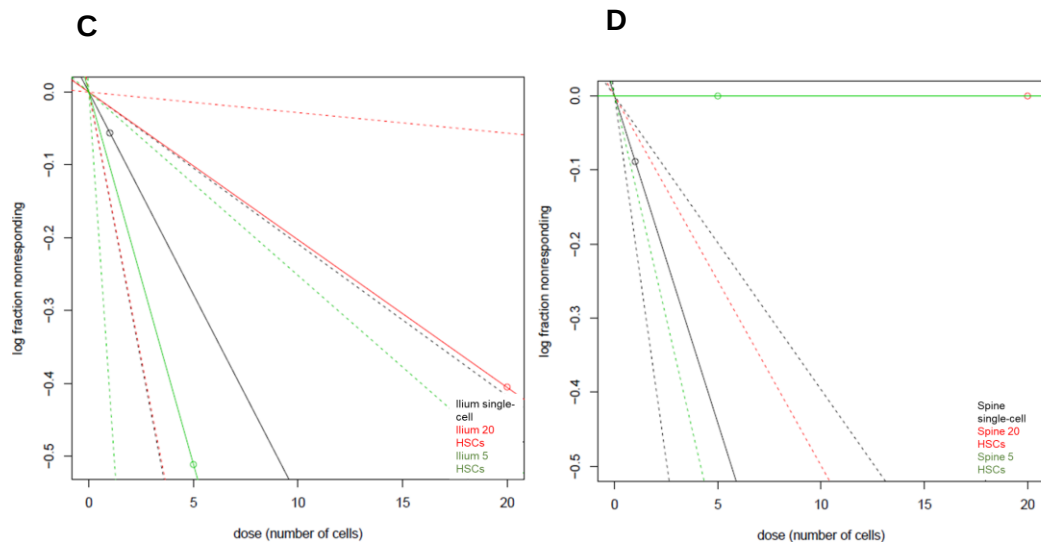


Figure 3.18. Limiting dilution model fitted to data from single-cell, 5- and 20-HSC dose transplant data in femur (A), tibia (B), ilium (C), and spine (D). The natural logarithmic fraction of non-responding (not repopulated mice) was plotted against the different numbers of HSCs tested throughout the transplantation assays. Solid lines (slopes) represent the mean value of the log-active cell fraction (repopulating cells). Dotted lines represent 95% confidence intervals (CI). The down-pointing triangle represents the HSC dose with zero negative values, i.e., all mice were repopulated. HSC frequencies were estimated using the 16-week chimaerism data across experiments for all bone marrow and HSC dose groups.

Table 3.4 ELDA Estimation of HSC frequency

Overall test for differences in stem cell frequencies between any of the groups

Chisq	DF	P.value
23.1	11	0.0171

Pairwise tests for differences in stem cell frequencies

Group 1	Group 2	Chisq	DF	Pr(>Chisq)
Femur_20HSCs	Spine_20HSCs	8.32	1	0.00393
Spine_20HSCs	Tibia_20HSCs	8.32	1	0.00393

3.4.4 Analysis of chimaerism by age of recipient

Due to the particularly large size of the single-cell experiment – where 291 primary recipients were injected across the different experimental dates – age of transplant recipients varied significantly, from 7 to 26 weeks of age. Hence, in order to explore the potential influence of recipient age in chimaerism levels or repopulation success across the four bone marrow HSC groups, age was included in the analysis as a variable which could contribute to differences

in the data. First, I analysed the chimaerism levels at 16 weeks observed in single-cell transplant recipients from all bones, where recipients were divided into two groups: young (7-16 weeks) and mid-aged (17-26 weeks) (Carrelha et al, 2018; Yamamoto et al, 2018; Yamamoto et al, 2013). No statistically significant differences were observed between young and mid-aged recipients of HSCs derived from all bone marrow groups analysed together (Figure 3.19A). Secondly, I analysed HSC contribution to the total WBC fraction at 16 weeks in recipients from femur, tibia and ilium HSCs, grouped by age in single-cell transplants (Figure 3.19B). While tibia HSCs displayed higher chimaerism levels in young recipients than ilium (0.0369) and spine (0.0466) HSCs in the same age group, there were no significant differences between chimaerism levels between age or bone groups.

I then analysed the number of successfully repopulated recipients by HSCs from different bone marrow groups categorised by age, in both single-cell transplants and LDAs (Table 3.5). Femur HSCs repopulated 30% of young recipients and 70% of mid-age recipients in single-cell transplantations. Two-thirds of tibia recipients were categorised as young, while only a third of mid-aged tibia HSC recipients were repopulated. Ilium HSCs maintained a uniform repopulating success rate across young and mid-aged recipients. On the other hand, the majority of repopulated spine HSC recipients were of a young age, while less than 20% of reconstituted recipients were mid-aged. Nonetheless, despite that spine HSCs seemingly performed at a higher efficiency in younger recipients, there were no repopulated animals in LDAs when they were transplanted with any of the different HSC doses (20 or 5 cells) from the spine. Importantly, all LDA transplant recipients were considered young (7-13 weeks old), with only 6 weeks variation between the youngest and oldest recipient. Contrastingly, in LDAs as in single-cell transplants, femur HSCs displayed the highest repopulating rate. Furthermore, tibia HSCs repopulated just over 50% of recipients in LDAs, while ilium HSCs successfully reconstituted over one-third of LDA recipients.

Thus, overall, the femur contained the highest frequency of long-term reconstituting and self-renewing HSCs in both single-cell transplant and LDA experiments, and was able to repopulate both young and mid-age recipients. Whilst spine HSCs successfully reconstituted recipients in single-cell assays were young, none of the young LDA recipients were repopulated, confirming that the spine harbours a low frequency of functional HSCs.

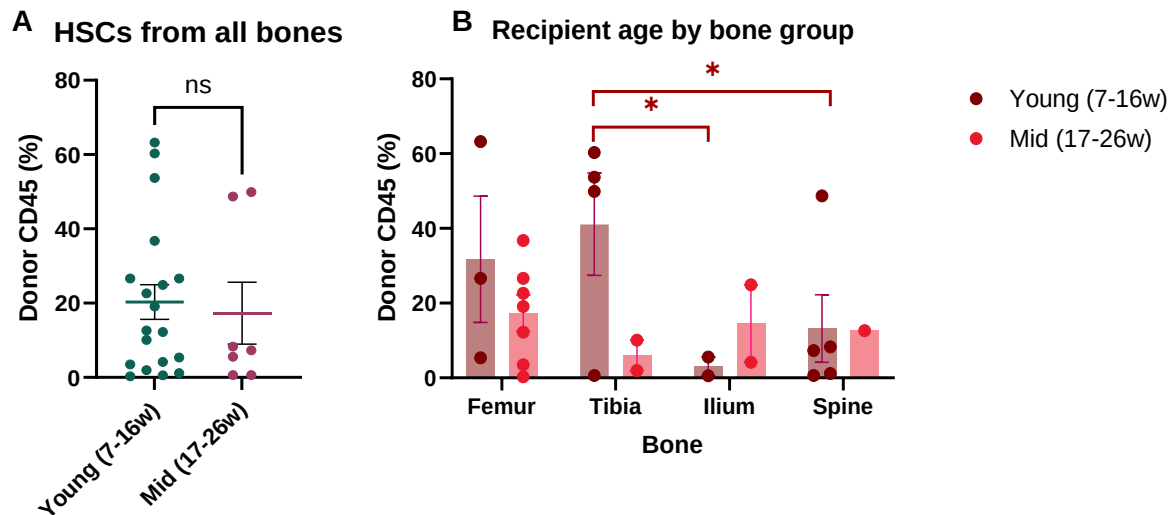


Figure 3.19. Analysis of chimaerism by recipient age in single-cell transplants. A) Chimaerism levels in successfully reconstituted recipients was analysed by dividing recipients into young (7-16 weeks) and mid-aged (17-26 weeks) recipients, taking into consideration recipients of HSCs from all four bones (femur, tibia, ilium and spine). Unpaired *t* test did not show any significant differences between recipient age groups. B) Analysis of chimaerism levels by age and bone marrow HSC group. Statistically significant differences were observed between young recipients of tibia HSCs and young recipients of ilium and spine HSCs. Nevertheless, no significant differences were observed between young and mid-aged recipients for any of the bone marrow HSC groups.

Table 3.5 Successfully repopulated recipient per bone marrow HSC group and age in single-cell transplant and LDA experiments.

Bone	Single-cell		LDA
<i>Successfully repopulated recipients</i>	Young (8-16 weeks)	Mid (17-26 weeks)	Young (7-13 weeks)
Femur	30%	70%	62.5%
Tibia	66.7%	33.3%	53.14%
Ilium	50%	50%	37.5%
Spine	83.3%	16.7	0%

Bone	Single-cell + LDA <i>Successfully repopulated recipients</i>
Femur	17.86%
Tibia	12.82
Ilium	8.53%
Spine	7.8%

3.5 Comparison of molecular profile of paired femur/tibia and ilium/spine groups affirmed the observed functional differences

The comprehensive transcriptomic data analysis, together with the series of transplantation assays, posed the femur and tibia as potentially the two locations in which the majority of long-term reconstituting HSCs reside. Furthermore, in light of the higher MoIO and RepopSig scores that HSCs from these two bones exhibited in our previous data analysis round, we reasoned that there should be global differences in gene expression patterns in femur and tibia HSCs pooled as a single group. We sought to answer this question by comparing the molecular profiles of paired femur/tibia and ilium/spine HSC groupings. For this, we applied the FindMarkers feature of the Seurat analysis pipeline, in order to identify DEG in each of these two groups. The resulting UMAP showed that most of the femur/tibia HSCs were preferentially clustered at the top of the HSC1 cluster that we had identified when analysing differential gene expression across all bones. Ilium/spine HSCs clustered underneath femur/tibia and were more abundant in the HSC2 cluster, suggesting that, pooled as groups, they were molecularly distinct, in a similar manner to when bones are compared individually. Moreover, the distribution of femur/tibia and ilium/spine across the 'small' cluster, further supports the observation that cells with the lower MoIO and Repop scores are found in these cluster, as well as exhibiting expression of mature cell lineage markers, as demonstrated above.

Finally, we sought to profile the expression of the specific genes which could potentially underscore these differences (Figure 3.19 and Table 3.5). The FindMarkers function identified four differentially expressed genes in the femur and tibia, namely, which were: *Rn18s-rs5* (18s RNA, related sequence 5) ($p = 5.01e-11$), *Rpl36* (ribosomal protein L36) ($p = 8.67e-07$), *Mocs1* (molybdenum cofactor synthesis 1) ($p = 1.16e-06$), and *Snx18* (sorting nexin 18) ($p = 1.17e-06$). No DEG were found in the ilium/spine HSC group, reinforcing the results from the Seurat FindAllMarkers analysis, in which HSCs from femur and tibia exclusively exhibited specific HSC gene expression patterns. These results suggested, therefore, that femur and tibia HSCs share molecular and functional similarities, since they both displayed high scores for functionally-defined gene signatures, as well as higher frequencies of long-term self-renewing HSCs. Therefore, these could be classified as the locations harbouring the largest proportions of long-term reconstituting HSCs defined by the most stringent method, serial transplantation, and thorough bioinformatic analysis.

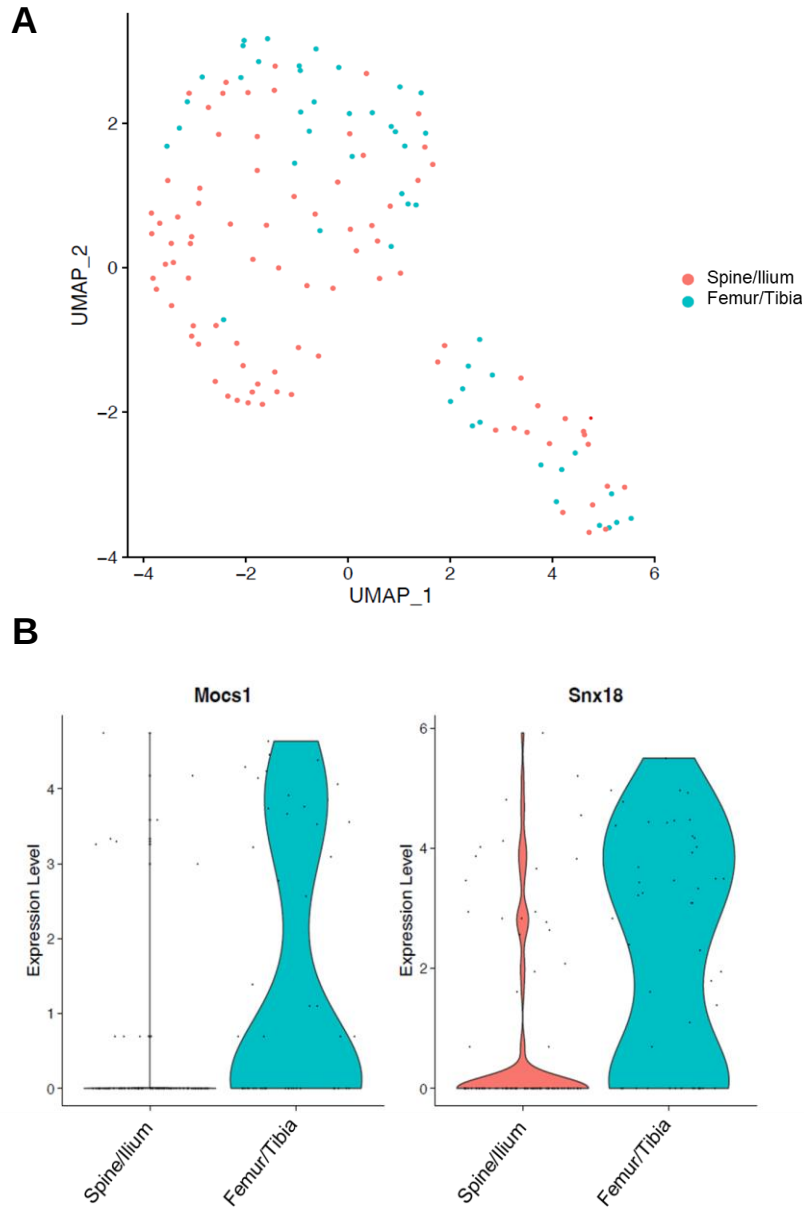


Figure 3.20. Comparison of the molecular profiles of femur/tibia and ilium/spine paired groups. A) UMAP plot of DGE analysis in the two groups, showing a relatively higher proportion of femur/tibia HSCs within the HSC1 cluster, while the HSC2 cluster was more enriched for HSCs from ilium/spine. B) Violin plots of two DE genes in femur/tibia HSCs relative to ilium/spine HSCs. A full list of the genes identified can be found in Table 3.4. The FindMarkers function of the Seurat pipeline was run to perform DE analysis between paired groups.

Table 3.6 Femur-Tibia vs ilium-Spine Comparison							
Marker	Q	avg_log2FC	p_val	pct.1	pct.2	cluster	gene
Rn18s-rs5	7.24E-07	0.433383	5.01E-11	1	1	Femur-tibia	Rn18s-rs5
Rpl36	0.012548	-0.77622	8.67E-07	0.958	0.976	Femur-tibia	Rpl36
Mocs1	0.016762	1.99032	1.16E-06	0.5	0.118	Femur-tibia	Mocs1
Snx18	0.016908	1.358547	1.17E-06	0.688	0.259	Femur-tibia	Snx18

3.6 Summary of findings

Throughout this chapter, I characterised the phylogenetic, molecular and functional features of niche-localised HSCs. In the phylogenetic analysis, murine HSCs were found to accumulate mutations with age, resembling the SBS1 mutational signature, which has been previously described in normal tissues and tumors in adult human patients (Alexandrov et al, 2020). These mutations then served as endogenous barcodes to trace the life histories of single-cell derived HSC clones retrospectively - from the time of tissue collection (which was at 6.7 months of age for our experimental animals) back to the fertilised egg, which was the ancestral cell from which all of the HSCs profiled were derived. Remarkably, there was consistent evidence that HSCs resided within their anatomical site of origin throughout adulthood, and it is likely that this occurred since the time that BM is colonised in embryogenesis, although this will be subject to further analysis of the phylogenetic data.

Having inferred tissue-specific clustering in HSCs, it was possible that clonal relatedness could also translate into unique molecular and functional features specific to HSCs from individual bones. I then moved on to profile gene expression by first comparing HSCs from all four BM locations, unveiling a specific femur and tibia HSC expression pattern, which was found neither in ilium nor spine HSCs. To further verify this, and to be able to predict functional performance, we analysed the gene signature scores for the MoIO and RepopSig, which inform about the repopulating capacity of freshly isolated and *ex vivo* expanded single HSCs, respectively. We found that HSCs from the femur and tibia exhibited higher scores than did HSCs from the ilium and spine. From this stage I then transitioned to assess the geographical implications of HSC origin on their functional performance.

In serial transplantation, femur- and tibia-derived HSCs demonstrated to have the greatest self-renewal and multilineage contribution capabilities, being able to actively contribute to the circulating WBC fraction, and also reconstituting the CD45⁺ cell fraction in BM and spleen of primary and secondary transplant recipients. Differentiation programmes identified in femur and tibia HSCs implied that these cells possess durable, serially transplantable potential, which was later confirmed by LDA assays. ELDA analysis showed that the highest frequency of long-term reconstituting HSCs was found in the femur and tibia (when recipients were transplanted with 20 HSCs). Conversely, the spine was estimated to have the lowest HSC frequency, while no major differences were estimated for ilium HSCs.

3.6.1 Summary of new contributions to the field

1. Residence within location of origin of HSCs derived from different bones, from time of bone marrow colonisation through to adulthood
2. Fundamental functional and molecular differences between HSCs from different bone marrow niches
3. Higher frequencies of long-term self-renewing HSCs in the bone marrow of femur and tibia, and lower frequency in spine bone marrow

3.6.2 Future experiments

1. To repeat single-cell transplantations in a reduced cohort (100-150 mice) using a different LT-HSC isolation strategy by flow cytometry
2. To repeat the scRNA-seq experiments to have at least 100 cells from each bone, adding up to a total of 400 cells, which would increase statistical power. Likewise, this could also circumvent the absence of specific clustering when comparing all four bone groups by dimensionality reduction.
3. To label cells prior to transplantation, in order to trace their progeny across primary and secondary transplants. Additionally, this would allow to identify a potential homing location of preference, i.e., whether donor HSCs from the femur preferentially engraft the femur bone marrow, or to any other location in the body.
4. To confirm that HSCs in fact remain embedded in their tissue of origin, I would reconstruct the phylogenies of a type of progenitor cells, such as megakaryocyte-erythroid progenitors (MEPs) or granulo-myeloid progenitors (GMPs). It is possible that progenitors are more migratory than HSCs since they are more likely to be mobilised in accordance with organismal demands, subsequently strengthening our results.

3.6.3 Potential implications of new insights from this Chapter

The findings presented throughout this chapter now shed light on previously unaddressed questions. First, we have demonstrated that HSCs preferentially reside within the tissue which they colonised at embryogenesis. This discovery could potentially impact how we understand somatic evolution in blood tissue, since the imprinted ‘memory’ in HSCs acquired from their tissue of origin might influence their degree of contribution to total blood output.

Likewise, we have shown fundamental functional differences between HSCs isolated from individual bone marrow locations, contributing to our understanding on the underlying causes for heterogeneity in the HSC pool.

These results could be highly clinically relevant, as HSCs from different anatomical locations might respond in different ways to specific kinds of stress. Haemorrhage could be an interesting example, as mentioned above, since in such extreme situations, HSCs from a particular region could be more actively and more substantially contributing to blood production in response to lost blood volume. Likewise, bone marrow injury or infections (Kelly et al, 2022) activate haematopoietic production, which is likely to be localised given that HSCs in femur and tibia are highly functional.

In addition, a recent review by Yang and colleagues (2024) presents a summary of HSC localisation studies in human subjects. Although the studies presented did not profile the functionality of HSCs isolated from different bone marrow locations, it was found that HSCs have anatomical ‘preferences’ even within the same bone.

Furthermore, molecular profiling of bone marrow cells in mice and humans has been demonstrated to differ between different bones of the skull, consequently exerting different effects on the healthy and diseased central nervous system (Kolabas et al, 2023). Therefore, understanding the cellular composition of the niche in different mouse bones could inform about particular features in bones which result more attractive to HSCs, both in physiological and pathological conditions.

To conclude, this body of work demonstrates that the location of origin has an impact on the clonal relationship between HSCs and their progeny, as well as their molecular features and their performance in serial *in vivo* functional assays. Thus, having understood the relevance of somatic mutations, their sequential acquisition and increased mutational burden with age, I decided to investigate the impact of acquired mutations on the competitive behaviour of HSCs by developing and establishing a novel clonal competition model, which will be the focus of the next chapter.

Chapter 4 - Development of a new *in vitro* clonal competition model and characterisation of new competitor types

Introduction to the chapter

In the previous chapter, I demonstrated the power of naturally acquired mutations as endogenous barcodes, which enable the reconstruction of life histories of single-cell derived clones. However, accumulation of mutations can disrupt the normal cellular processes which sustain and contribute to tissue homeostasis, resulting in the development of cancer. In cancer, genetically mutated cells must impair, suppress and/or outcompete their normal counterparts. This is often associated with uncontrolled proliferation and evasion of the immune response. One of the mechanisms by which cancer cells achieve all of this is through the phenomenon of *cell competition*. This process was originally described in *Drosophila melanogaster* (Bryant & Schneiderman, 1969; Garcia-Bellido & Merriam, 1971), to be then profiled in mouse embryos three decades later (Moreno & Basler, 2004); and eventually, in haematopoietic stem and progenitor cells over the past two decades (Marusyk et al, 2010; Bondar & Medzhitov, 2010). During mammalian embryonic development, cell competition serves as a quality control mechanism for tissue formation, in which defective cells - impairment in differentiation, mitochondrial dysfunction or karyotypic abnormalities - are eliminated through apoptotic cell death by competitor cells (Nichols et al, 2022). As a fitness-selection mechanism, it can be exploited by malignant cells to exterminate their healthy counterparts, thereby enabling the proliferation and expansion of selected mutant clones, which will eventually lead to tumour invasion and migration (Yang et al, 2025).

A substantial fraction of the cell competition models have been developed *in vivo*, including genetic mosaics of *Drosophila* and mouse embryos (Baker et al, 2021), with only a handful of *in vitro* assays to profile the competitive activity of cultured cell lines (Aflalo & Weinstein, 1990). Nevertheless, the crucial role of competition in myeloid malignancies has been highlighted in both pre-leukaemic and leukaemic disorders, as evidence points at the outperformance of healthy clones by their malignant counterparts as a potential major driver of disease onset and evolution (Tsai & Lindsley, 2020).

In this chapter, I present the development and application of a novel *in vitro* clonal competition model, through which I profiled the competitive activity of multiple clone types at large scale, allowing us to perform several independent experiments which were reproducible and produced consistent results. By grounding this model on a split culture approach, we were

able to profile the dynamics of cell competition between wild-type and pre-leukaemic (TET2 LOF) HSCs throughout a 2 stage 28-day expansion period. Collectively, this work represents 300 uniquely expanded and competed clones from each genotype, and, for the first time in the field, led to the identification of a distinct pre-leukaemic competitor cell phenotype which actively suppresses the growth of wild-type cells, termed “TET2-super competitors”.

4.1 Single HSC expansion cultures can be split with homogeneous cellular and molecular outputs

Given that cancer is primarily a genetic disease that originates from a single cell, we set out to understand how single cell-derived pre-leukaemic clones might shape the competitive landscape of HSC clonal dynamics. Our experimental design for this chapter therefore involved a preliminary experiment starting with clonal cultures whereby single clones of one genotype were grown and then put in competition against multiple independent clones of another genotype. To establish this system, we first needed to ensure that the variability between split cultures was minimal. We therefore took, single ESLAM cells from a C57BL/6J female mouse and sorted them into 96-well plates in F12-PVA based 28-day expansion conditions (Wilkinson et al, 2019). At day 14 of culture, single-cell derived clones were split across four wells in equivalent proportions - each new well receiving 25% of the contents from the original well, and the culture was continued until its completion at day 28.

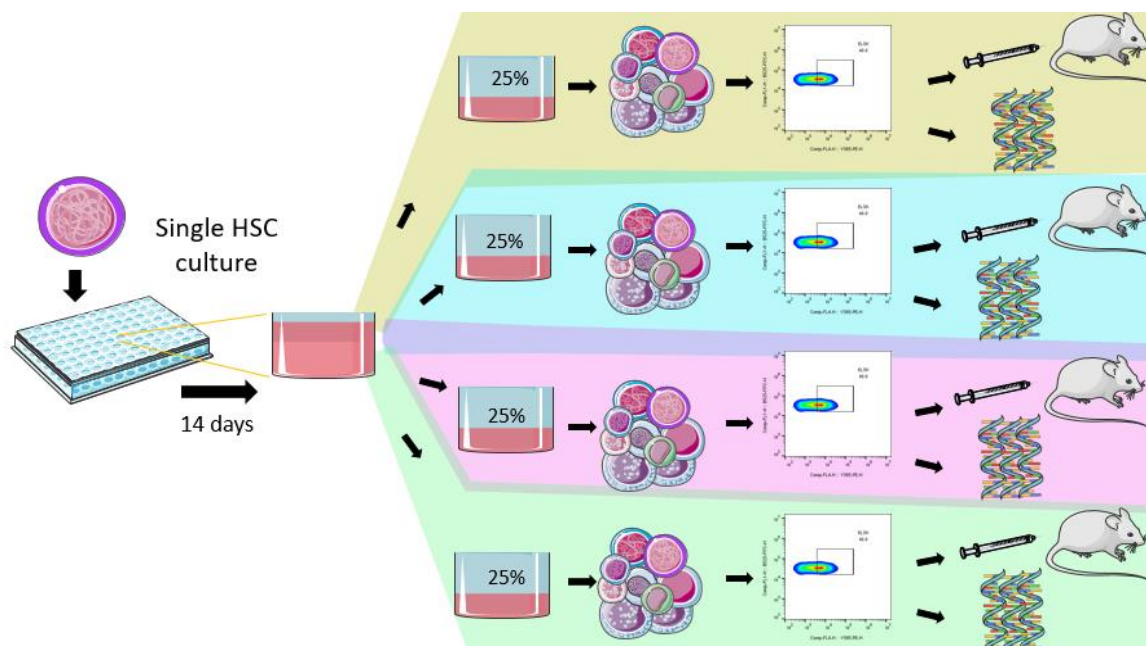


Figure 4.1 - Experimental design for split cultures from single-cell derived clones. Single ESLAM HSCs were isolated from wild-type mice and cultured in F12-PVA based expansion cultures for 14 days. Clones were split into four equal fractions (25% of original well contents); each fraction was transferred and distributed across four wells

in new culture plates, which were expanded for 14 more days. Clones were harvested for phenotypic, molecular and functional profiling at the 28-day endpoint of the culture.

4.1.1 Functional and phenotypic profiling of split clones demonstrated they preserved the properties from the clone they originally derived

To investigate the similarity between split clones, cells were harvested for flow cytometry analysis (25% of well contents), single-cell RNA sequencing (25% of culture) and for *in vivo* functional assays (50% of clone contents) at the end of the culture (Figure 4.2). Flow cytometry analysis showed that split clones shared similar frequencies of phenotypic HSCs (ELSK = EPCR⁺ CD45⁺ Lin⁻ Sca-1⁺ c-Kit⁺), as well as comparable number of viable cells per well (Figure 4.2A and Table 4.1). It has been previously shown that phenotypic HSC content of <20% provides a reliable metric parameter to identify long-term reconstituting cells from *ex vivo* expanded single-cell derived clones (Che et al, 2022). Chimaerism levels at 16 weeks in phenotypic HSC recipients are equivalent to those displayed by recipients of freshly isolated HSCs in limiting dilution assays (Wilkinson et al, 2019). Accordingly, in transplantation, split clones were demonstrated to have roughly equivalent reconstitution capacity in each of their corresponding recipients, i.e., split clones shared similar levels of multilineage reconstitution potential. Interestingly, even though not all clones were able to repopulate the recipient (which normally occurs in HSC transplantation experiments from single cell derived expansion clones) this was a feature which was also shared across other clones within the same split group. Hence, clones which derived from a functionally capable clone preserve the same potential even after splitting, and clones which derived from less functionally capable clones, would not gain any advantage after splitting. This also correlated with the proportion of ELSK cells in each of the split clones - clones with a starting low proportion of ELSK cells would not significantly change for any of the splits, and vice versa - clones with higher content of ELSKs would demonstrate it across all split cultures.

A

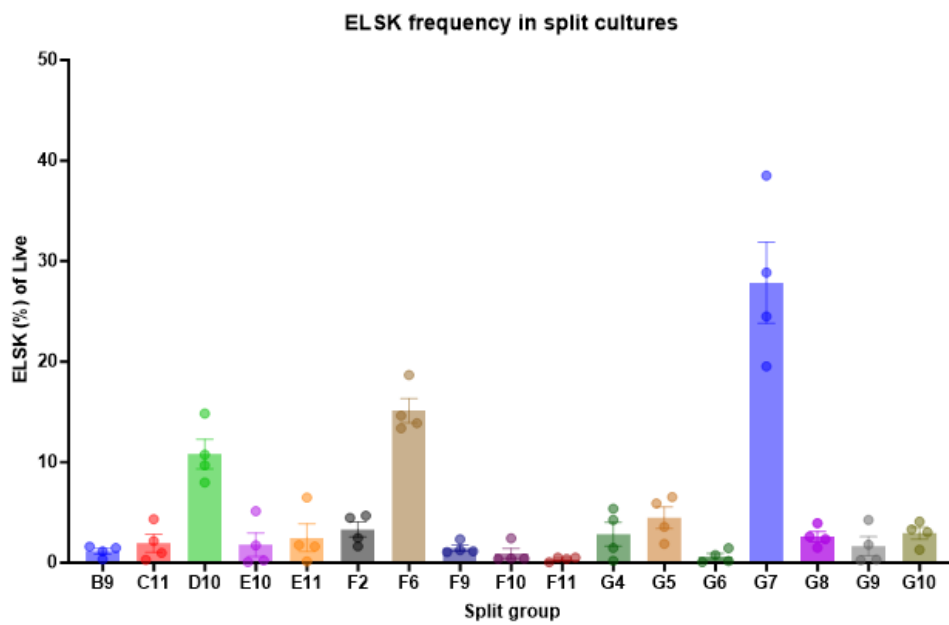
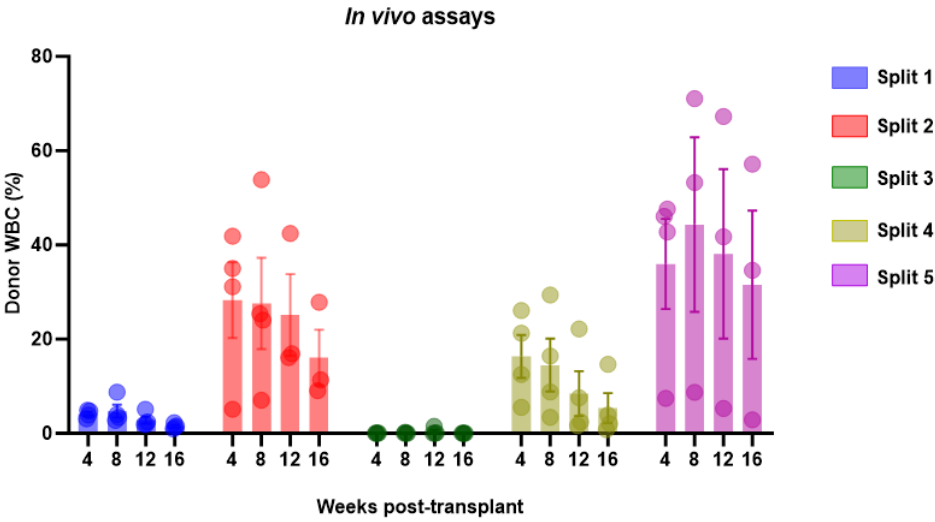


Table 4.1 ELSK frequency - Mean and SD in split clones

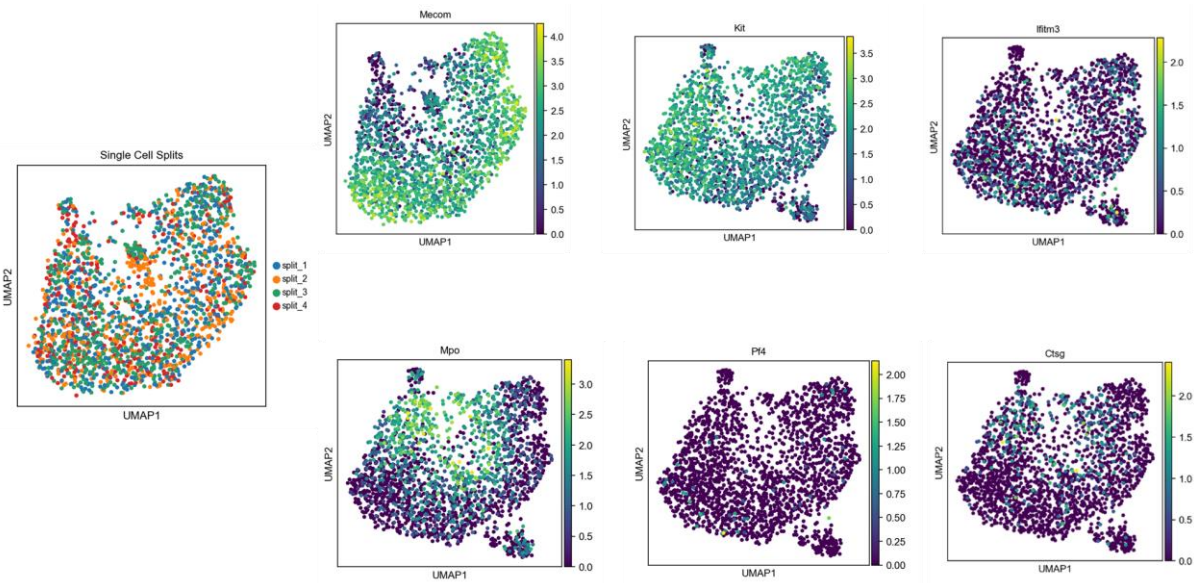
Split clone	Mean	SD
B9	1.143	0.5659
C11	1.943	1.776
D10	10.82	2.913
E10	1.798	2.344
E11	2.5	2.753
F2	3.313	1.5
F6	15.14	2.406
F9	1.453	0.597
F10	0.915	1.01
F11	0.362	0.217
G4	2.83	2.395
G5	4.473	2.148
G6	0.62	0.634

G7	27.8	8.06
G8	2.595	1.006
G9	1.653	1.874
G10	2.933	1.182

B



C



D

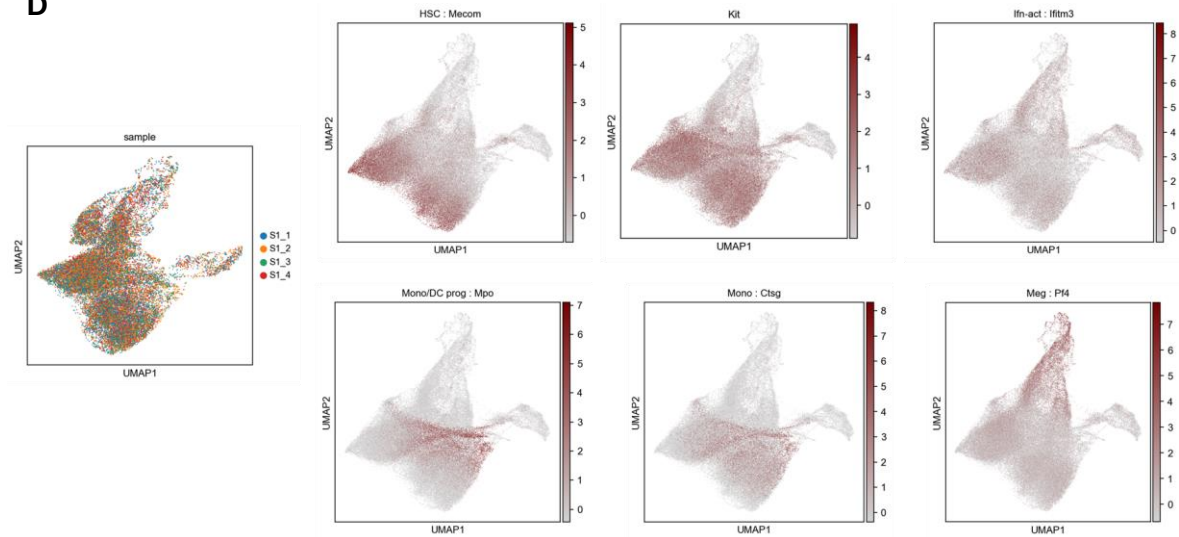


Figure 4.2. Phenotypic, functional and molecular profiling of split cultures. A) ELSK frequency of Live at the 28-day culture endpoint (14 days after splitting), each bar represents a split group derived from a single-cell clone; each dot represents individual splits derived from such clones. B) Relative donor contribution to total white blood cell fraction in recipients of split clones. Each dot represents an independent biological replicate ($n = 3$ per split, 5 split clones transplanted in total). C) Single-cell RNA sequencing data from one split clone. To the left, a UMAP plot shows heterogeneous gene expression across four split clones. To the right, UMAP plots show expression of individual genes: *Mecom*, *Kit*, *Ifitm3*, *Mpo*, *Pf4* and *Ctsg*. Colour scale represent low (blue) to high (yellow) expression values. D) Molecular profiling of split bulk cultures. Data shown corresponds to one split culture (4 splits from that original culture). In a similar way to scRNA-seq data, UMAP to the left displays gene expression across all splits, and UMAPs to the right show individual gene expression, red colour scale represents low (white) to high (red) gene expression. Hence, these data demonstrate the rough molecular equivalency of both clonal and bulk split cultures.

4.1.2 Single-cell and bulk transcriptomic profiling revealed that split clones are molecularly equivalent to their original clone

Next, sets of split clones were molecularly profiled to affirm the results from the *in vitro* and *in vivo* characterisation. Split clones from the sample with the highest ELSK content, which corresponds to well G7 in the original split culture plate, were selected for scRNA-seq, with its four corresponding split clones (Figure 4.2C). Dimensionality reduction analysis was performed on the scRNA-seq data - the results revealed heterogeneity across each of the four splits derived from the original clone, but this heterogeneity was observed in all four split cultures. As an illustrative example, expression of *Mecom*, *Kit*, *Ifitm3*, *Mpo*, *Pf4* and *Ctsg* - which have been previously been reported as important for the identification of HSC in both human and mice (Yokomizo et al, 2022; Shin et al, 2014; Calaminus et al, 2012; Wellington et

al, 2020; Strobl, 1993; Wartman et al, 2012) - was observed to be evenly distributed across samples with all splits displaying similar transcript levels to the original clone (identified as split 1), implying that split clones were similarly comprised regarding cell types and roughly equivalent from a molecular point of view. Further supporting this, is the recent description of *Mecom* in driving HSC functionality (Voit et al, 2022), according nicely with the strong functional performance of all splits derived from this clone in transplantation (Figure 4.2B).

We next broadened our molecular profiling by performing bulk RNA-seq profiling of four split clone groups (n=16 in total), which were collected from a subsequent validation experiment (Figure 4.2D). Heterogeneous gene expression was observed for four splits derived from bulk HSC cultures. Furthermore, when analysing the expression of individual genes, including *Mecom*, *Kit*, *Pf4*, *Ifitm3*, *Mpo*, and *Ctsg* within each sample group, it was shown that each of the split clones had similar expression levels of each gene. This then confirmed that both single-cell and bulk derived split cultures displayed heterogeneous gene expression.

Comprehensive phenotypic, functional and molecular profiling allowed to conclude that split clones were equivalent in phenotypic HSC content, long-term reconstitution capacity and gene expression, respectively, to the clone from which they originally derived. Altogether, these findings highlighted the applicability of this assay to study different aspects of HSC clonal dynamics. Particularly, since it facilitated the profiling of a single-cell derived clone distributed across different wells, it was then suitable to model competition between clones of different genotypes *in vitro*. This then enabled the transition to the next phase of the experiment - the design and execution of clonal competition cultures of wild-type and TET2-mutant HSCs.

4.2 Cell competition assays using wild-type and TET2-mutant derived clones

Having demonstrated that split clones maintain the phenotypic, molecular and functional properties of their clone of origin, the method was next optimised with the aim of designing a novel *in vitro* model of clonal competition, which has not yet been reported for studying competitiveness of HSC clones carrying different mutations. The experimental setup consisted of single-cell initiated cultures, separately sorting single ESLAM HSCs from wild-type (CD45.1) and TET2 KO (CD45.2) mice into 96-well plates in order to expand them for the first 14 days of F12-PVA-based expansion conditions. (Figure 4.3). Cloning efficiency - the formation of clones in each of the wells where a single HSC was sorted - was verified by examining the plates under the microscope prior to splitting and combining wild-type and TET2 clones. In addition, this time, a 'competition matrix' was designed, in which wild-type and TET2^{-/-} HSC clones were split and mixed in equal proportions: individual clones from each genotype were

split four ways as in the initial split culture experiments, seeding 25% of the original clone across four wells in a new plate.

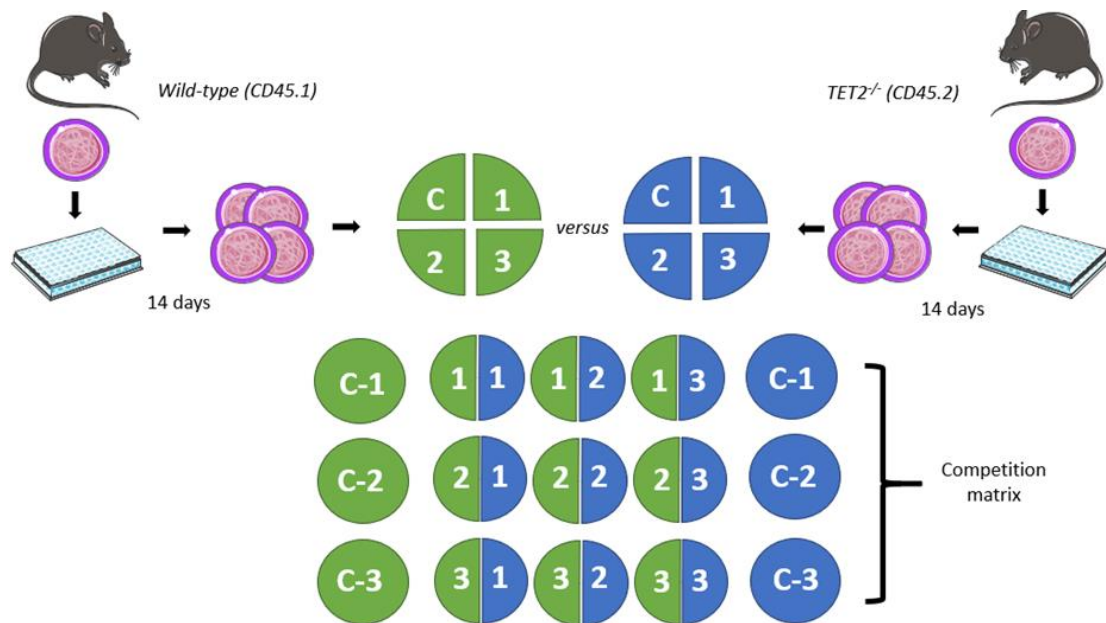


Figure 4.3 - Experimental design of a clonal competition model. Single ESLAM HSCs were isolated from wild-type mice (expressing CD45.1) and TET2^{-/-} mice (expressing CD45.2) and cultured separately in F12-PVA based expansion conditions for 14 days. As with split cultures, clones were fractionated into four equal portions (25% of clone contents each). For competition assays, equivalent fractions (quadrants 1, 2 and 3) of wild-type clones were mixed with TET2 split clones, thus creating unique competition combinations across a 'competition matrix' composed of 9 competition wells, three wild-type and three TET2 unmixed control (represented by quadrants C) clones which did not compete (C1, C2, C3 of wild-type or TET2, respectively). Cells were harvested at the end of the culture for downstream applications (28 days).

Each competition matrix consisted of three control wells of wild-type and three control wells of TET2 split clones, which were not mixed and not set in competition, but were preserved untouched throughout the experiment. The competition wells had an equal proportion of wild-type (50%) and TET2 (50%) clones, i.e., they were mixed at a 1:1 ratio. For the initial experiment, we calculated the number of live cells seeded in each competition well: 5.424×10^3 cells (derived from medium-sized clones) and 1.562×10^4 cells (derived from large-sized clones) were mixed with clones from the other genotype which were equivalent in size. For the subsequent experiments, we continued to use clones of similar size and cell number. Thus, a 3 x 3 competition matrix consisted of nine wells with unique clones competing against each other, plus three control wells of each genotype. After splitting and mixing, the competition cultures were allowed to expand for further 14 days; the total duration of the culture was 28 days (Figure 4.3).

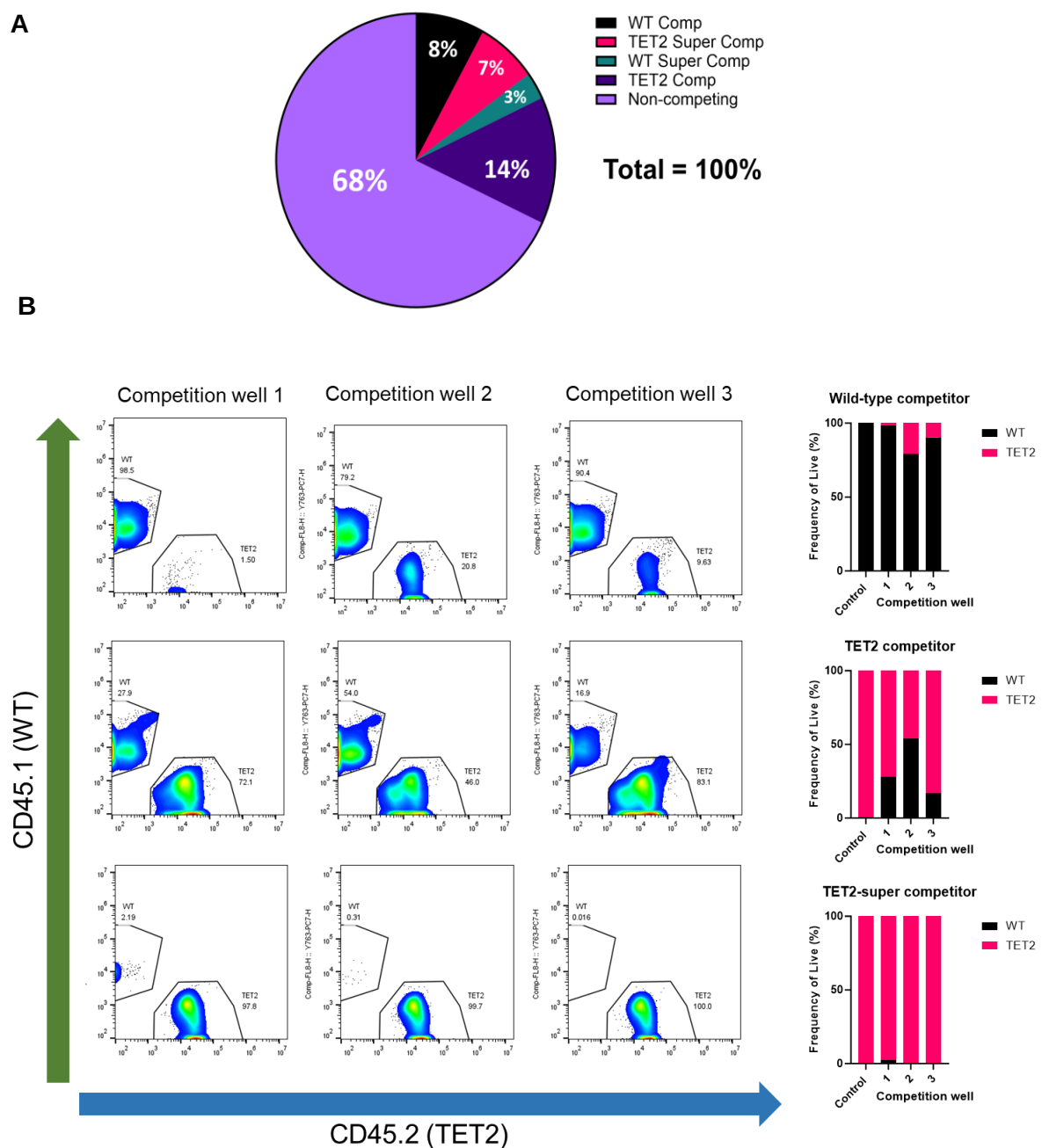
The results presented in this chapter correspond to three independent cell competition experiments. For the first two experiments, I collected and expanded clones for flow cytometry, in which I primarily analysed the proportion of live cells derived from wild-type or TET2 clones in competition wells, ensuring that these frequencies corresponded to the original clones from which they derived. Bulk poly(A) RNA-sequencing was performed on clones from the three experiments. Having thus obtained sufficient data to make more informed decisions, I performed transplantation assays and analysis of HSPC populations in competition cultures (together with the untouched control wells) from clones collected on the third experiment, as well as mass spectrometry analysis of cell culture media supernatants from competitor clones. In this way, phenotypic, molecular, functional and proteomic data were generated and analysed for the different types of competitor clones.

4.2.1 Identification of a TET2-super competitor clone *in vitro*

To verify the feasibility of this new approach, and to characterise the competition dynamics between wild-type and TET2^{-/-} clones, clone composition was analysed by flow cytometry at the end of the culture (Figure 4.4). Assuming that all clones had a relatively comparable competitive ability across genotypes, we would have expected a 50:50 wild-type: TET2 ratio present in competition wells, only if there is no advantage of one genotype over the other. Therefore, the main criteria to determine the competitive advantage of any given clone, be it wild-type or TET2^{-/-}, were: 1) the proportion of total viable cells had to be above 50% across all three competition wells in the matrix for each clone; 2) the control clone from which the competitor derived must be viable (at least 50% viability of total cells); 3) the control clones corresponding to all rival clones for one particular clone must be equally viable (at least 50% viability). I analysed 100 competition matrices in total, their sum equating to 300 wild-type and 300 TET2^{-/-} individual clones, and therefore, 900 unique competition wells in total.

Clones were mostly viable and expanded as normally expected for clonal cultures in the F12-PVA-based system. Despite this, the majority of clones (68%) did not meet the competitor clone criteria defined above (Figure 4.4 A). Overall, 3.7% (n = 11) of wild-type and 7.7% (n = 23) of TET2^{-/-} outgrew their respective counterparts by a 2:1 ratio or higher and were classified as 'Wild-type competitors' or 'TET2 competitors'. Upon closer inspection of the competitor cells, it became clear that the vast majority of TET2^{-/-} competitors had very strongly outcompeted their WT counterparts these clones comprised around 7.3% (n = 22) of the total TET2^{-/-} clones analysed, which were able to outcompete all their respective wild-type rivals within the competition matrix by a 70%:30% proportion, and this was statistically significant (p < 0.0001). Notably, in some instances, wild-type cells were completely purged, with the final

culture containing 100% of TET2 live cells and virtually no detectable wild-type live cells in the competition wells. Nevertheless, controls corresponding to each of the outcompeted wild-type clones were viable and had expanded normally throughout the course of the culture. The unique suppressive capacity of these particular TET2^{-/-} clones led to us terming them '*TET2-super competitors*', denoting the ability to eliminate rival clones, or suppress their expanding capability. Further analysis revealed a small fraction of the wild-type clones (2.3%) which outcompeted the TET2 rival clones by a similar proportion as the TET2 super competitors did ($p < 0.0001$). Nevertheless, these wild-type 'super competitors' did not perform as consistently and effectively as did the TET2-super competitors (Figure 4.4 B).



C

Competition wells

Dot plot showing the frequency of live cells (%) for WT (black dots) and TET2 (pink dots) across three competition wells: WT Comp, TET2 Comp, and TET2 super-comp. The y-axis represents the Frequency of Live (%), ranging from 0 to 100. Horizontal lines indicate the mean and standard deviation for each group. Statistical significance is indicated by p-values above the groups.

Legend: WT (black dots), TET2 (pink dots)

Significance values (p-values):

- WT Comp vs TET2 Comp: <0.0001
- WT Comp vs TET2 super-comp: <0.0001
- TET2 Comp vs TET2 super-comp: <0.0001
- WT Comp vs TET2 super-comp: <0.0001
- WT Comp vs TET2 Comp: 0.0104
- TET2 Comp vs TET2 super-comp: 0.0110
- WT Comp vs TET2 super-comp: <0.0001
- WT Comp vs TET2 Comp: <0.0001
- TET2 Comp vs TET2 super-comp: <0.0001
- WT Comp vs TET2 super-comp: <0.0001

D

Competition wells

Normalized live cell count

WT

TET2

WT Comp

TET2 Comp

TET2 Super Comp

0.0229

0.0071

0.0311

<0.0001

<0.0001

<0.0001

Condition	Strain	Mean Normalized Live Cell Count (approx.)
WT Comp	WT	22,000
	TET2	5,000
TET2 Comp	WT	15,000
	TET2	20,000
TET2 Super Comp	WT	5,000
	TET2	28,000

Figure 4.4 Identification of a new TET2-super competitor clone. A) Proportions of competitor types classified as non-competitors (did not overcome rival clones), wild-type or TET2 competitors (overcame by 50% or more live cell relative frequency), wild-type or TET2 super competitors (overcame by 70% or more). B) Flow cytometric profiles of CD45.1 (wild-type) and CD45.2 (TET2) live cells in a competition matrix, i.e., competition wells 1, 2, and 3. Barplots represent frequency of live of their corresponding competitor type (top: wild-type, middle: TET2, bottom:

TET2-super competitor). TET2-super competitors annihilated their wild-type counterparts consistently and frequently across all experiments. C-D) Live cell frequency (C) and normalised cell count (D) across competition assays in wild-type, TET2 and TET2-super competitor clones. Dotted lines on Live cell frequency (C) set at 70% (red) and 30% (blue) to represent the established cutoffs to define a supercompetitor clone. All TET2-super competitor Live cell frequencies are above the 70% line. Two-way ANOVA was performed to determine statistical significance across groups.

4.2.1.1 TET2-super competitors yielded the largest number of viable cells among all competitor types

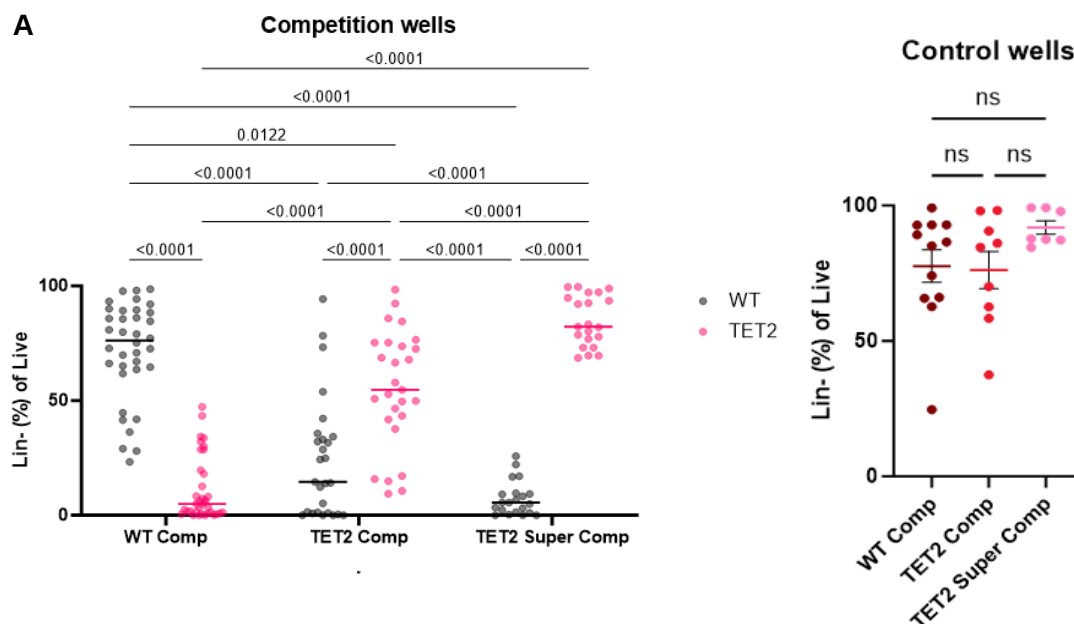
Characterisation of new types of competitor clones requires to determine the total number of cells from each genotype would normally serve as an informative parameter, in addition to supporting data from live cell frequency analysis. Therefore, I then moved on to analyse the total number of viable cells in all competition wells. Normalised cell counts supported the results obtained from the analysis of live cell frequency (Figure 4.4), with significantly higher absolute numbers of TET2-super competitor cells than wild-type cells ($p < 0.0001$), with an average of 3.7×10^4 total TET2-/- cells, in contrast to an average of 4.93×10^3 wild-type cells remaining in competition wells in the presence of a TET2-super competitor. Wild-type competitors, on the other hand, preserved an average of 3.4×10^4 cells in all competition wells, leaving a remnant of 1×10^4 TET2-/- cells. Interestingly, the number of live TET2-super competitors was significantly higher in the competition wells when compared to wild-type competitors ($p = 0.008$). Within the TET2 competitor condition, there were no significant differences in the absolute counts of live cells between wild-type and TET2-/-, with an average of 1.45×10^4 live cells from wild-type and 2.09×10^4 TET2 viable cells in the competition wells, which were significantly lower when compared to wild-type ($p = 0.052$) and TET2-super competitors ($p < 0.0001$).

4.2.1.2 Phenotypic profiling of competition cultures unveils significantly elevated content of phenotypic HSCs (ELSKs) in TET2-super competitors

To dissect the phenotypic profile of each competitor type, the frequencies and numbers of three main populations: Gr-1⁻ CD11b⁻ Lineage-negative (Lin⁻), Lin⁻ Sca-1⁺ c-Kit⁺ (LSK) and EPCR⁺CD45⁺LSK (ELSK) (Figure 4.5) populations were calculated. The replacement of traditional lineage markers such as CD4, CD8, CD45R, TER119, Ly6G/C and CD127 (Wilkinson et al, 2019) with CD11b and Gr-1 within the ELSK strategy has previously been demonstrated to yield phenotypic HSCs (pHSCs) which are able to serially reconstitute transplant recipients (Che et al, 2022).

In every condition, all three types of competitors showed significantly higher numbers of Lin⁻ than their corresponding rival clones ($p < 0.0001$), with TET2-super competitors containing the highest frequency of Lin⁻ cells (average 80% of Live) of all three competitor types (Figure 4.5A). In addition, Lin⁻ frequencies in individual control clones (not set in competition) were fairly similar across wild-type, TET2 and TET2-super competitors, showing no statistically significant differences among the competitors.

Frequencies of LSK and ELSK populations of wild-type cells across all three competition conditions were significantly lower than TET2 competitors or super competitors (Figure 4.5 B-C). Moreover, within the wild-type competitor condition, both wild-type and TET2^{-/-} displayed low frequencies and absolute counts of LSK and ELSK cells. In individual control wells, as well as in competition wells, TET2-super competitors displayed significantly higher counts of the LSK population than the wild-type competitors ($p = 0.045$), as well as a higher proportion than TET2 and wild-type competitors ($p = 0.003$) (Figure 4.5B). When comparing ELSK numbers in control versus competition wells of TET2-super competitors, no significant differences were found, indicating that - in accordance with the principle of phenotypic equivalency of split cultures - ELSK output does not vary between untouched clones and competing clones (Figure 4.5D).



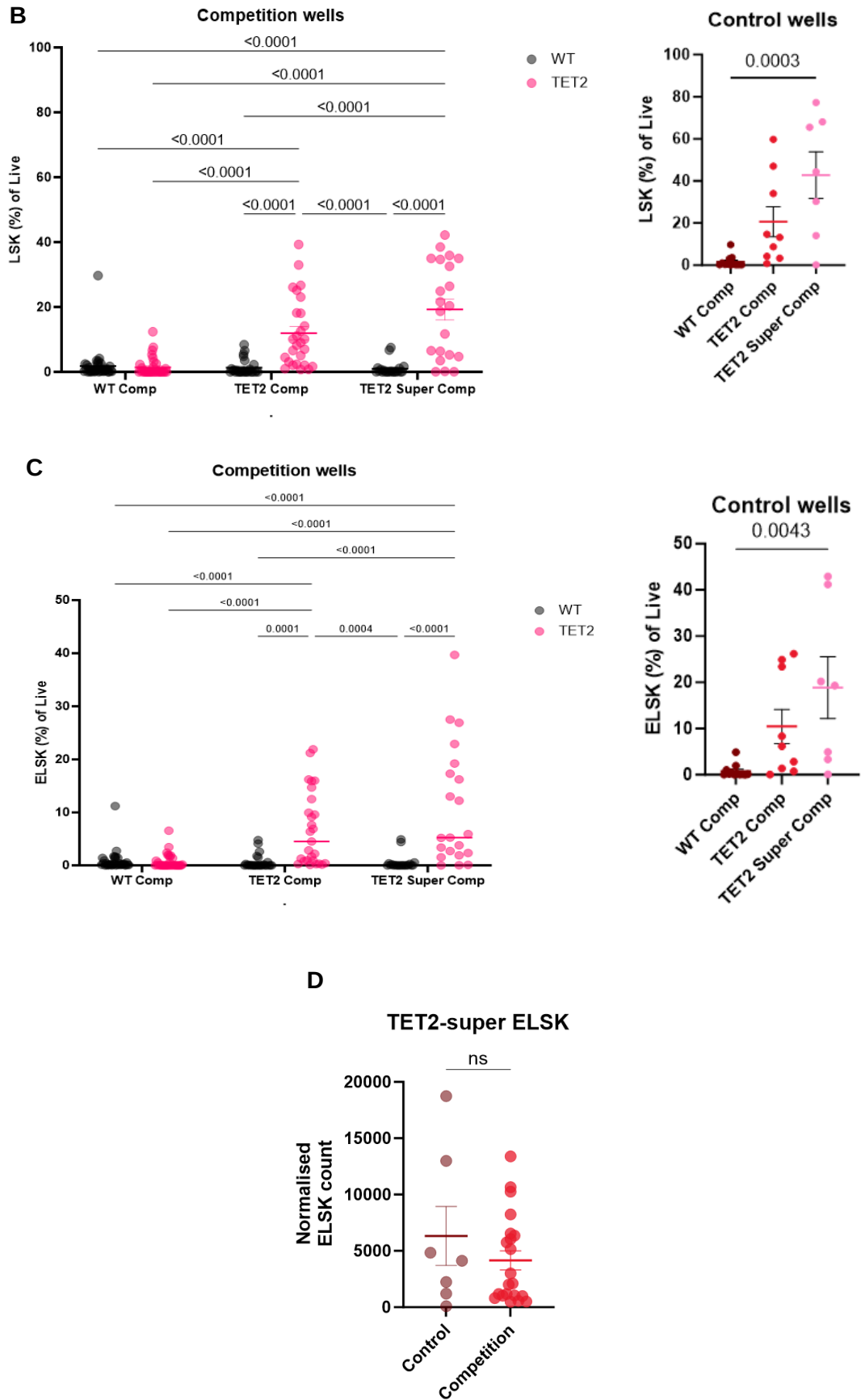


Figure 4.5 Phenotypic analysis of competitor clone composition. A) Frequency of Lin⁻ population of total live cells in competition wells (left) and control (not set in competition) wells (right). B) LSK frequencies in competition (left) and control wells (right). C) Frequency of ELSKs out of live total cells in competition wells (left) and control wells (right). D) Comparison of normalised ELSK number in control versus competition wells of TET2-super competitors, showing no significant differences. Two-way ANOVA analysis was applied to calculate statistical significance when analysing multiple groups in competition wells, and one-way ANOVA was performed to calculate statistical significance for individual groups in control wells.

4.2.1.3 A high ELSK yield was observed for TET2-super competitors in both untouched controls and within the competition matrix

I then performed a comprehensive comparison of ELSK numbers across competitor types in both control and competition wells (Figure 4.6). As expected, TET2-super competitor clones displayed higher numbers of ELSK cells, averaging to 4.9×10^3 cells in the control wells (Figure 4.6C), compared to 3.25×10^3 in TET2 competitors (Figure 4.6B) (no statistically significant difference) and an average of 144 ELSKs in wild-type competitors (Figure 4.6A) ($p = 0.0241$). Within the competition matrix, TET2-super competitor ELSK numbers averaged to 4.15×10^3 cells, which indeed did not differ significantly from the average number of ELSKs found in the control wells, thus displaying uniform numbers between split clones derived from the original control clone. In the same way, wild-type competitors contained an average of 239 ELSKs in competition wells, and TET2 competitors averaged to 1.98×10^3 ELSKs. These numbers, and by consequence, frequencies, were much lower in wild-type competitors when compared to the results from the original wild-type split expansion cultures (Figure 4.2). Average ELSK frequency (out of Live cells) was 1.7% and 0.55% in non-competed control and competition wells of wild-type clones. What was more enticing, however, was the increased ELSK fraction in TET2-super competitors than wild-type ($p < 0.0001$) and TET2 competitors ($p = 0.0005$) (Figure 4.5C), which posed the question of whether the phenomenon of super competition was to be ascribed to intrinsic or extrinsic cell factors.

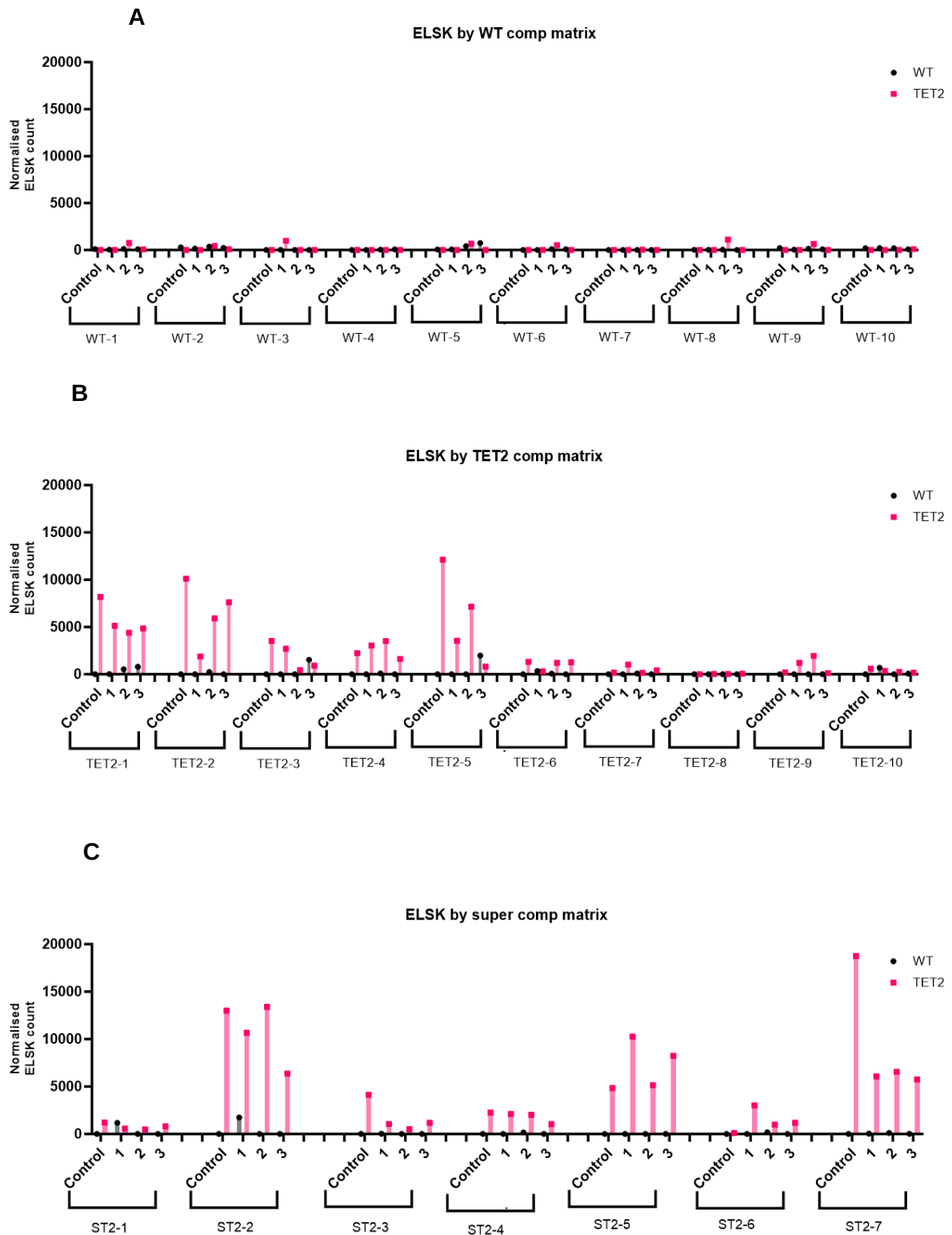


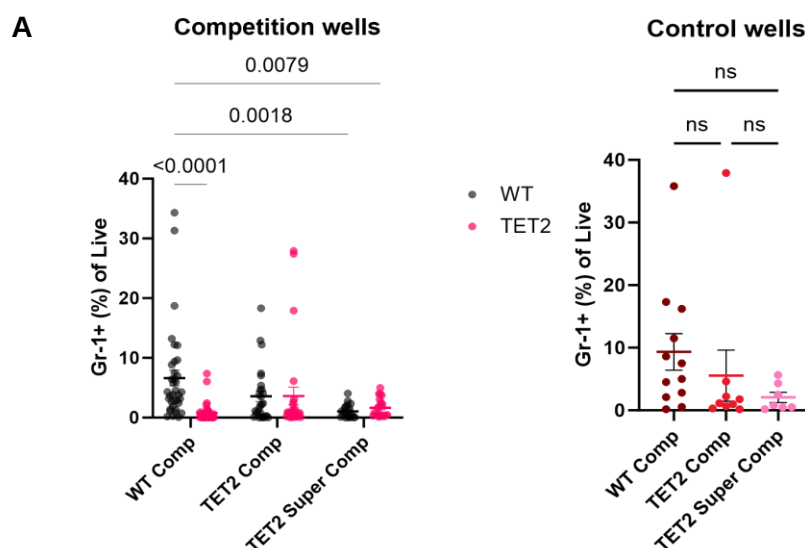
Figure 4.6. Normalised ELSK counts in control and competition wells represented by individual barplots for each clone analysed. Data was organised by competitor type: wild-type (A), TET2 (B) and TET2-super (C). Control = untouched, control well; 1, 2, and 3 = competition wells 1, 2, and 3, respectively. Wild-type ELSK numbers were the lowest among the three types of competitors (144 ELSKs in control and 239 in competition wells). Remarkably, TET2-super competitors displayed the highest number of ELSKs in both control (6.321×10^3 ELSKs)

and competition (4.158×10^3 ELSKs) wells, which were likewise significantly higher than in TET2 competitor clones. Black colour bars represent wild-type and pink bars represent TET2 ELSKs.

4.2.1.4 Analysis of the mature cell fraction in competition and control wells unveiled a higher frequency of Gr-1⁺ granulocytes in competing wild-type clones

To determine if TET2-super competitors were able to maintain a more enriched stem cell pool in the cultures, both in control and competition clones, and to discard the possibility that the evidently larger number of total live cells would result in a larger fraction of differentiated cells, the lineage positive (Lin⁺) fraction was analysed separately (Figure 4.6). Frequencies of single-positive populations - either for Gr-1 (Ly6G/C) or CD11b (Mac-1) - were calculated in competition and in control wells. When set in competition, wild-type clones had the largest proportion of Gr-1⁺ cells, and was significantly higher than the Gr-1⁺ frequency in TET2-super competitors ($p = 0.0079$). No significant differences were observed between competitors in regards to the CD11b⁺ fraction, either in competition or in the control clone wells. These data showed that TET2 and TET2-super competitors were able to maintain the purity of the phenotypic HSC population within both control and competition wells in a more efficient manner than did the wild-type clones, which explains the reduced frequency and number of ELSK cells produced by these clones.

Hence, the fact that TET2-super competitors had a reduced presence of differentiated cells in cultures and an elevated frequency of phenotypic HSCs, suggested that they were likely to display a distinctive functional performance in transplantation assays in comparison to wild-type and TET2 competitor clones. To assess this, I transitioned to the functional profiling of competitor clones, which will be described in the following section.



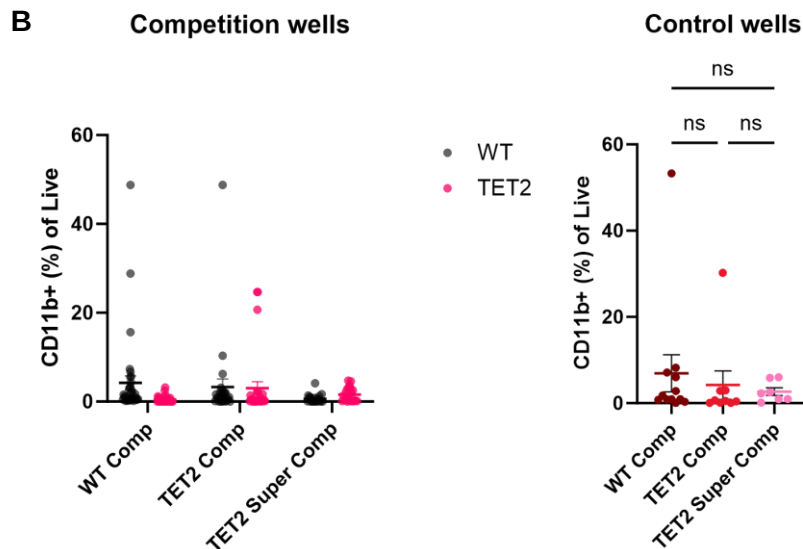


Figure 4.7. Analysis of mature cell populations (Lin⁺) in competitor clones. A) Gr-1⁺ (granulocytes) frequencies calculated out of total live cells in competition (left) and control (right) wells. While wild-type competitors displayed increased Gr-1⁺ cell frequencies in competition relative to TET2 and TET2-super competitors, frequencies remained at comparable levels in the untouched, control clones across competitor types. B) CD11b⁺ (monocytes) frequency of live cells in competition (left) and controls (right). No significant changes were observed in CD11b⁺ frequencies for any of the competitors, either in competition or isolation (controls). Biological replicates of the wild-type (n = 5) and TET2^{-/-} (n = 3) were used for bulk isolation for this experiment. Two-way ANOVA was applied to calculate statistical significance across multiple groups in competition wells, and one-way ANOVA for analysing individual groups in control wells.

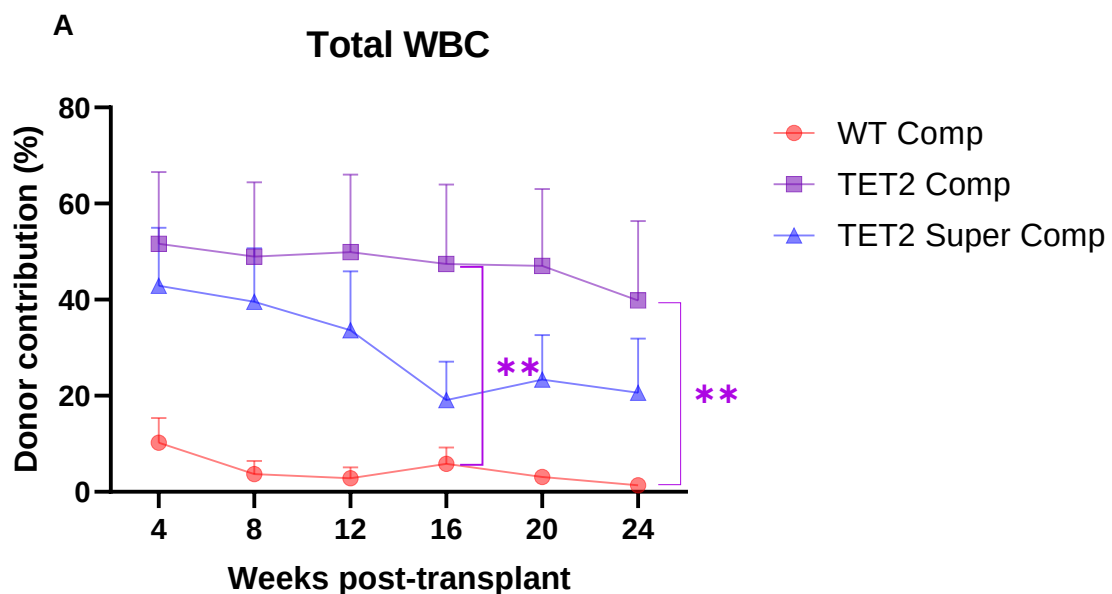
4.3 Transplantation assays of competitor clones revealed that TET2-super competitors did not have significantly increased numbers of functional HSCs

To determine the long-term reconstitution potential of the different types of competitors, *in vivo* transplantations were performed. Once the selection of competitor clones based on the criteria outlined above had been completed, I harvested 30% of the total clone contents of each control corresponding to the selected clones, in order to obtain purely wild-type, TET2 or TET2-super competitor only samples for transplantations. In total, 12 recipients were injected with wild-type competitor clones, 9 recipients with TET2 competitors and 7 recipients with TET2-super competitor clones. Of the 28 recipients, three died due to causes unrelated to the experiment (specifically, cage flooding) at 4 and 12 weeks post-injection (TET2 competitor group) and 16 weeks post-injection (TET2-super competitor group). Peripheral blood samples were routinely collected every four weeks starting at week 4 and concluding at week 24 post-injection to monitor multilineage donor contribution of at least 0.5% to the total circulating white blood cell (WBC) fraction, including granulocytes-monocytes, B and T cells. Overall, 65.5% of

transplanted clones showed multilineage engraftment in recipients: 58.3% of wild-type competitors, 75% of TET2 competitors achieved, and 85.7% of TET2-super competitors (Figure 4.8A).

Regardless of not exhibiting significantly elevated ELSK content in competition cultures, TET2 competitors steadily displayed the highest levels of multilineage engraftment throughout the transplant experiment, achieving its highest peak at 16 weeks, with a very slight decline by week 24 of transplant. TET2-super competitors, in contrast, did not attain such high levels of chimaerism, experiencing a notable decrease 4 months post-transplant. Notwithstanding, these competitor clones were equally capable of sustaining long-term haematopoiesis as the rest of the competitor types, and chimaerism levels were mildly elevated after 16 weeks.

Regarding contribution of the different competitor types to mature, circulating blood lineages, it was found that TET2 and TET2-super competitors displayed significant multilineage engraftment, while wild-type competitor contribution was minimal (Figure 4.8B). For instance, the donor-derived granulo-myeloid (GM, Ly6G⁺ CD11b⁺) fraction was significantly increased in TET2 competitors consistently. Moreover, TET2-super competitor level of B-cell lineage contribution was nearly equivalent to that of TET2 competitors, notably increased at 8 weeks, and then mildly decreasing and stabilising throughout the experiment until 24 weeks. Hence, wild-type competitors were not able to contribute significantly to blood production in the recipients, whilst both the TET2 and TET2-super competitors were. Although TET2 and TET2-super relative contribution did not differ one from the other, TET2 competitors were the better-performing clones in functional transplantation assays overall.



B

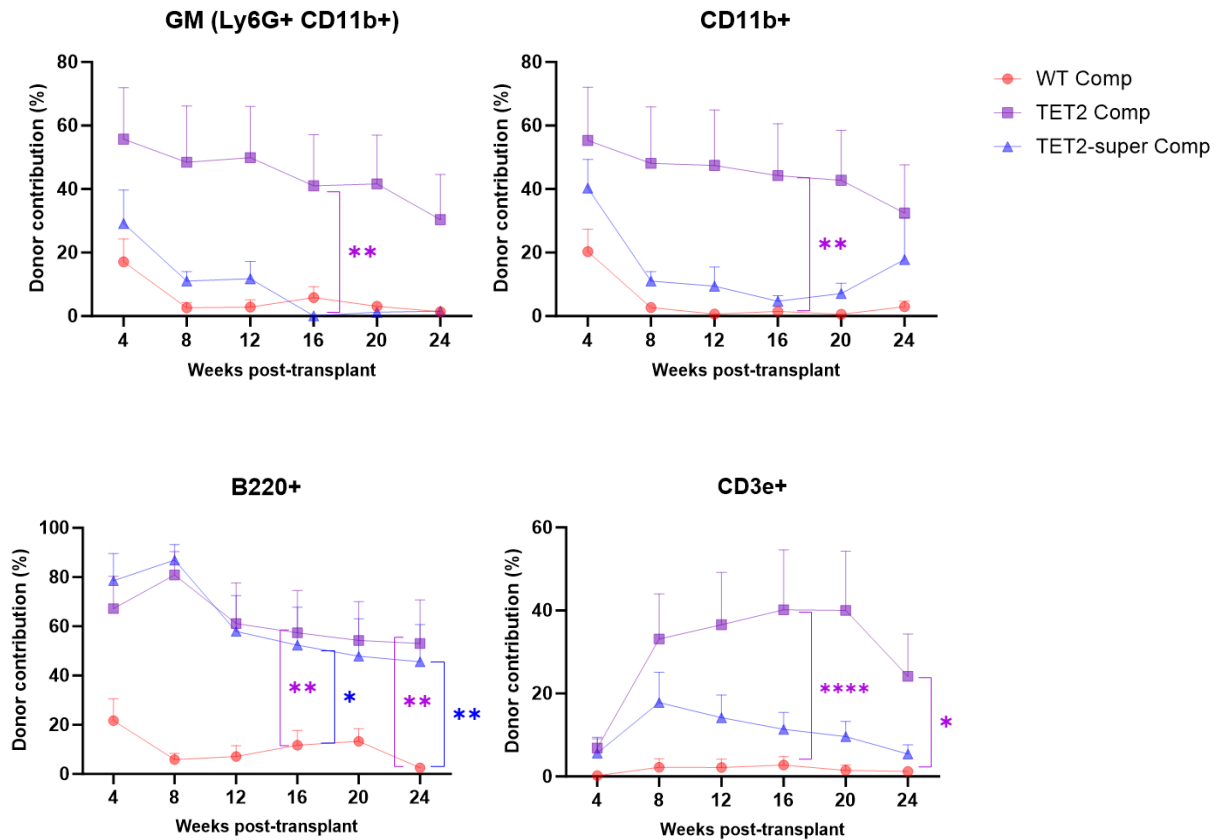


Figure 4.8. Functional assessment of competitor clones. A) Total donor contribution to circulating WBC fraction by competitor type. Comparison of chimaerism levels at 16 weeks revealed significant differences between wild-type and TET2 competitors ($p = 0.0021$) and 24 weeks ($p = 0.0061$). No statistically significant differences were observed between wild-type and TET2-super competitors. B) Frequencies of donor-derived granulo-myeloid (Ly6G+ CD11b+), monocyte (CD11b+), B-cell (B220+) and T-cell (CD3e+) fractions in transplant recipients. Two-way ANOVA tests were applied to calculate significant differences between experimental groups across the different timepoints. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

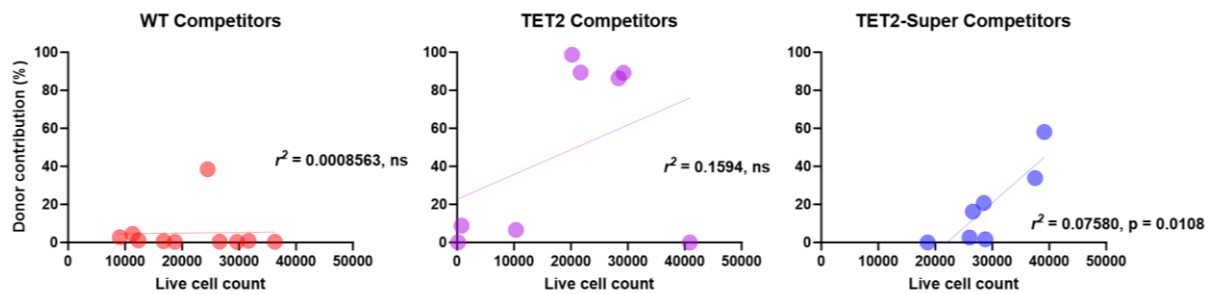
4.3.1 Donor chimaerism significantly correlated with ELSK total cell count in TET2-super competitor clones

It has previously been reported that a frequency of >20% ELSK population out of the Live cell compartment in cultured HSCs is a strong indicator of good functional performance *in vivo* (Che et al, 2022). To validate this statement for the different competitor types, I analysed the correlation between donor chimaerism detected in the recipient mice with three parameters: 1) The total number of Live cells in competition wells, 2) Frequency of CD45.1 (wild-type) or CD45.2 (TET2) cell in competition wells and 3) The total number of ELSK cells in competition wells (Figure 4.9). Indeed, linear regression analysis showed that there was a significant correlation between total live cell number (Figure 4.9A) ($r^2 = 0.07580$, $p = 0.0108$) and total ELSK number (Figure 4.9B) ($r^2 = 0.6798$, $p = 0.0225$) with chimaerism levels at 16 weeks in

TET2-super competitors exclusively. There was no significant correlation between ELSK number and donor chimaerism for wild-type and TET2 competitors.

On the other hand, while there was likewise a stronger correlation between ELSK number and chimaerism in the TET2 competitors ($r^2 = 0.7165$, $p = 0.008$), there was no significant correlation with total number of Live cells (Figure 4.9B). This could have been due to large-sized clones being produced by TET2-super competitors in competition, which at the same time results in increased ELSK numbers overall. Furthermore, as mentioned above, ELSK frequency was significantly higher in TET2-super competitor control wells compared to wild-type competitor controls, in addition to being significantly higher in competition than in wild-type or TET2 competitors. Lastly, in accordance with the phenotypic analysis and results from donor chimaerism in peripheral blood, no significant correlations were found for the wild-type competitors between relative chimaerism levels and live cell (Figure 4.9A) or ELSK count (Figure 4.9B).

A



B

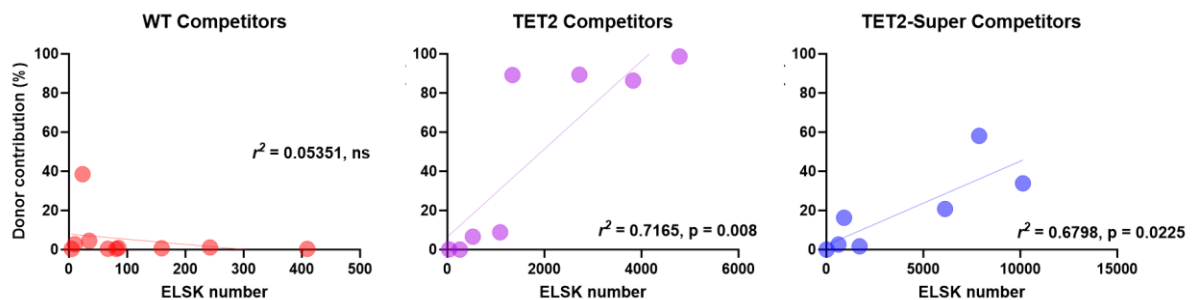


Figure 4.9. Linear regression model to calculate the correlation between relative donor chimaerism with competitor clone Live (A) and ELSK (B) counts. A simple linear regression model was fit to the data for each analysis. A) Correlation between total live cell count and relative donor contribution to total WBC fraction, showing a statistically significant correlation for TET2-super competitors exclusively. B) Linear regression computed a statistically significant correlation between ELSK number and relative donor chimaerism. r^2 , coefficient of determination.

4.3.2 TET2 and TET2-super competitors comparably reconstituted the bone marrow and spleen of recipients

At the end of the transplantation experiment, total chimaerism along with stem/progenitor cell populations (HSC/HSPC) and mature lineages were analysed in two main haematopoietic tissues from the recipients: BM and spleen. As suggested by the peripheral blood analysis results, wild-type competitors showed limited engraftment levels in both tissues, therefore not contributing to any other cell populations analysed. In contrast, donor engraftment in spleen between TET2 and TET2-super competitor clones was notably similar. In the BM, TET2-super average competitor engraftment was 39.58%, almost 2-fold lower than TET2 competitors (average of 62.4% across all recipients), but still significantly elevated relative to wild-type competitors (Figure 4.10A).

Detectable donor chimaerism of the LSK fraction was exclusively observed for TET2 competitors in the BM; however, in the spleen, TET2-super competitors reconstituted a reduced, but important proportion of the LSK fraction (Figure 4.10B). None of the competitor types contributed to the ESLAM fraction, neither in the BM nor the spleen. However, mature lineage output by TET2 and TET2-super competitors in both tissues was comparable, with both contributing significantly to the GM fraction. In a similar way to blood chimaerism data, the frequency of the GM fraction (Figure 4.10C) calculated from donor CD45⁺ cells was significantly elevated in TET2 ($p < 0.0001$) and TET2-super ($p = 0.0034$) competitor recipients in BM and spleen (TET2, $p = 0.0005$; TET2-super, $p = 0.0173$) than in wild-type competitors.

In summary, results from the *in vivo* functional assays from different types of competitors revealed that both TET2 and TET2-super competitors were able to reconstitute the circulating WBC fraction, as well as the BM and spleen of recipient animals. Contrastingly, wild-type competitors did not show the same functional behaviour, which could have been due to the low ELSK numbers detected in wild-type wells at the end of the competition assays, both in untouched controls and in competition wells. In light of these results, we reasoned that the super-competitive behaviour of TET2 clones was plausibly derived from non-cell autonomous mechanisms, i.e., it did not exclusively rely on the high ELSK output in culture, but it must have been supported by another type of mechanism which would result in the elimination of wild-type rival clones. Therefore, I performed molecular profiling of all competitor clones, which will be described in the next section.

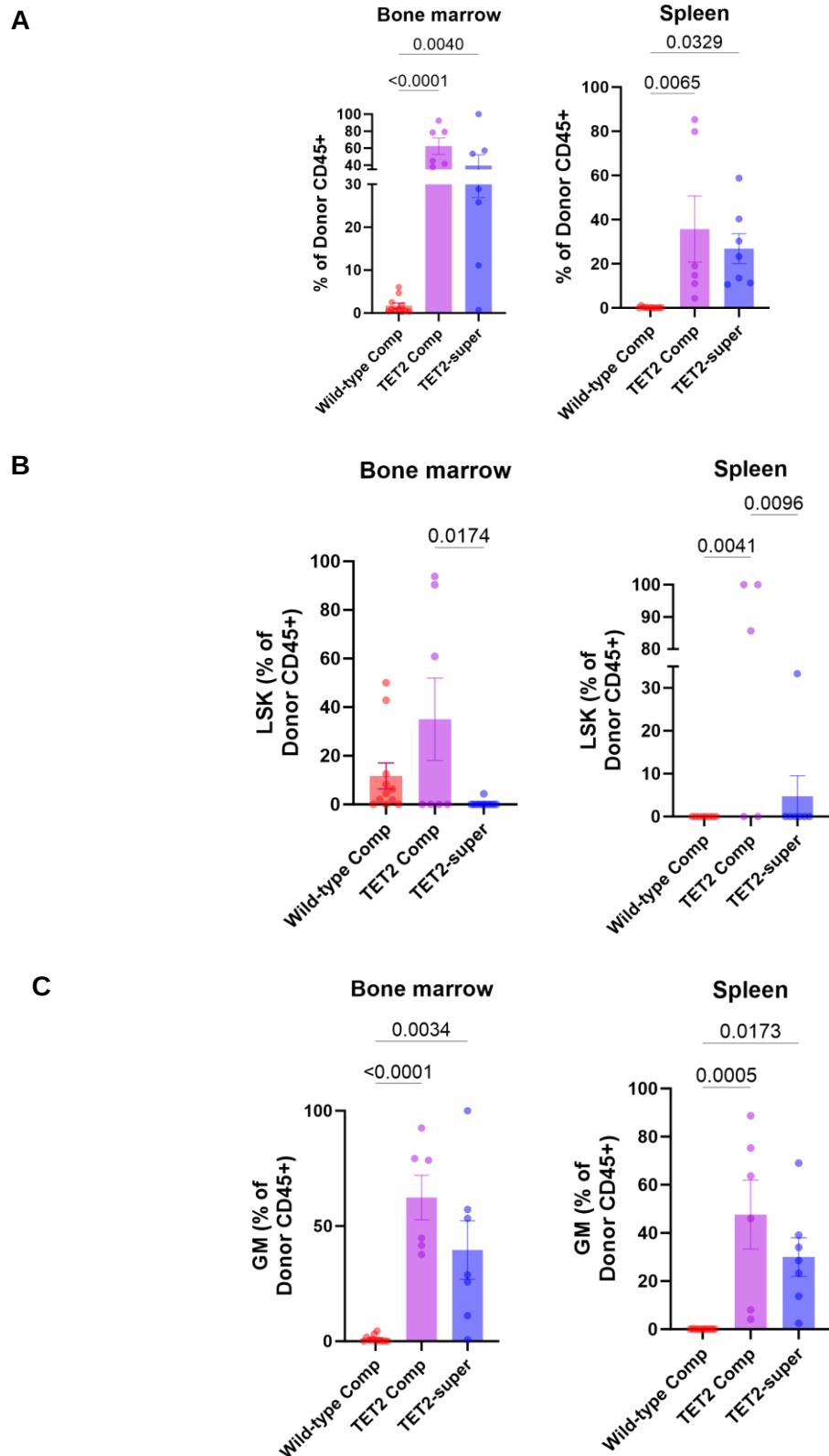


Figure 4.10. Analysis of donor chimaerism in bone marrow and spleen of recipients. A) Relative donor chimaerism in the bone marrow (left) and spleen (right) was higher in recipients of TET2 and TET2-super competitor clones than in wild-type clone recipients, which could be attributed to the low output of ELSKs by wild-type competitors in culture. B) Frequency of donor-derived LSK and C) GM cells in bone marrow and spleen of transplant

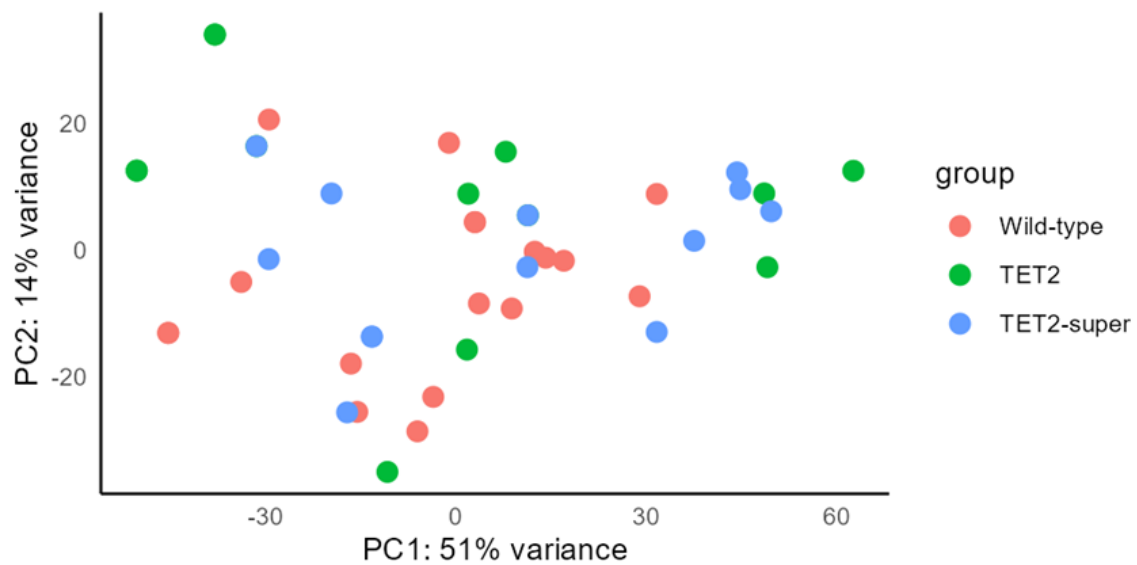
recipients. While TET2 competitors made substantial contributions to the LSK fraction in the bone marrow, neither TET2, nor any other type of competitor was able to reconstitute the LSK population in the spleen. GM frequencies of donor CD45+ cells remained comparable in TET2 and TET2-super competitor recipients on both haematopoietic tissues, while wild-type competitor relative contribution remained the lowest of all three. One-way ANOVA analysis was performed to calculate statistical significance across groups.

4.4 Comparison of molecular profiles between competitor types revealed specific molecular features in the TET2-super competitor clones

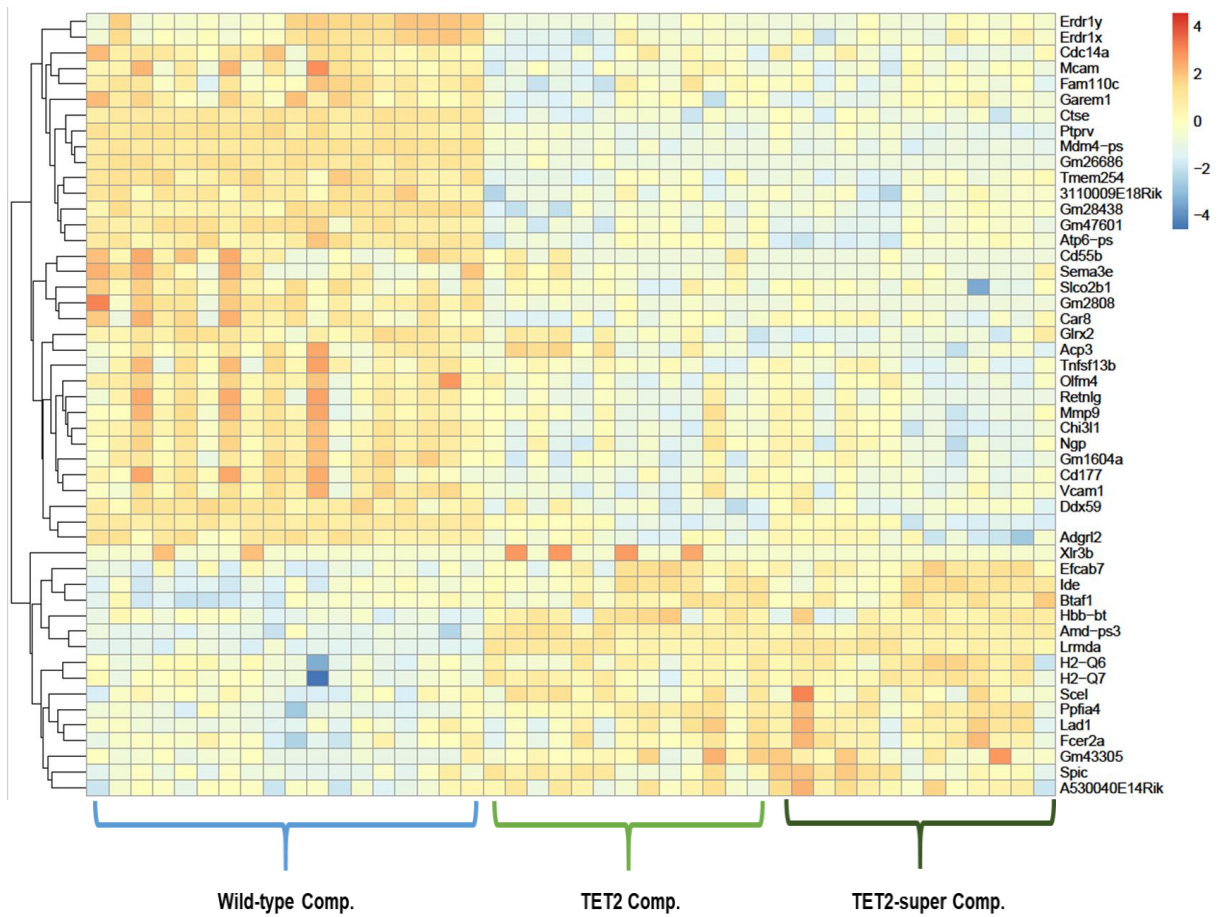
In order to investigate the molecular mechanisms by which the TET2-super competitors were impairing the growth of wild-type HSCs, I performed and analysed bulk molecular data from all three competitor types. After library preparation and quality control assessment, a total of 34 competitor clones were sequenced: 18 wild-type competitors, 13 TET2 competitors and 13 TET2-super competitors, from three independent cell competition experiments. Raw transcript counts were normalised to transcripts per million; batch correction was applied to the data to discard any potential batch effects, as each independent experiment was performed on different dates. No batch effects were found in the dataset across experimental dates. A global PCA analysis revealed no specific clustering of samples by competitor type or by date of the experiment, rather, competitor clones displayed a seemingly scattered distribution (Figure 4.11A). This could have been due to insufficient number of replicates analysed, although it is equally possible that a mixture of cell types contained in the culture wells at the time of sample collection hindered the clustering of samples when performing the dimensionality reduction analysis. Despite the lack of specific clustering by competition subtype in global analyses, differential gene expression analysis was performed to interrogate the transcriptome of each of the competitor types and to make paired comparisons between them. Initially, the DESeq2 experimental design consisted in comparing every competitor type against wild-type competitors as the reference control group. In a first-pass analysis of each competitor type expression profiles, log2-transformed gene counts of the one-hundred most highly expressed genes were filtered from the dataset (Figure 4.11B). Results from this analysis suggested that there was a larger number of highly expressed genes in the wild-type competitors, than in the TET2 and TET2-super competitors. However, when analysing the expression of individual genes detected to be highly expressed in the TET2-super competitors, there were three genes with significantly higher log2-transformed counts: *Lad1* ($p = 0.0020011$ vs wild-type), *Ide* ($p = 0.0000065$), although this was also highly expressed in TET2 ($p = 0.0000210$ vs wild-type), and *Cep85*, which was significantly higher than in both wild-type and TET2 ($p=0.0482932$ vs TET2 and $p=0.0013707$ vs wild-type) (Figure 4.11C). Of note, *Cep85* encodes for the centrosomal protein 85, which although this specific protein has not been directly implicated in HSC function, the centrosome has been shown to have an important role in defining the invaginations which constitute the lobulated shape of early myeloid progenitors (Biedzinski et

al, 2020), as well as being crucial for erythroid cell differentiation (Tatrai & Gegely, 2022). This was suggestive that the super-competitors were likely to express molecules associated with cells of the myeloid lineage. However, most of the top 100 highly expressed genes were found in the wild-type and TET2 competitors, with only a small fraction (described above) of genes present in the TET2-super competitors. Therefore, to formally compare the molecular profiles of competitor clones, I conducted a global gene expression analysis across all samples by competitor type.

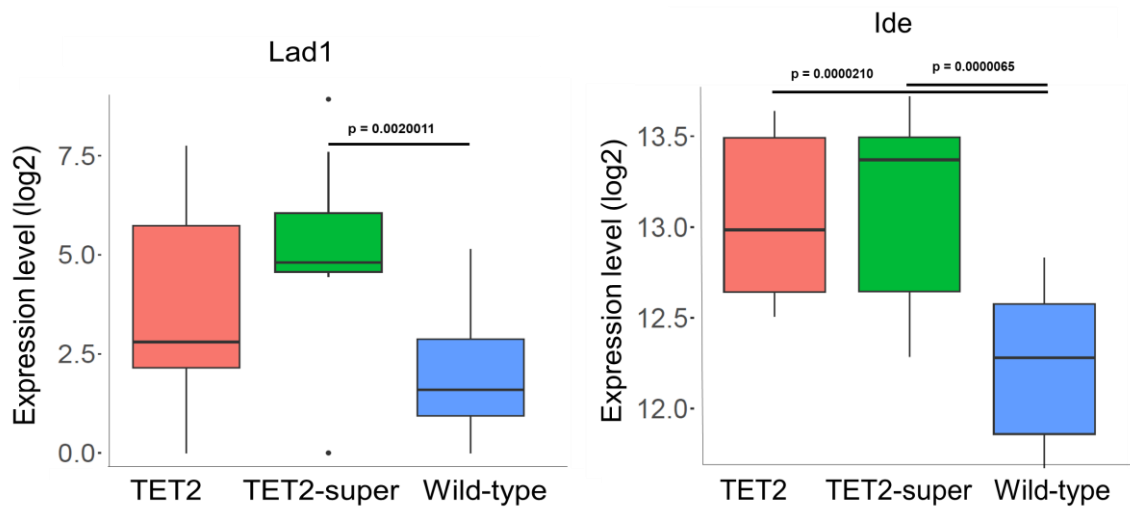
A



B



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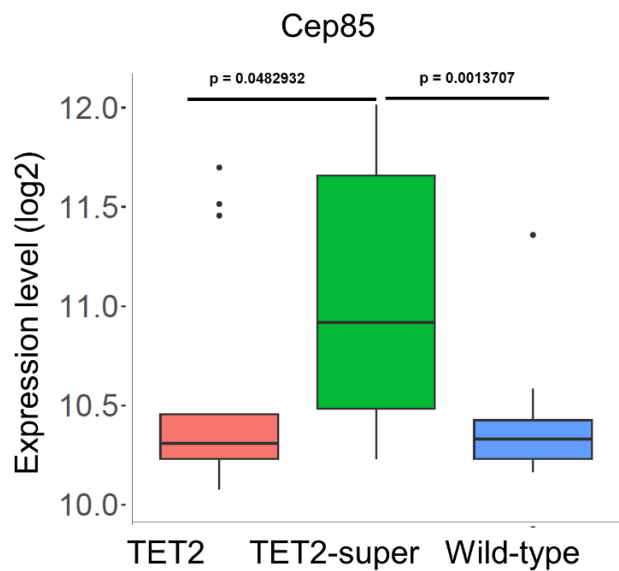


Figure 4.11 RNA-sequencing of competitor clones. A) PCA of clones by type (left) and data of experiment (right), showing the two first principal components. B) Heatmap visualisation of top highly expressed genes across all three competitor types (top 50 genes are shown for visualisation purposes). Red colour scale indicates log2-transformed and normalised gene counts from lowest (blue) to highest (red). C) Boxplots of individual gene expression from log2-transformed data. One-way ANOVA was applied to calculate statistical significance between individual groups. D) Volcano plots of differential gene expression analysis in wild-type versus TET2-super (41 differentially expressed (DE) genes), TET2 versus TET2-super (4 DE genes) and TET2 versus wild-type competitors (166 DE genes).

4.4.1 DGE analysis identified genes implied in structural organisation and cell adhesion in TET2-super competitors versus wild-type competitor clones

A summary of the results from the DGE expression analysis between competitor types is presented in Table 4.2. Notably, of the 41 genes detected in the TET2-super competitors (Figure 4.12A), *Abca6* ($\log_2FC=2.034$, $p=0.006164$) and *Ptcra* ($\log_2FC=3.77$, $p=0.007383$) had likewise been found to be highly expressed when analysing individual log2-transformed gene count data only. However, another member of the ABC transporter family - *Abcg3* was detected among the differentially expressed genes. Two genes related to the structural organisation of the cytoskeleton, *Lad1* - ladinin 1, an anchoring filament protein (Moon et al, 2020) - and *Map7d2* - MAP7 domain containing 2, involved in microtubule organisation in neurons (Kikuchi et al, 2022) - were upregulated in TET2-super competitors ($p = 0.0003$, $\log_2FC = 3.5968$ and $p = 0.04555$, $\log_2FC = 1.3456$, respectively). Interestingly, *Adgrg5* - an adhesion G-protein coupled receptor family II member which interacts with extracellular matrix (ECM) proteins - was also upregulated in the super competitors ($p = 0.014509$, $\log_2FC = 2.4623$). Molecules implicated in immune regulation and function such as Histocompatibility Q regions loci 6 and 7 (H2-Q6 & H2-Q7), *Cd160*, *Cd4*, *Fcer2a* and *Cd209a* (SIGNR-1), as well

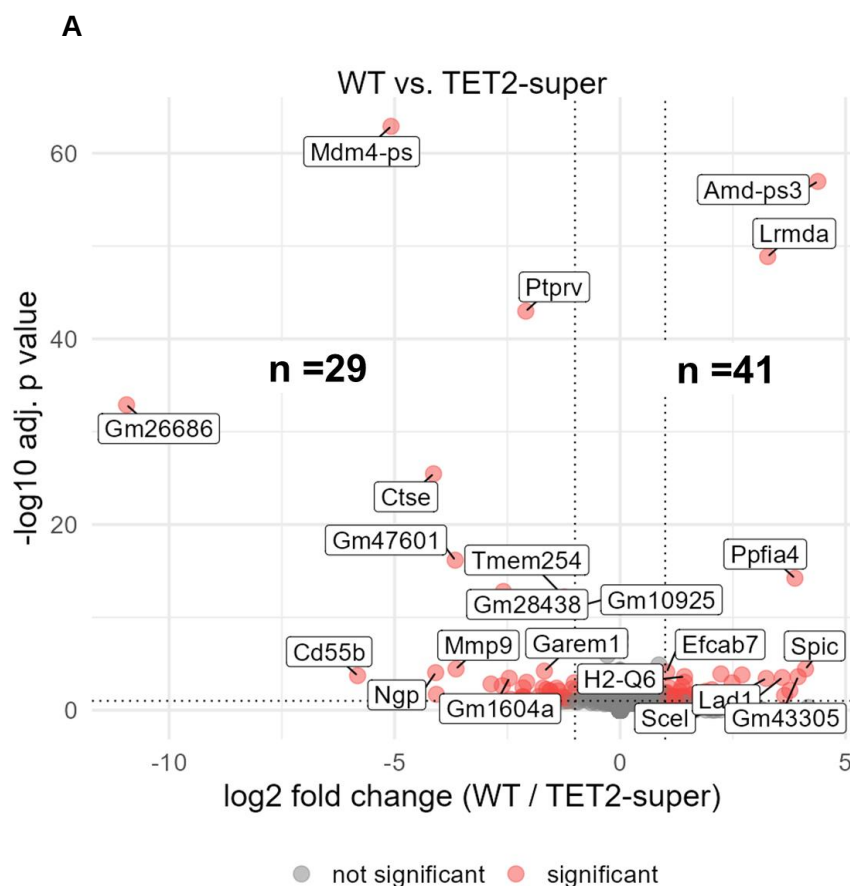
as genes associated with ion binding activity, such as *Efcab7* and *Scel*, which enable calcium ion binding and metal ion binding activity, respectively (Cui et al, 2024; Champlaud et al, 1998).

Table 4.2 RNA-seq analysis of competitor types		
Comparison	DE genes	Total genes identified
WT vs TET2-super	29 (WT), 41 (TET2-super)	20,942
TET2-super vs TET2	4 (TET2-super), 3 (TET2)	20,493
WT vs TET2	100 (WT), 166 (TET2)	20,493
Cutoffs set at: $\log_2$ fold-change > 1, p (adjusted) < 0.05		

Intriguingly, comparison between wild-type and TET2 competitors revealed the upregulation of 166 genes in the TET2 competitors (Figure 4.12C). In contrast, there were only four differentially expressed genes obtained from the comparison between TET2 and TET2-super competitors (Figure 4.12B): the 4930558J18Rik RIKEN project cDNA gene (Bono et al, 2002), which has no ascribed function; *Capn11* (Calpain 11); *Synm* (synemin); and *Ikzf3* (IKAROS family Zinc finger 3), all of which were upregulated in the TET2 super competitors. Analysis of the top 100 expressed genes in TET2 and TET2-super competitors revealed that the largest proportion of highly expressed genes are found in the TET2 competitors, which corresponded to only 22% of genes. Gene expression of only four of these genes was found to be significantly elevated in the TET2-super competitors: *Ikzf3*, *Itgae* (Integrin subunit alpha E), *Sp140l1* (SP140 nuclear body protein Like 1) and *Hoxb3os* (Homeobox B3 and B2, opposite strand).

Next, the Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment analysis (Table 4.3) identified five enriched pathways in TET2 super / wild-type competitor comparison (false discovery rate (FDR) < 0.05), and no pathways enriched in the TET2 super / TET2 competitor comparison (Table 4.3). KEGG pathway enrichment analysis was performed on all genes resulting from the DESeq2 analysis, and no filtered datasets were used. The most highly enriched of these pathways was mmu04514, corresponding to cell adhesion signalling

molecules ($p < 0.000176$, fold enrichment 11.70007), including genes related to the major histocompatibility complex II (H2-Q7 and H2-Q6); *Itgb2l*, the integrin β -2 subunit (*Itgb2l*), also known as leukocyte-associated antigen CD18; junctional adhesion molecule 2 *Jam2* (CD322); and Neogenin-1 *Neo1*, implicated in angiogenesis and cell adhesion (Renders et al, 2021). The latter, however, was shown to be downregulated in TET2-super competitors by DESeq2 differential gene expression analysis. Furthermore, three genes within the programmed cell death protein 1 ligand, PD-L1, pathway were found to be enriched in TET2-super competitors: CD4, *Batf3* and *Tlr9*. Because of the limited number of genes in these pathways, no strong conclusions could be drawn from the molecular data. However, it was clear that the TET2-super competitors had a specific molecular profile, defined by the expression of genes implicated in ECM protein binding, metal ion binding, cellular adhesion and cytoskeleton organisation.



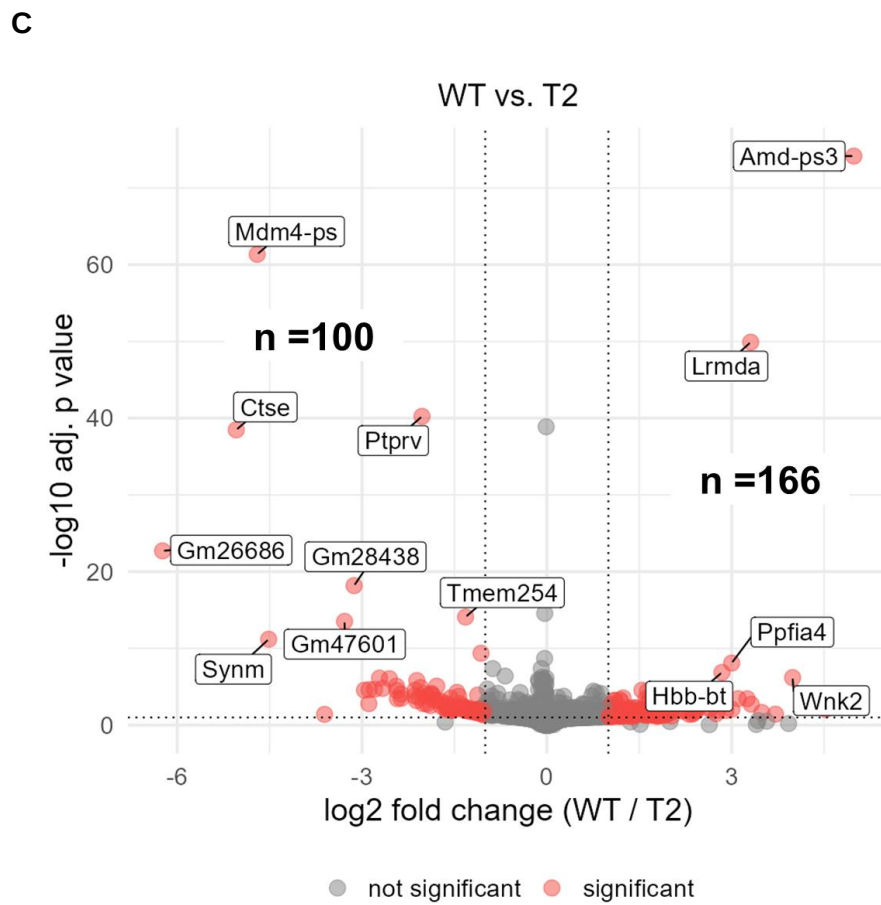
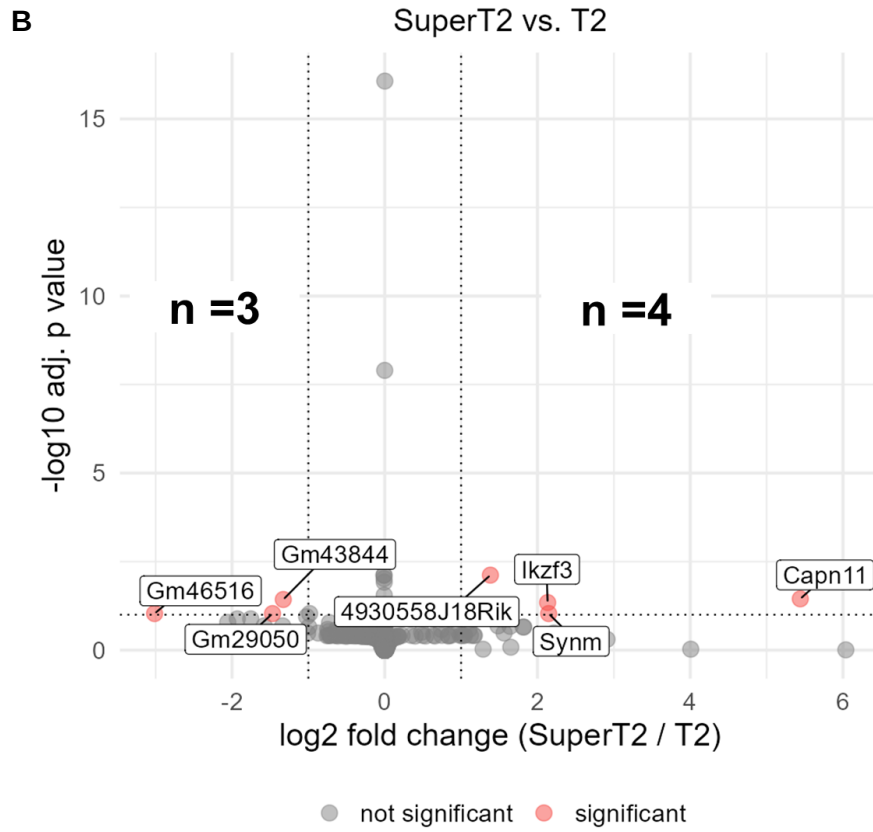


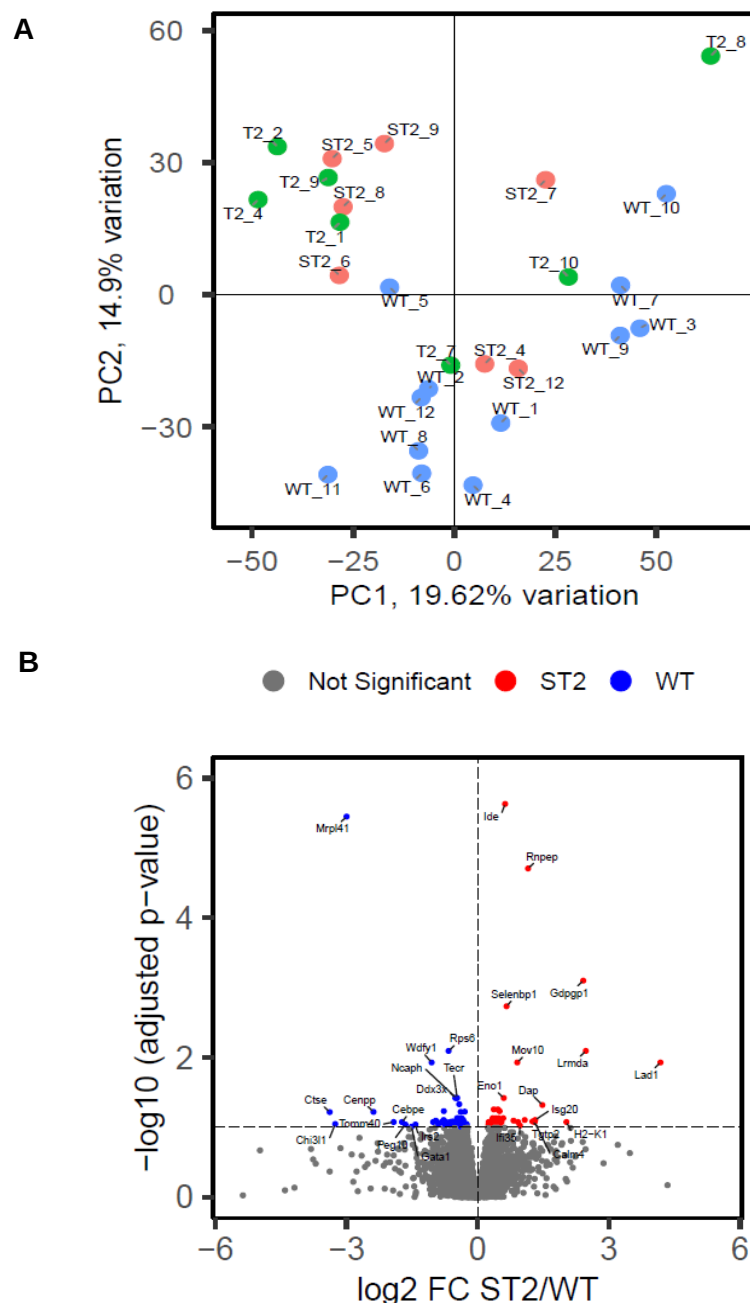
Figure 4.12. Differentially expressed genes in wild-type versus TET2-super (A), TET2-super versus TET2 (B) and wild-type versus TET2 (C) competitor clones. A total of 41 DE genes were identified in TET2-super competitors when compared to wild-type clones. These genes comprised molecules implicated in cell adhesion pathways (*Adgrg5*), ECM binding (laminin-1, integrin β -2 subunit), insulin degradation (insulin-degrading enzyme) and ion binding (scellin). Comparison between TET2-super and TET2 competitors revealed only 4 DE genes in TET2-super competitors, which could be due to a mixture of cell types present in the wells in either of these competitors, resulting in fewer differences at the transcriptome level. The highest number of DE genes was identified in the wild-type versus TET2 competitor comparison. KEGG pathway analysis (Table 4.3) identified 5 enriched pathways in TET2-super competitors in relation to wild-type clones, which supported the DGE analysis findings. DESeq differential gene expression analysis, $\log_2FC < 1$, p (adjusted) < 0.05 .

Table 4.3 KEGG pathway enrichment analysis - TET2-super versus wild-type competitors (FDR < 0.05)								
KEGG ID	category	subcategory	Description	Fold Enrichment	p.adjust	qvalue	DE genes	Gene symbol
mmu04514	Environmental Information Processing	Signaling molecules and interaction	Cell adhesion molecules - Mus musculus (house mouse)	11.70007	0.000176	0.000131	7	Itgb2l, CD4, Neo1, H2-Dma, Jam2, H2-Q7, H2-Q6
mmu05166	Human Diseases	Infectious disease: viral	Human T-cell leukaemia virus 1 infection - Mus musculus (house mouse)	7.267713	0.005133	0.003817	6	Itgb2l, CD4, Neo1, H2-Dma, H2-Q7, H2-Q6
mmu04612	Organismal Systems	Immune system	Antigen processing and presentation - Mus musculus (house mouse)	13.75575	0.005133	0.003817	4	CD4, Neo1, H2-Dma, H2-Q7, H2-Q6
mmu04640	Organismal Systems	Immune system	Hematopoietic cell lineage - Mus musculus (house mouse)	12.73138	0.005534	0.004115	4	Fcer2a, CD4, CD55b, H2-Dma
mmu05235	Human Diseases	Cancer: overview	PD-L1 expression and PD-1 checkpoint pathway in cancer - Mus musculus (house mouse)	10.19957	0.022307	0.016588	3	CD4/Batf3/Tlr9

4.5 DIA-MS analysis of conditioned media from competitor clones was in line with the findings from RNA-seq analysis

Thus far, the molecular and functional data were strongly suggestive of a non-cell autonomous mechanism by which the TET2-super competitors gained massive advantage against wild-

type controls. Therefore, after completing the analysis of RNA-sequencing data, I collected tissue culture media from competitor clones from the third independent cell competition assay. Culture media were analysed by data-independent acquisition (DIA), a global mass spectrometry (MS)-based proteomics methodology (Li et al, 2021). Media supernatant from days 26 and 28 of culture were pooled and profiled together, as clones secrete more proteins into the media at later timepoints in the culture (Daniel Bode, *personal communication*). In total, 26 competitors were profiled: 12 wild-type, 7 TET2 and 7 TET2-super competitors (Figure 4.13). There were no technical issues when running the samples on the timsTOF instrument (see Methods). Between 5500-6000 protein groups were reported across all samples, an indicator of good sample quality.



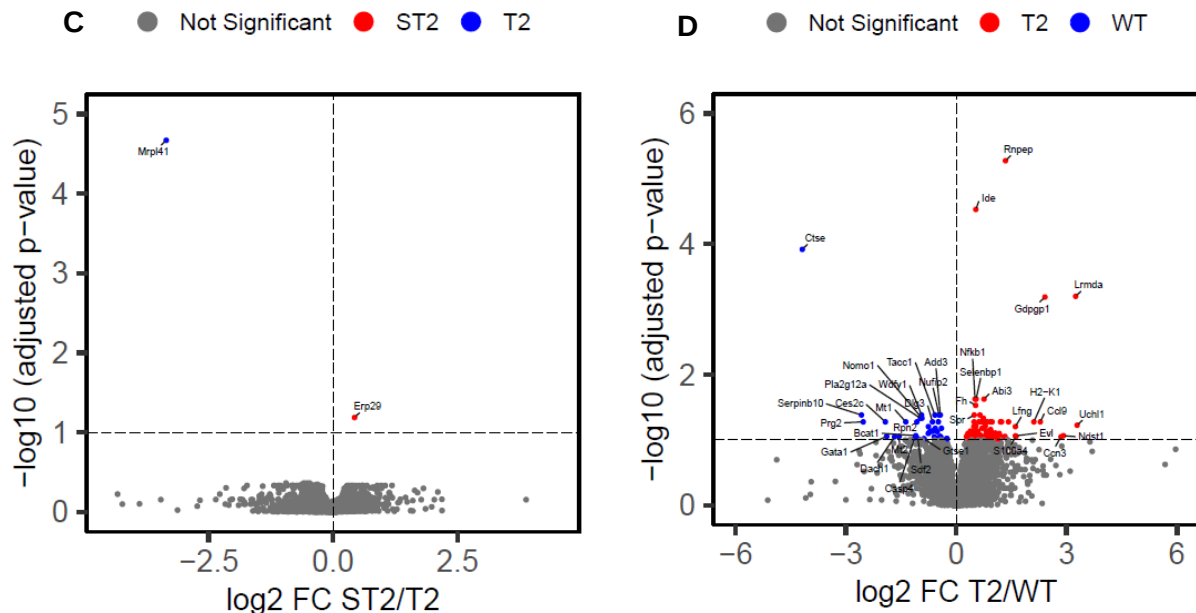


Figure 4.13. DIA-MS analysis of competition culture media. A) PCA loadings plot of the 26 competitor samples profiled (12 wild-type, 7 TET2 and 7 TET2-super), no specific clustering by competitor type was observed. PCA analysis was performed on normalised protein count data by the TMM approach (see Methods). B) Volcano plots representing differentially expressed proteins in: TET2-super versus wild-type (B), TET2-super versus TET2 (C), TET2 versus wild-type (D) competitors. 22 proteins were found to be highly expressed in TET2-super competitors in relation to wild-type competitors, but only one protein was differentially expressed in the supercompetitors compared to TET2 competitors. Despite there being a number of proteins found in both the TET2-super and TET2 competitors when compared to wild-type competitors, unique proteins like ISG20, IFI35, ladinin-1 were detected in the super competitors only.

Prior to analysis, peptide abundance raw data were quantified and then normalised (see Methods section), and so a PCA analysis was performed on the normalised data. Despite the lack of evident clustering of samples, seven of the twelve wild-type clones preferentially clustered together, while the rest of the wild-type clones were widely distributed across the plot (Figure 4.13A). TET2 and TET2-super competitors did not form any specific clustering.

4.5.1 TET2-super competitor clones secreted molecules implicated in insulin metabolism, ECM binding and interferon- γ signalling into culture media

Next, differential protein expression analysis was performed on the normalised data for all competitor clones. Comparison between TET2-super and wild-type competitors revealed the presence of 22 significantly highly expressed proteins in the super competitors with cutoff set at $p < 0.1$, and $\log_2 FC > 0.5$ (Figure 4.13B). Agreeing nicely with the molecular profiling data, ladinin-1 and *Lrmda* (leucine-rich melanocyte differentiation associated) were both highly

expressed in the TET2-super competitors. Moreover, proteins involved in insulin processing (*Ide*, insulin degrading enzyme) and glucose metabolism (GDP-D glucose phosphorylase 1) were also upregulated in the super competitors. Upregulation of selenium binding protein 1 (*Selenbp1*) observed in this dataset, together with high expression of sciellin inferred from the RNA-seq data, suggested that metal ion binding proteins could be a relevant feature of TET2-super competitors. Interferon-stimulated gene 20 kDa protein (*Isg20*) and interferon-induced 35 kDa protein homolog upregulation were also suggestive that interferon signalling might mediate the super competitive behaviour of the TET2 clones. Lastly, as observed in the RNA-seq dataset, two regions (D and K regions) of the histocompatibility 2 class I complex were differentially expressed in the super competitors.

Analysis of differentially expressed proteins between TET2 and TET2-super competitors (Figure 4.13C) revealed that only *Erp29* (endoplasmic reticulum protein 29) was significantly upregulated and *Mrpl41* (mitochondrial ribosomal protein 41) was significantly downregulated ($p > 0.1 \log_2FC < 0.5$). No more proteins with statistically significant expression values were detected in this comparison. In contrast, 15 proteins were differentially expressed between wild-type and TET2 competitors (Figure 4.11D), some of which were also detected in the TET2-super competitors. None of the interferon- induced and stimulated proteins or ECM binding laminin-1 were found to be upregulated in the TET2 competitors, in contrast to super competitors. Notably, *Ctse* (cathepsin e) was found to be downregulated in the wild-type competitors when compared to both TET2 and TET2 super competitors, which is of interest since cathepsin E deficiency has previously been implicated in impaired cell adhesion mechanisms in macrophages (Tsukuba et al, 2009).

Having thus completed comprehensive profiling of competitor clones from phenotypic, functional, molecular and proteomic data, I then moved on to perform functional *in vitro* testing of targets selected from the transcriptomics and proteomics.

4.6 *In vitro* target validation revealed a potential mechanism for TET2-super competitive behaviour by the upregulation of cell adhesion pathways

Considering there were 41 differentially expressed genes and the 22 upregulated proteins detected in the TET2-super competitors in relation to wild-type clones, it was necessary to establish a set of criteria to select targets for *in vitro* testing. First, targets which were found to be upregulated in both the molecular and proteomic data were considered. Second, targets which were identified in the five KEGG enriched pathways from the molecular data analysis. Third, to select out of these targets, those implicated in relevant signalling pathways to myeloid

malignancies. Therefore, since cell adhesion was most highly enriched KEGG pathways in TET2-super competitors, and given that the genes encoding for the Q6 and 7 regions, as well as D and K chains of the histocompatibility class I complex were found to be upregulated in both datasets, this pathway was selected as one of the targets. Since two interferon-stimulated proteins, ISG20 and IFI35, together with the CD160 antigen, which upregulates class II interferon production, interferon signalling was selected as the second target pathway to carry out *in vitro* validation of these data.

Given that pathways and not specific targets were selected, I resorted to the previously published data by Che, Bode and colleagues (2022), who characterised the response of clones with high ELSK content to a group of Rho-GTPase inhibitors. Rho-GTPases have been implicated in the sensing and response to microenvironmental cues by HSCs, which facilitate interactions with other cells in the BM niche and regulate quiescence (Yang et al, 2007; Ghiaur et al, 2006; Cancelas & Williams, 2009). Thus, Rho-GTPase inhibitors, CASIN, Rhosin and NSC23766 were selected to target cell adhesion in culture. Cdc42 member of the Rho-GTPase family which controls many cellular processes, including cell motility, migration, microtubule organisation and cell cycle transition (Duan et al, 2021). CASIN is a Cdc42 activity specific inhibitor, and has been shown to rejuvenate aged HSCs, as well as guiding malignant HSCs towards the endosteum of the BM (Grochowski et al, 2023).

On the other hand, in order to then target interferon signaling, the fact that both ISG20 (Deymier et al, 2022) and IFI35 (Jian et al, 2018) are stimulated and induced, respectively, by type II interferon, commonly referred to as IFN- γ , in addition to CD160 being involved in the positive regulation of IFN- γ production (Tu et al, 2015), this was then selected as the fourth target molecule to test. In this way, I selected three Rho-GTPase inhibitors and recombinant addition of IFN- γ for validation through functional *in vitro* tests. The initial hypothesis was that inhibition of Rho-GTPases, which mediate cell adhesion, would not impair TET2 HSC growth, since they were found to overexpress molecules involved in this biological process. Likewise, addition of large concentrations of recombinant IFN- γ should not affect TET2 HSCs, if these were in fact resilient to the prolonged inflammatory environment in culture. Hence, the overall aim of these tests was to find a plausible molecular basis for TET2 super competition.

For these experiments, bulk expansion cultures of wild-type and TET2 ESLAM HSCs were performed as bulk cultures produce more robust data when testing different molecules, whether it be inhibitors or recombinant proteins (Che et al, 2022; Juan Rubio-Lara, *personal communication*). Concentrations of each of the Rho-GTPase inhibitors to be tested were based on the reported concentrations previously tested and validated by Che et al (2022) on

wild-type HSCs in F12-PVA based expansion system: CASIN: 2, 10 and 20 μ M; Rhosin: 1, 10 and 50 μ M; NSC23766: 5, 50 and 200 μ M. A validated IFN- γ *in vitro* concentration on mouse HSCs was reported by de Bruin and colleagues (2013) to be 20 ng/mL, which was the intermediate concentration selected. Thus, IFN- γ concentrations to be tested were: 2, 20 and 200 ng/mL.

Regarding Rho GTPase inhibitors specifically, these are their main features:

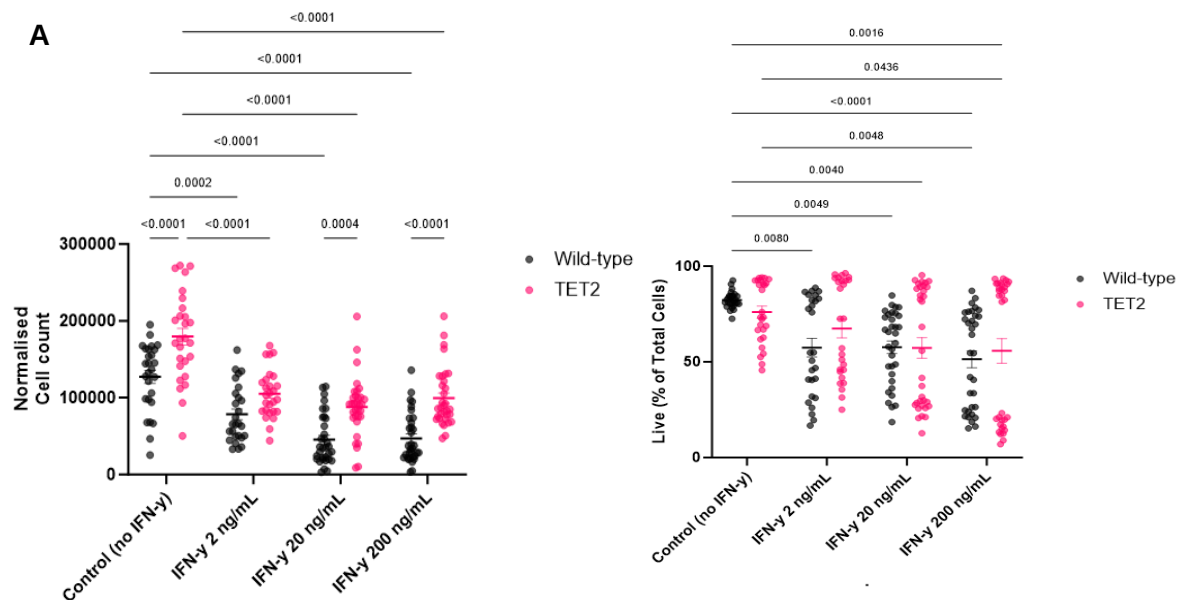
1. CASIN, the Cdc42 activity specific inhibitor (or Pirl-1 related compound 2 (Peterson et al, 2006)) as the name implies, inhibits the Rho GTPase family member Cdc42, which in HSCs multiple cell surface receptor signals, including CD117, 1 integrin and CXCR4, which are all essential for cytoskeletal dynamics and cell adhesion (Liu et al, 2019; Florian et al, 2012).
2. Rhosin, the Rho-A specific inhibitor (Shang et al, 2013) (also known as G04), bind the Tr58 region in RhoA which disrupts its interaction with GEFs, resulting in inhibition of platelet aggregation induced by thrombin (Dandamudi et al, 2023). In HSCs, RhoA inhibition has dual roles, as its activation has been found to retain HSCs in the G0 phase of the cell cycle, thereby affecting their self-renewal potential (Jaganathan et al, 2013), while also been reported as locking HSCs in the active phases of the cell cycle, enhancing HSC/HSPC expansion and proliferation (Ghiaur et al, 2006).
3. Lastly, the NSC-23766 compound is a specific inhibitor of the small Ras protein, Rac1 interaction with GEFs (Gao et al, 2004), which has been described as essential for regulating HSC polarisation, actin polymerisation and migration (Yang et al, 2001), as well as engraftment and homing to the niche (Hu et al, 2020).

Therefore, testing these three targets on both wild-type and TET2 HSC cultures would provide evidence on whether cell adhesion would be one of the mechanisms underscoring super-competitive behaviour. Likewise, by inducing an environment like the one induced by IFN- γ mediated inflammation, was expected to result in wild-type death, and overall survival of the TET2 HSCs.

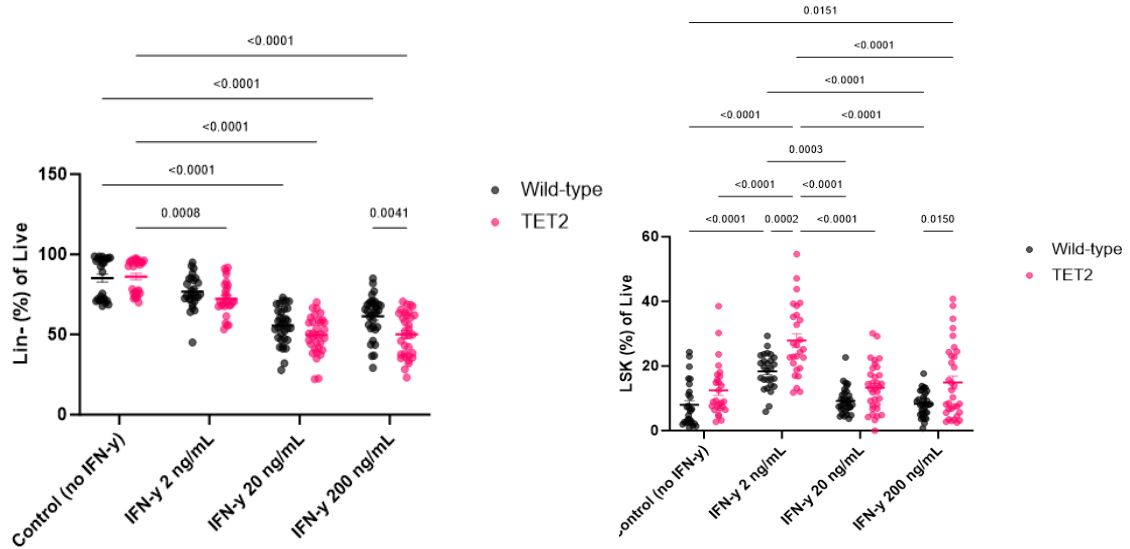
4.6.1 Wild-type and TET2-mutant HSC proliferation was not significantly affected by IFN- γ

IFN- γ is expressed by T lymphocytes, natural killer (NK) and natural killer T cells in response to virus and mycobacteria, although it has also been implicated in the impairment of haematopoiesis (de Bruin et al, 2014). Here, a recombinant IFN- γ protein was added in three

different concentrations (Figure 4.14) to bulk wild-type and TET2-mutant HSC cultures on the first media replacement (5 days post-sort). Total culture well contents were harvested and analysed by flow cytometry at the 28-day endpoint of the culture. Phenotypic analysis of IFN- γ treated wild-type and TET2-mutant HSCs showed a significant decrease in the normalised live cell numbers in wild-type cultures across all three doses (2, 20 and 200 ng/mL) of IFN- γ tested (Figure 4.14A). It was noted that TET2 live cell numbers were significantly increased in the control (untreated) control wells ($p < 0.0001$), averaging to 1.71×10^5 live cells per well. This could be attributed to the enhanced self-renewal capacity which is characteristic of TET2-mutant HSCs (Ko et al 2011), thereby producing more cells in culture. TET2 live cell number was uniform under all IFN- γ concentrations tested, with an average between 8×10^4 to 1×10^5 live cells per well. Nonetheless, these numbers were significantly decreased in comparison to the control condition ($p < 0.0001$) by an almost 50% reduction in the total number of live cells. Wild-type live cell numbers under the lower concentration averaged to 6.5×10^4 cells, but these decreased by almost a half when treated with 20 and 200 ng/mL IFN- γ (3.6 and 3.9×10^4 cells, respectively). Further analysis of live cell proportion (%) in these cultures, showed significant reductions in the three IFN- γ conditions respect to the control condition, but no significant differences between wild-type and TET2 in any of the conditions tested.



B



C

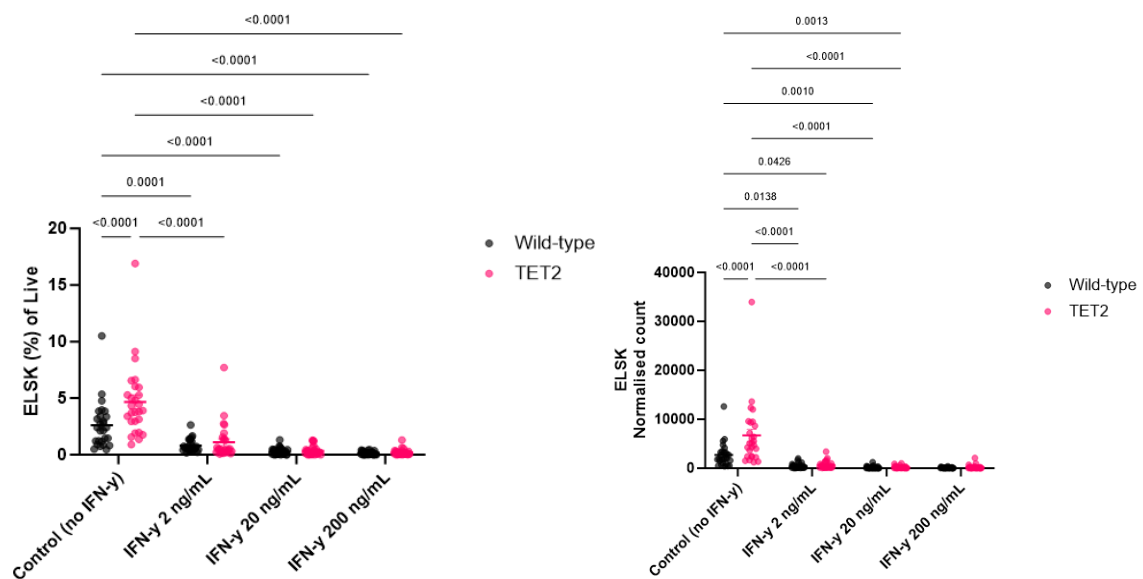


Figure 4.14 Analysis of wild-type and TET2 cultures treated with IFN-γ. A) Normalised cell numbers and B) Live frequencies of wild-type and TET2 cells. Although wild-type cell numbers were reduced under control conditions in comparison to TET2, there were no statistically significant differences in live cell frequencies for any of the genotypes at any concentration tested. B) Lin- (left) and LSK (right) frequencies of total live cells. An increase in LSK frequency in the TET2-mutant HSC cultures was observed at all concentrations tested. C) ELSK frequencies (left) and normalised counts (right) under all IFN-γ treatment conditions. There were very low frequencies and numbers of ELSK detected for both genotypes, regardless of the IFN-γ concentration. Two-way ANOVA was performed to calculate statistical significance between multiple groups across different IFN-γ concentrations.

4.6.1.1 The ELSK compartment was negatively affected by IFN- γ treatment in both wild-type and TET2^{-/-} HSC cultures

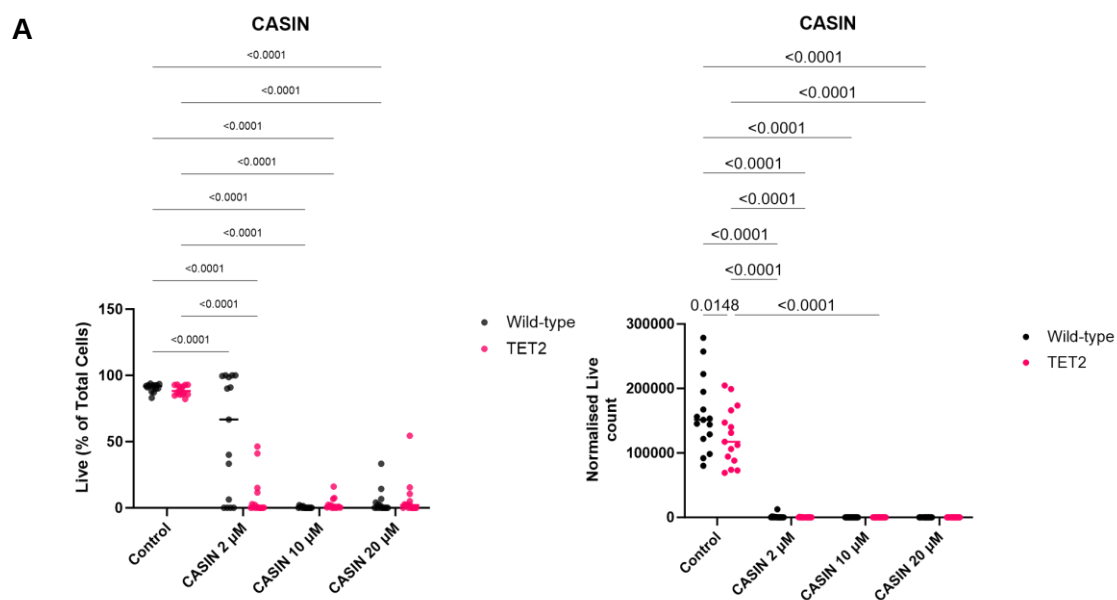
A closer inspection of the stem and progenitor cell populations revealed variable results in wild-type and TET2 cultures. Frequency of Lin⁻ cells (Figure 4.14B) was remarkably similar under control conditions for both genotypes, which was resembled in cultures treated with the lower concentration. Although there was a slight decrease in TET2 Lin⁻ frequency at the highest IFN- γ dose, there were no relevant differences in the other concentrations tested. Analysis of LSK frequencies (Figure 4.14B) revealed an overall increase in TET2-mutant cells compared to wild-type cells treated with the low ($p = 0.0002$) and high concentrations ($p = 0.0150$), but not the intermediate concentration (non-significant). Strikingly, ELSK frequencies and number were very low for both genotypes under all IFN- γ treatment conditions, but not in the controls. The average number of wild-type ELSKs in the control condition was 2×10^3 cells per well, and 6.1×10^3 cells for TET2 ELSKs. Nevertheless, ELSK numbers were in the order of hundreds across all concentrations tested for both genotypes, except for the low concentration (Figure 4.14C), where some of the TET2 wells contained between 1.3 to 3.3×10^3 ELSKs, but this was the case for only a fraction of the samples. These results suggest that IFN- γ did not affect growth of wild-type HSCs in culture as expected, given the observed expression of interferon-induced and stimulated proteins in TET2-super competitors, since there was still a significant number of wild-type live cells growing under IFN- γ treatment conditions. Furthermore, the depletion of ELSK cells from cultures indicated that the addition of IFN- γ at any concentration hints at a possibly reduced functional potential in both genotypes, suggesting that interferon signaling might mediate wild-type annihilation by TET2-super competitors in an inflammatory environment. However, TET2-super competitors were shown to have increased numbers of ELSK cells, which is the opposite of what was observed in this *in vitro* validation tests.

4.6.2 Inhibition of Cdc42 activity was lethal to wild-type and TET2 HSCs, and inhibition of RhoA slightly reduced TET2^{-/-} HSC cell viability at high concentrations

Given the upregulation of cell adhesion and the high expression of cell adhesion and anchoring proteins were found in both the transcriptomics and proteomics datasets for the TET2-super competitors, the working hypothesis for the experiments described in this section was that CASIN, Rhosin and NSC-23766 should not, in principle, affect TET2 HSC growth or proliferation, but they should be able to impair wild-type growth. Thus, I performed bulk cultures and tested these three inhibitors at the concentrations stated above.

Analysis of normalised live cell counts of wild-type and TET2-mutant cultures, showed an average number of 1.51×10^5 cells in wild-type and 1.17×10^5 cells in TET2 control (untreated) conditions. Nevertheless, all CASIN doses tested were lethal to HSC cultures of both genotypes, as there were no live cells detected in any of the conditions for neither wild-type nor TET2 cultures (Figure 4.15A). Live cell frequency analysis confirmed the lethal effect of CASIN treatment on these cultures. Although some wells were observed to show 100% viability, this was likely a technical artifact, as numbers of viable cells were depleted for all conditions. As a result, there were no Lin-, LSK or ELSK cell populations left in the wells. These data indicate that inhibition results in lethality to bulk HSC expansion cultures, regardless of genotype or dose added to the culture media, which also suggests that TET2-super competitors are equally as susceptible to Cdc42 inhibition as wild-type competitors would be.

Treatment with Rhosin, on the other hand, yielded different results than for CASIN (Figure 4.15B). Live cell frequencies were comparable between wild-type and TET2-mutant, with a noticeable drop in the TET2 HSCs at the highest dose tested (50 μ M). Here, surprisingly, TET2 live cell frequency was reduced almost 2-fold lower than for wild-type HSCs, yet not statistically significant. Normalised live cell numbers (Figure 4.15B) were unchanged for both wild-type and TET2-mutant HSCs when treated with the lowest Rhosin dose (1 μ M). Surprisingly, cell numbers were significantly elevated under treatment with 10 μ M Rhosin, but dramatically dropped when treated with the highest dose of 50 μ M. These data indicated that both wild-type and TET2-/- expanded HSCs were both affected substantially by high concentrations of Rhosin, and none of them were resilient to the inhibition of RhoA, which discarded the activity of such enzyme as essential for sustaining supercompetitive behaviour.



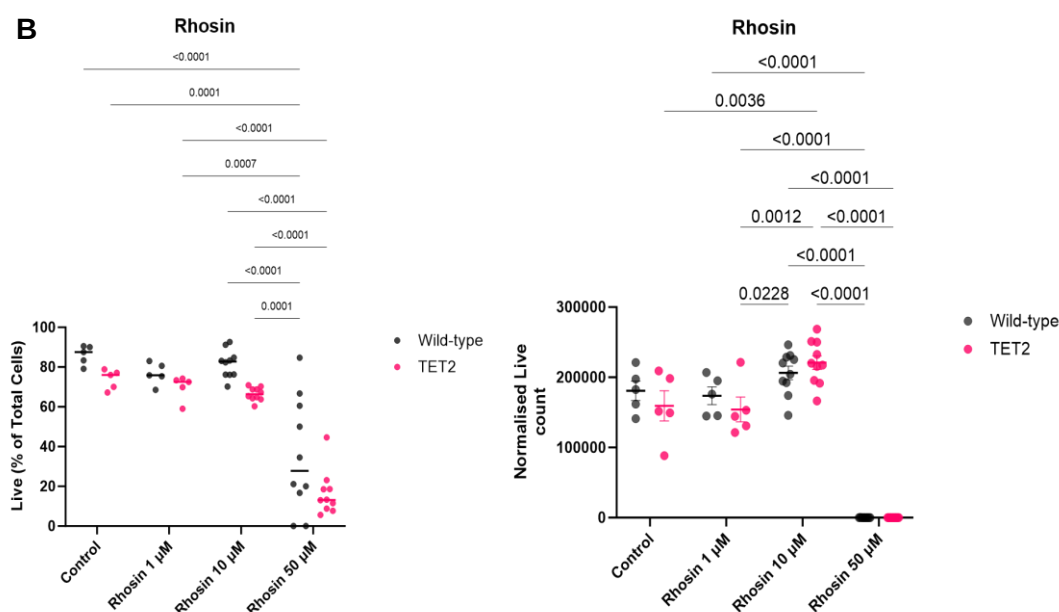


Figure 4.15 CASIN and Rhosin treatment affected viability of both wild-type and TET2^{-/-} HSCs. A) CASIN was lethal at all doses tested. Live cell frequency (of total cells) (left) and cell numbers (right) were depleted throughout doses in both genotypes. B) Rhosin negatively affected cell viability (left) in wild-type and TET2^{-/-} HSC cultures, with a slightly more pronounced effect over TET2-mutant cells at 50µM. Nonetheless, live cell numbers (right) were completely depleted at the same dose in both genotypes, although the low (1µM) and intermediate (10µM) doses did not produce any noticeable effects on viability. Two-way ANOVA was performed to calculate statistical significance across groups and inhibitor doses tested.

4.6.2.1 Inhibition of the Rac1-GEF interaction by NSC-23766 impaired wild-type, but not TET2, HSC growth *in vitro*

The third and final inhibitor tested was the NSC-23766 compound, inhibiting the Rac1/GEF interaction. Normalised live cell counts treated with 5 and 50µM of NSC-23766 were roughly equivalent to the untreated controls, meaning that these doses did not alter wild-type or HSC expansion (Figure 4.16). However, in response to the highest NSC-23766 dose tested, 200µM, TET2-mutant live cell counts were significantly increased in reference to wild-type cells ($p < 0.0001$) (Figure 4.16A). This number was also higher than for the untreated TET2 controls, although not statistically significant. Frequencies of the lineage-negative compartment did not display any significant differences across doses tested for any of the genotypes, except for a significant reduction in wild-type cells treated with 5µM NSC-23766 compared to the untreated control ($p = 0.0003$). In the same manner, normalised LSK counts (Figure 4.14B) were significantly elevated in TET2 cells treated with 200µM NSC-23766, with an average of 1.46×10^5 LSK cells per well, much higher than for any other treatment or control condition, in both wild-type and TET2 cultures. Further analysis revealed that - in spite of wild-

type and TET2 ELSK frequencies (Figure 4.16B) remaining unchanged across treatment conditions compared to the controls - normalised wild-type ELSK counts were significantly decreased in the low ($p = 0.081$), intermediate ($p < 0.0001$) and high ($p < 0.0001$) doses in reference to the untreated control. Average ELSK number in the untreated wild-type control was 1.8×10^4 cells per well, decreasing to 5.6×10^3 in the low dose, 838 in the intermediate dose, and 2.7×10^3 in the high dose of NSC-23766 treatment. Contrastingly, TET2 ELSK numbers remained unchanged across all treatment conditions, starting with an average number of 1.4×10^4 ELSKs in the controls, followed by 1.4×10^4 , 5.7×10^4 , and 1.11×10^4 cells in the low, intermediate and high doses.

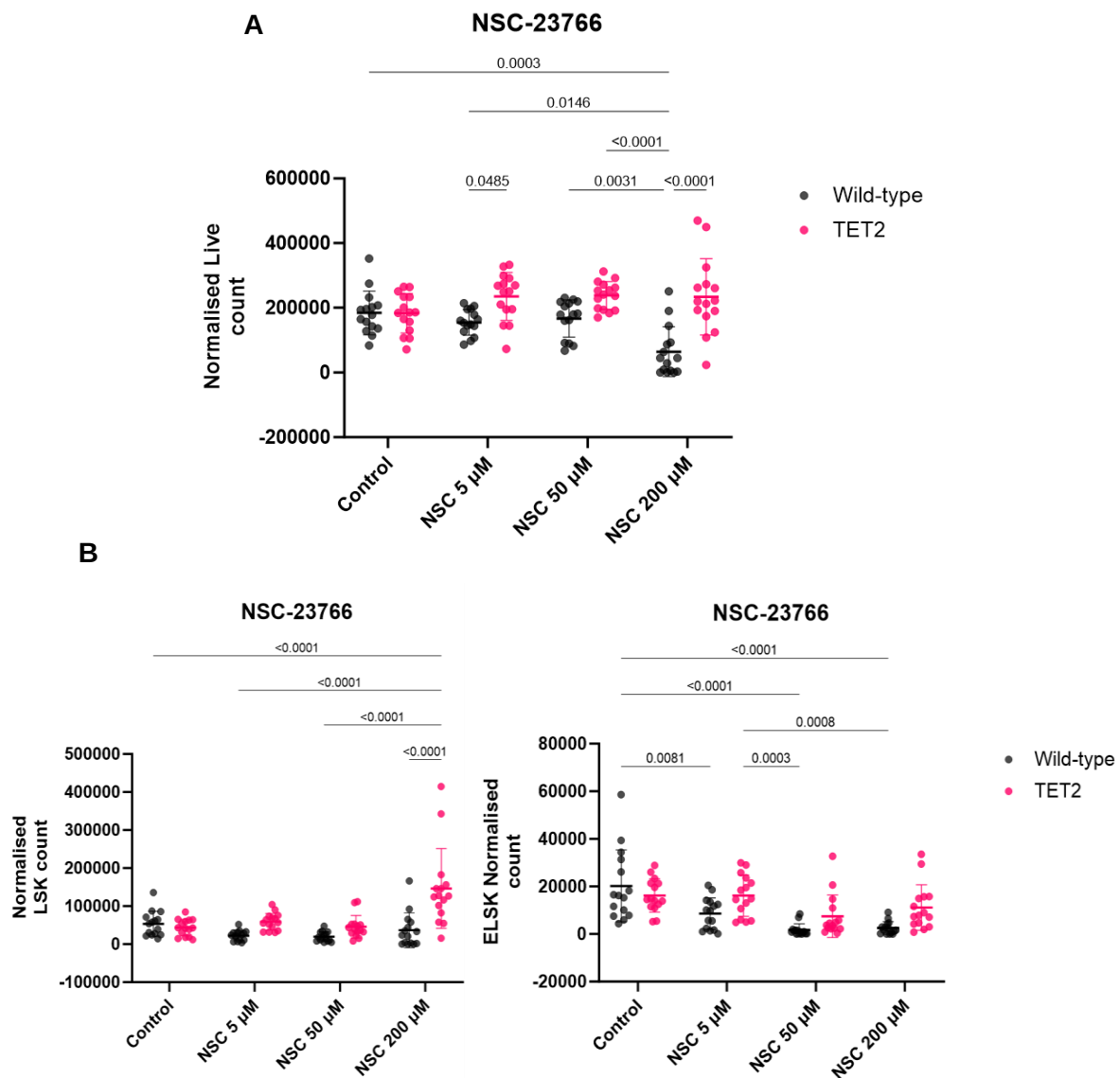


Figure 4.16. NSC-23766 treatment ameliorated wild-type HSCs but TET2^{-/-} cells remained unaffected at all doses tested. A) Normalised live cell counts in wild-type and TET2 cultures treated with three different doses of NSC-23766. Live cell number was particularly higher in TET2 than wild-type cultures at the highest concentration (200µM). B) Normalised LSK (left) and ELSK (right) counts in NSC-23766 treated cultures. A significant increase

in LSK numbers was observed in TET2 cultures at 200 μ M. While ELSK counts did not vary significantly between wild-type and TET2, wild-type ELSKs were decreased in a dose-dependent manner, while TET2^{-/-} ELSKs remained resilient. Two-way ANOVA was performed to calculate statistical significance between multiple groups.

4.6.2.2 TET2^{-/-} HSCs remain resist the Rac1/GEF inhibitory effect of NSC-23766 in a dose-dependent manner

Of the four different molecules tested, although NSC-23766 did not produce major changes in the number of phenotypic HSCs in the TET2 cultures, it evidently inflicted substantial reductions on wild-type ELSK numbers in culture. Therefore, one more experiment was performed to test higher concentrations of NSC-23766, in order to determine the highest concentration tolerated by TET2-mutant HSCs in which they could still expand and show an equivalent number of ELSK cells as they did under the untreated and low dose conditions in the first test. These concentrations would still have to ameliorate wild-type HSC expansion and lower ELSK yield. From lowest to highest, the three concentrations tested were 50, 200 and 400 μ M of NSC-23766 on bulk wild-type and TET2 HSC cultures (Figure 4.17).

In TET2^{-/-} HSC cultures, treatment with 50 μ M NSC-23766 resulted in an increase of live cells, from 9.8×10^4 in the controls to 1.4×10^5 , an increment of nearly 5×10^4 cells (Figure 4.17A). As expected, the 200-uM dose did not impact viability of TET2^{-/-} HSCs, undergoing a very slight reduction in comparison to the low dose (8.66×10^4 viable cells on average). Conversely, viability of wild-type HSCs was substantially ameliorated by the 200-uM of NSC-23766, where live cell numbers were essentially depleted (mean of 20 live cells), in comparison to the controls (3.7×10^4 cells). TET2 live cell numbers were significantly higher than wild-type cells at the 50 μ M ($p = 0.0103$) and 200 μ M ($p < 0.0001$) doses. Nevertheless, the 400-uM dose resulted to be lethal for both wild-type and TET2^{-/-} HSCs, as no viable cells were found in the wells, thereby suggesting that, whereas TET2 deficiency makes *ex vivo* expanded HSCs resilient to low and intermediate doses of NSC-23766, it is not sufficient to evade the detrimental effects caused by the highest experimental dose tested. The TET2^{-/-} HSPC/HSC compartment was largely unaffected by the 50 and 200-uM doses, but as a result of the detrimental effects of the 400-uM dose, cultures were also depleted from LSK and ELSK cell entirely (Figure 4.15B). Remarkably, LSK numbers were not decreased by NSC-23766 treatment, but were maintained stably and consistently at the 50 and 200-uM doses. Although ELSK numbers were reduced at the 200-uM dose, this was not statistically significant relative to the number of ELSKs in the control wells (Figure 4.17B).

These results demonstrated that, while doses above 200 μ M of NSC-23766 *in vitro* impaired both wild-type and TET2^{-/-} HSC expansion, TET2^{-/-} HSCs were able to counteract the Rac1/GEF inhibition by NSC-23766 in a dose-dependent manner. In accordance with the

molecular and proteomics data, it is highly likely then, that the supercompetitive behaviour of the subset of TET2 clones we profiled relies, in part, on the overexpression of molecules involved in cell adhesion mechanisms mediated by the small Ras protein family of the Rho GTPases, as well as their interaction with GEFs, which could impair wild-type HSC expansion but not TET2. This could, therefore, confer a selective competitive advantage to the later, which could contribute to the phenomenon of supercompetition.

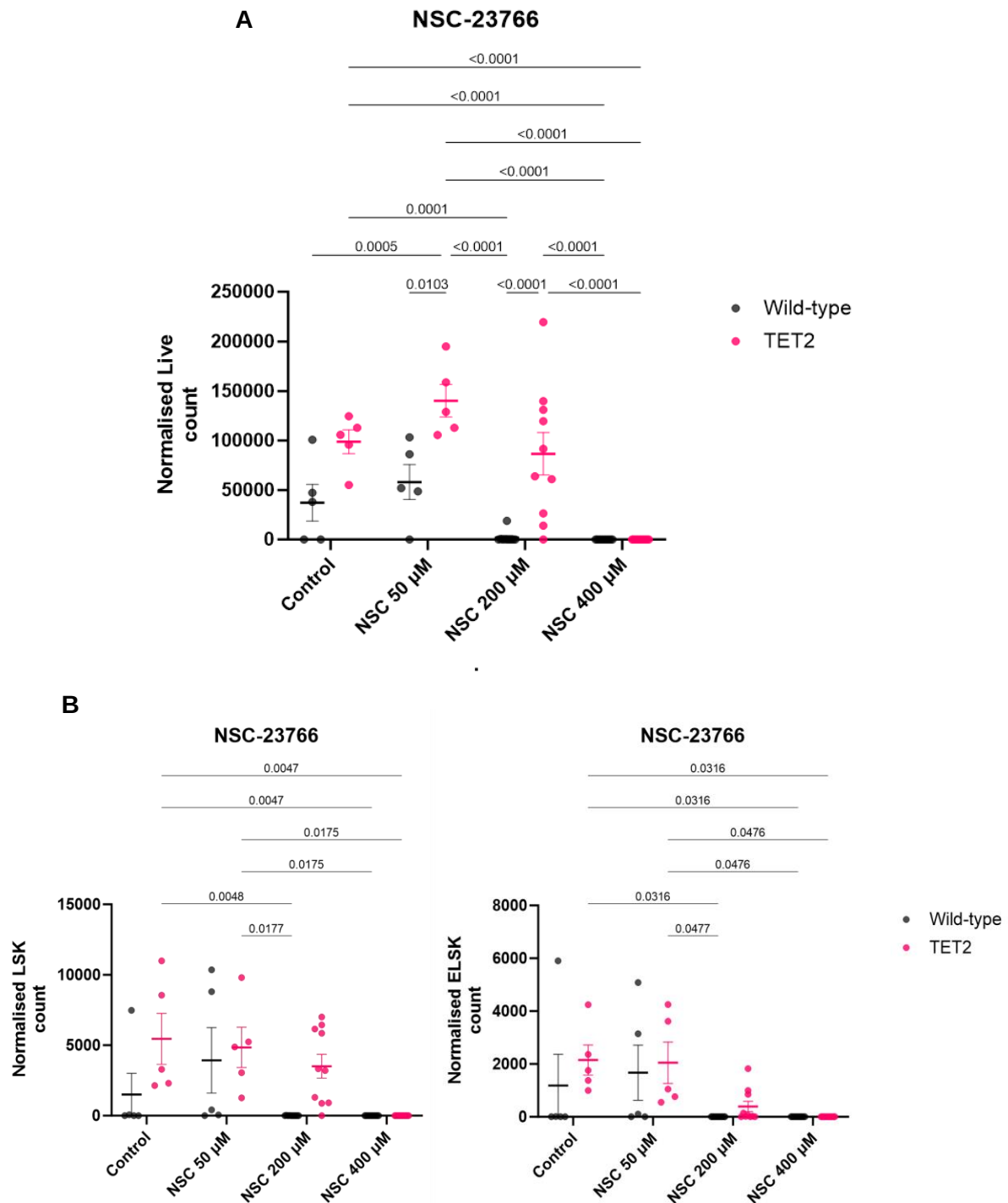


Figure 4.17. TET2^{-/-} HSCs resist NSC-23766 inhibitory effects in a dose-dependent manner. A) Normalised live cell counts in wild-type and TET2^{-/-} HSC cultures (n = 15 sample wells per condition per genotype) treated with three different doses of NSC-23766. The 400µM dose was detrimental to both genotypes, and as a result, depleted the culture of viable cells. B) LSK (left) and ELSK (right) cell counts in wild-type and TET2^{-/-} HSC cultures. The LSK compartment of TET2 was largely unaffected by the 50-uM and 200-uM doses, while wild-type LSKs underwent a dramatic reduction at 200µM NSC. A similar trend was observed on ELSK numbers. Albeit noticeable, reduction of TET2^{-/-} ELSKs was not statistically significant in relation to the controls at the intermediate dose. Two-way ANOVA was performed to calculate statistical significance between multiple groups.

4.7 Summary of findings

In this chapter, I described the development and application of a novel *in vitro* clonal competition model, through which we unveiled the existence of a previously undescribed type of TET2 competitor clone - the *TET2-super competitors*. By leveraging state-of-the-art methods for HSC *ex vivo* expansion, we established a single-cell derived 'split' clone culture system. Extensive phenotypic, molecular and functional profiling of such split cultures demonstrated the robustness of the method in which we were able to take equal fractions of a single clone and, in principle, profile its competitive activity against multiple clones of a different genotype. We then designed a clonal competition assay, whereby single-HSC derived clones were split and mixed across nine wells in an arrangement we termed 'competition matrix', all done in a 96-well plate format, which allowed us to conduct these assays at scale. In total, I profiled 300 individual competitor clones, which equates 900 individual competition wells, which all harboured unique clones competing against each other, adding great statistical power to our assay.

Throughout three vastly comprehensive experiments, we identified a subset of TET2 competitor clones, which outperformed their rival wild-type clones and outnumber them by a 70:30% proportion, although in many instances they annihilated them, leaving no traces of wild-type cells in the competition wells. These newly identified TET2-super competitor clones produced the highest number of ELSK cells in culture, and these numbers were comparably high in both the untouched, control wells and the competition wells. Transplantation assays demonstrated that, while TET2 and TET2-super competitors did not differ significantly in their ability to repopulate myeloablated recipients, there was strong correlation between the ELSK output and relative donor chimaerism, which still distinguished these clones functionally. Molecular profiling of competitor clones aligned with mass spectrometry analysis of conditioned media obtained from these same clones, whereby molecules implicated in structural organisation, ECM binding and cell adhesion mechanisms, as well as interferon-γ signalling and insulin and glucose metabolism. Through a series of *in vitro* experiments for

validation of targets, I showed that, while Cdc42 inhibition was lethal to TET2^{-/-} cultured HSCs and RhoA inhibition did not produce any significant effects, inhibition of the Rac1/GEF interaction was detrimental to wild-type, but not TET2 in a dose-dependent manner. Likewise, the LSK and ELSK compartments were mostly unaffected, maintaining consistent numbers across different doses of the Rac1/GEF inhibitor, NSC-23766.

Indeed, these TET2-super competitor clones comprise a rare subset of competitors, which had not been reported before, but can now be profiled at scale thanks to the applicability and versatility of the *in vitro* clonal competition method. Further experimental work will be necessary to ascribe this remarkable activity to more specific signalling pathways, or even single molecules, which enhance malignant clone expansion, while at the same time, impairing normal, healthy clones, which is particularly relevant for both pre-leukaemic disorders, such as clonal haematopoiesis, and overt leukaemia.

4.7.1 Summary of new contributions to the field

1. An *in vitro* model of clonal competition at high scalability, in addition to increasing statistical power thanks to clone splitting, creating unique competition matrices
2. The identification of a TET2-super competitor clone, with superior performance, in many instances completely suppressing wild-type HSC growth
3. Higher expression of cell adhesion related genes in TET2-super competitors, which allow them to circumvent the impairment of Rho GTPase activity in a dose-dependent manner

4.7.2 Future experiments

1. To profile competition cultures at different timepoints, e.g., three, seven and ten days after setting up competition assays to understand how TET2 competitor clones outgrow their wild-type rivals over time
2. To treat wild-type and TET2 HSCs with TET2-super and wild-type competitor supernatants, respectively, to study the potential role of secreted proteins in the enhancement of competitive behaviour
3. To analyse the molecular profile and secreted proteins at an early and midpoint in the culture, to determine at which point in time there are significant gene expression changes or secreted proteins which might be hindered by the end of the culture
4. To sort and transplant labelled ELSK cells from competition cultures, instead of transplanting the entire content of the well

5. To profile ELSK cells from a representative fraction of the competition wells corresponding to the distinct types of competitor clones, in order to determine whether the supercompetitive behaviour could be ascribed to the ELSK fraction

4.7.3 Potential implications of the new insights from this Chapter

The work described in this chapter demonstrates the power of the latest HSC expansion culture systems and how these are suitable to study competition dynamics between HSC clones of different genotypes. Cell competition had traditionally been modelled *in vivo*, which provides valuable information on how competing cells behave in a living organism. However, one of the main limitations of this approach is scalability, as performing hundreds of transplantation experiments is resource intensive and requires at least four months to be completed. Attaining higher applicability and scalability, our approach is grounded in the split culture methodology, which increases the statistical power of the assay, at the same time allowing to profile the competitive capacity of one individual clone against three unique clones. TET2 loss-of-function mutation is frequently detected in patients with clonal haematopoiesis, CML, MDS, AML and in MPNs, many times co-occurring with the JAK2 V617F mutation. Importantly, TET2 together with DNMT3A, is one of the main driver mutations in clonal haematopoiesis, which is a myeloid disorder common in the elderly (Wang et al, 2022; Buscalet et al, 2017). The future impact of my work relies on the identification of a TET2-mutant subset which drives the elimination of normal, healthy cells, thus promoting the expansion of malignant cells, which is crucial to understanding somatic evolution in myeloid cancers. Likewise, since TET2 is found to be co-occurring with mutations which drive disease (Heyes et al, 2023; Mason et al, 2017; Chan & Majeti, 2013), new experiments could be conducted where TET2-mutant HSCs are set against HSCs carrying mutations in common co-mutations in AML, MDS or CH.

Thus, I have demonstrated that somatic mutations have a profound impact on the biology of HSCs, which can change the way they interact with other clones *in vitro*. In the next chapter, I will integrate the study of pre-leukaemic mouse models (*in vivo*) to understand the synergy of concomitant mutations, along with molecules secreted in the BM niche. In Chapter 3, I demonstrated the crucial importance of the niche in determining HSC properties, although in the forthcoming Chapter 5, I will take the approach of studying a single molecule, and how this impacts disease evolution in a novel triple-mutant mouse model.

Chapter 5 - Cell-extrinsic regulation mechanisms in the development of Myeloproliferative Neoplasms

Introduction to the chapter

Throughout Chapter 3, I characterised normal HSCs from different BM niches *in vivo*, showing the impact of the niche on molecular and functional properties of HSCs. At the same time, work in Chapter 3 likewise reinforced the use of somatic mutations as endogenous barcodes to reconstruct the phylogenies of HSCs. Moving onto Chapter 4, I exploited the power of somatic mutations to characterise the competition dynamics between preleukaemic and normal HSCs. This is how this body of work led into the research comprising the final Chapter of this Dissertation - the development and characterisation of a novel triple-mutant MPN mouse model to explore the crucial role of the BM on MPN disease biology, together with concomitant mutations in JAK2 and TET2.

Patient heterogeneity in MPNs is one of the most puzzling questions in the field of haematological malignancies. The molecular mechanisms underlying MPN disease have been partially elucidated by the identification of the three MPN driver genes: JAK2, CARL and MPL, it still remains unclear how clinical presentation between MPN subtypes can have such a high degree of variability (Grabek et al, 2020; Lee et al, 2020). Likewise, the fascinating question of how the different cellular compartments across the niche (Grockowiak et al, 2023) interact with normal and malignant HSCs, and how they sustain a chronic state of inflammation, has not been fully addressed. One of the main challenges is to faithfully recapitulate the MPN phenotype induced by the presence of concomitant mutations *in vivo*, and at the same, to profile the role of a single secreted molecule on disease phenotype. The current state-of-the-art in MPN mouse models is comprised by the knock-in JAK2 V617F mouse (referred to as JAK2^{R/R}, *R* denoting recombinant allele), which exhibits the severe red blood cell phenotype characteristic of PV in human patients, with haematocrit levels close to 100% (Li et al, 2014). In addition, the TET2 knock-out (KO) model (TET2^{-/-}) developed by the Rao laboratory (Ko et al, 2011), has allowed determination of the impact of TET2 loss of function mutations on MDS, AML and MPNs. The report of PV cases negative for JAK2 V617F mutations (Geetha et al, 2010), as well as mouse model data (Li et al, 2014), have demonstrated that, in some instances, standalone JAK2 homozygous mutations require the acquisition of other mutations to initiate disease. In addition, the impact of co-occurring JAK2 and TET2 mutations on diagnosis, prognosis and leukaemic evolution (Saint-Martin et al, 2009; Ortmann et al, 2015), has prompted the development of double-mutant mouse models

(Shepherd et al, 2018), which have permitted the characterisation of the phenotype induced by JAK2/TET2 homozygosity.

Recent reports have underlined the key role of inflammatory cytokines in myeloproliferative disorders (Wang & Zuo, 2019; Mendez Luque et al, 2019; Chen et al, 2021; Xiong et al, 2024), raising the possibility of exploiting the utility of single molecules as prognostic markers or targets for personalised therapy in MPNs. This could also help resolve the prevailing MPN patient heterogeneity, and consequently improve clinical management of the disease.

In this Chapter, I will begin by describing the findings which grounded the basis for my research, which consisted of serum cytokine profiling on a large MPN patient cohort, followed by the generation of a double mutant JAK2/IP10 mouse model. Then, I will describe the experimental approaches I undertook to generate an allelic series of MPN mouse models, in which the role of JAK2/TET2 homozygous mutations together with complete loss of IP-10 was profiled and characterised, focusing on the rescue of the severe red cell phenotype driven by JAK2 V617F. Finally, I will present data from two independent cytokine profiling experiments on a vast array of MPN mouse models, which establish the framework for future studies.

5.1 Comprehensive serum cytokine profiling of patients and mouse models of myeloproliferative neoplasms

Due to the largely prevailing problem of MPN disease heterogeneity, our lab performed a large screen on over 400 MPN patients. Patients were profiled for the expression of 38 serum cytokines and chemokines. Results from an initial cohort consisting of 185 patients led to the identification of a cytokine expression pattern in MPN patients, characteristic for each of the disease subtypes. To expand on these findings, 10 molecules (EGF, Eotaxin, GRO-alpha, TNF-alpha, IL-1RA, TGF-alpha, IL-6, IL-8, IP-10 and IFN-gamma) were selected based on disease specificity, significantly higher serum levels and decreased patient survival for at least one of the three MPN subtypes. The 10-plex array analysis in a larger cohort revealed that the expression levels of CXCL10 or IP-10, was shown to be directly correlated with disease severity and higher risk of AML transformation. This was observed in patients diagnosed with primary myelofibrosis (PMF) (Figure 5.1C, from Obro et al, 2020). Furthermore, patients harbouring both JAK2 and TET2 homozygous mutations had increased levels of circulating IP-10, which might explain its overexpression in PMF patients, having been previously identified as a pro-inflammatory cytokine (Figure 5.1C).

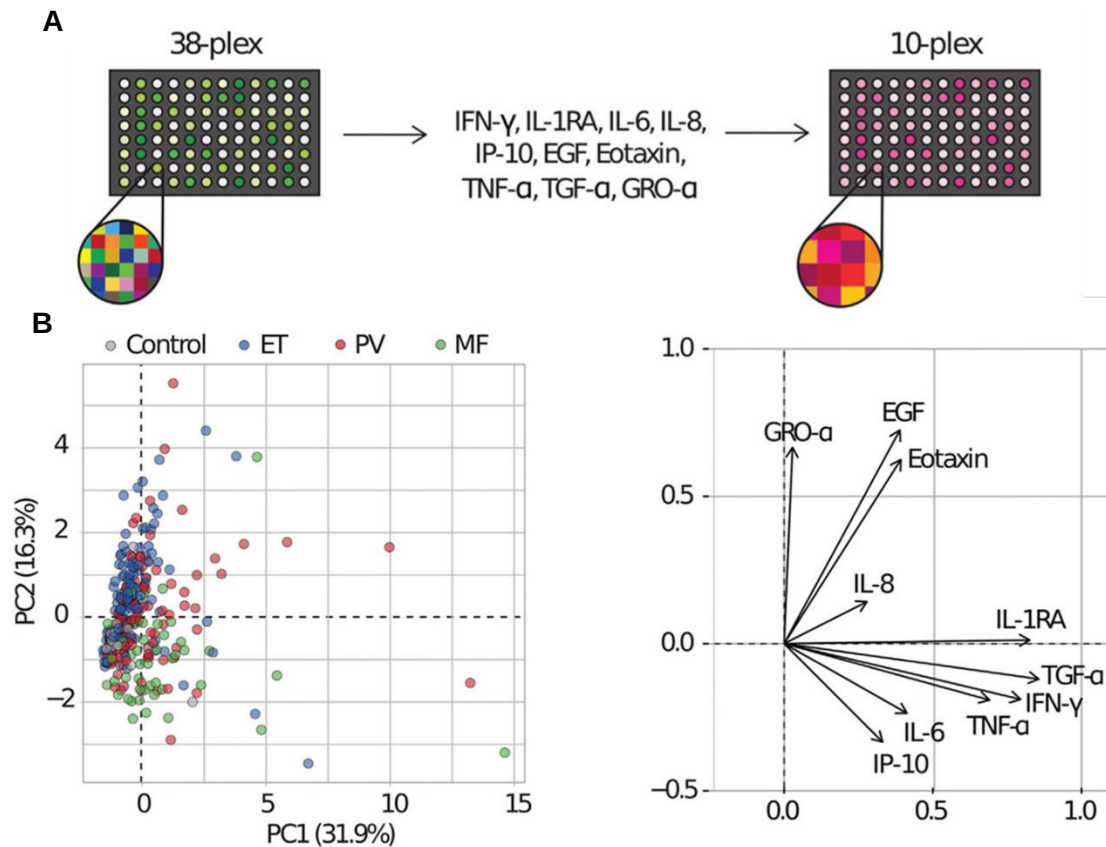


Figure 5.1. Serum cytokine screen of MPN patient cohorts overview. A) Schematic overview of the 38-plex cytokine screen initially performed, followed by a 10-plex experiment for validation of identified targets strongly correlated with MPN disease. C) Principal component analysis revealed molecules associated with disease severity, such as Eotaxin, EGF and Gro- α . In addition, CXCL10 or IP-10, was associated with JAK2 V617F mutations and myelofibrosis. Figure adapted from Obro et al, 2020.

Having identified cytokine expression patterns in patients which were informative for disease phenotype, we were eager to understand whether MPN mouse models would be appropriate to study these effects further. Therefore, a 32-plex serum cytokine and chemokine analysis was performed on several mouse models of MPNs: CALR^{del52}, JAK2 V617F, TET2, and JAK2/TET2 double mutant, as well as individual sets of wild-type littermate controls. Results from this experiment largely paralleled what had been observed in the MPN patient cohorts (Figure 5.2A) - IP-10 expression was comparable in wild-type and CARL^{del52} mice. However, significantly increased serum IP-10 levels were detected in mice of the TET2^{-/-} and JAK2^{V617F/V617F} TET2^{-/-} genotypes (Figure 5.2B), suggesting that abnormally high levels of IP-10 were related to having both copies of JAK2 and TET2 mutated, and even more prominently when both copies of both genes were mutated which is akin to the dysregulated IP-10 expression observed in MPN patients.

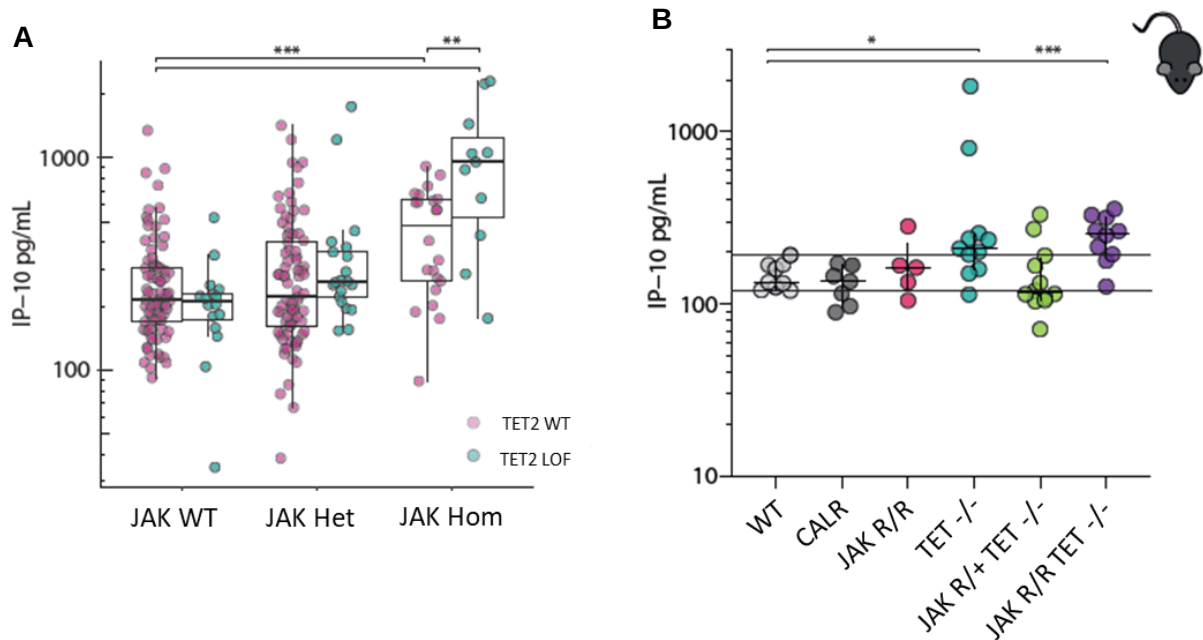


Figure 5.2. Profiling of serum IP-10 in MPN patients and MPN mouse models. A) IP-10 expression in patients according to JAK2 V617F and TET2 mutational status (WT = wild-type, Het = Heterozygous, Hom = Homozygous mutations for JAK2 or TET2. B) IP-10 expression profiling in wild-type, CALR^{-/-}, JAK2^{-/-}, TET2-single mutant and JAK2/TET2 double mutant mouse models. Bars show mean with SEM. Mann-Whitney U-test was performed to calculate statistical significance. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $p < 0.0001$. Figure adapted from Belmonte et al, 2024.

5.1.1 IP-10 deficiency in the host microenvironment does not prevent the development of a PV-like phenotype

To understand the role of IP-10 as an environmental factor in MPN disease onset and maintenance, an IP-10 knockout mouse model was acquired to conduct *in vivo* assays (Figure 5.3). Transplantation assays were undertaken, whereby IP-10 knockout and wild-type recipients received a transfer of 25 ESLAM cells isolated from JAK2^{V617F/V617F} TET2^{-/-} donors, a number of MPN HSCs sufficient to produce a robust and transplantable PV-like phenotype (Shepherd et al, 2018). Disease monitoring by analysis of peripheral blood cell counts showed an increase in the Haematocrit (%) at 12 weeks post-transplant in IP-10 recipients in relation to WT recipients (Figure 5.3). Likewise, Haemoglobin (g/dL) and Platelet ($10^3/\text{mm}^3$) counts were elevated in both recipient conditions, suggesting that IP-10 loss in the host microenvironment on its own was not sufficient to halt disease onset in transplantation setting.

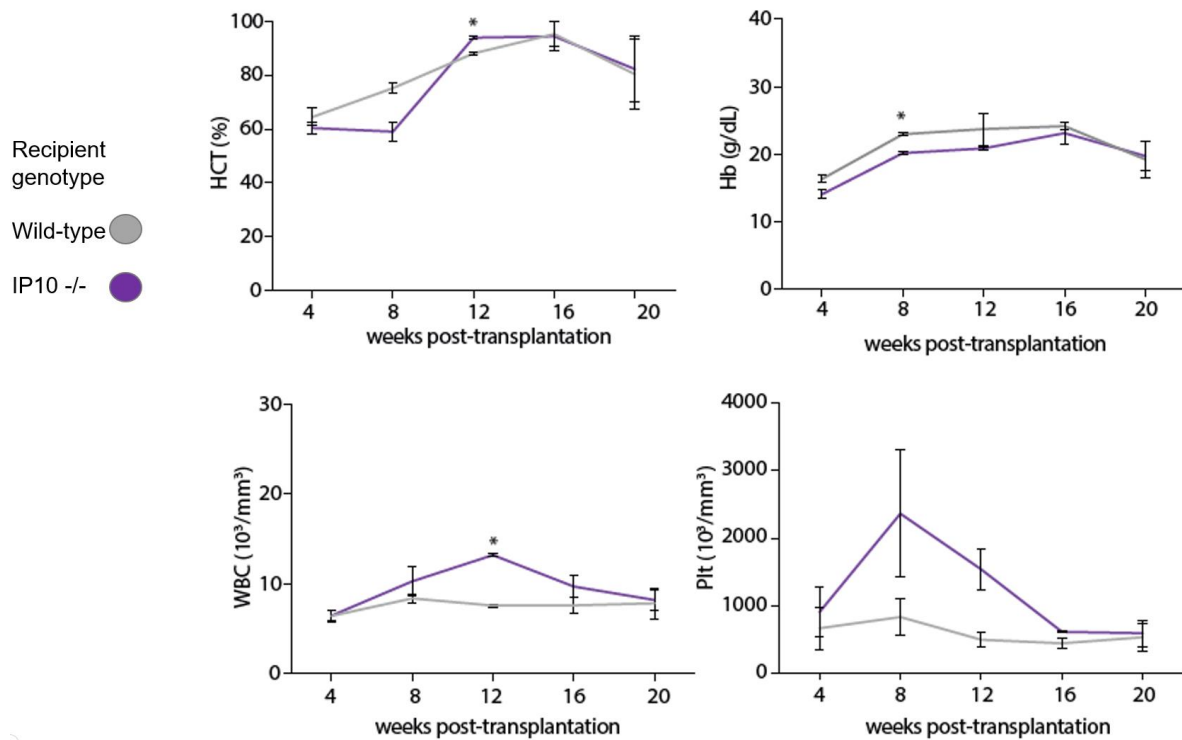


Figure 5.3 IP-10 is not sufficient to initiate disease in a transplantation setting. Wild-type and IP-10^{-/-} recipients were injected with 25 JAK2^{V167F/V617F} TET2^{-/-} ESLAMs. Serial blood samples were withdrawn from recipients every 4 weeks, from 4 to 20 weeks post-transplantation. Haematocrit, haemoglobin, WBC and Platelet counts are shown. Blood parameters remained at comparable levels for both wild-type and IP-10^{-/-} recipients, suggesting that IP-10 is not required for disease onset in a mouse transplantation model of MPN. Bars show mean with standard error of the mean (SEM). Unpaired t-test: * p<0.05; ** p<0.01; ***p<0.001; p<0.0001.

5.1.2 Loss of IP-10 in JAK2-homozygous mice triggers a mild reduction of the strong red cell phenotype

To overcome the possibility of the single- or double-mutant transplanted cells or their progeny re-introducing IP-10 into the host microenvironment, a new breeding line was established by crossing the IP-10 knockout mice with JAK2^{V617F/+} mice to produce a model where it was possible to study the implications of complete IP-10 loss in the context of JAK2 homozygosity. Disease progression was monitored by peripheral blood phenotyping from 8 to 16 weeks of age in wild-type, TET2, JAK2, IP-10-single mutant and JAK2/IP10-double mutant mice (Figure 5.4). Haemoglobin counts were slightly decreased in JAK2^{V617F/V617F} IP-10^{-/-} mice when compared to JAK2^{V617F/V617F} mice at 16 weeks of age, however, other parameters indicated that the phenotype in these double-mutant mice resembled the one displayed by JAK2 homozygous mice. Therefore, IP-10 loss in the presence of JAK2 homozygosity did not ameliorate the MPN phenotype in mice. Notwithstanding, previous findings from the patient and MPN mouse model serum cytokine screen, whereby we detected the highest serum IP-

10 levels in JAK2/TET2 concomitant homozygous mutations, we reasoned that loss of TET2 should therefore impact disease onset and development in and MPN mouse model. We then moved on to generate an allelic series of MPN mouse models, which will be described in the next section.

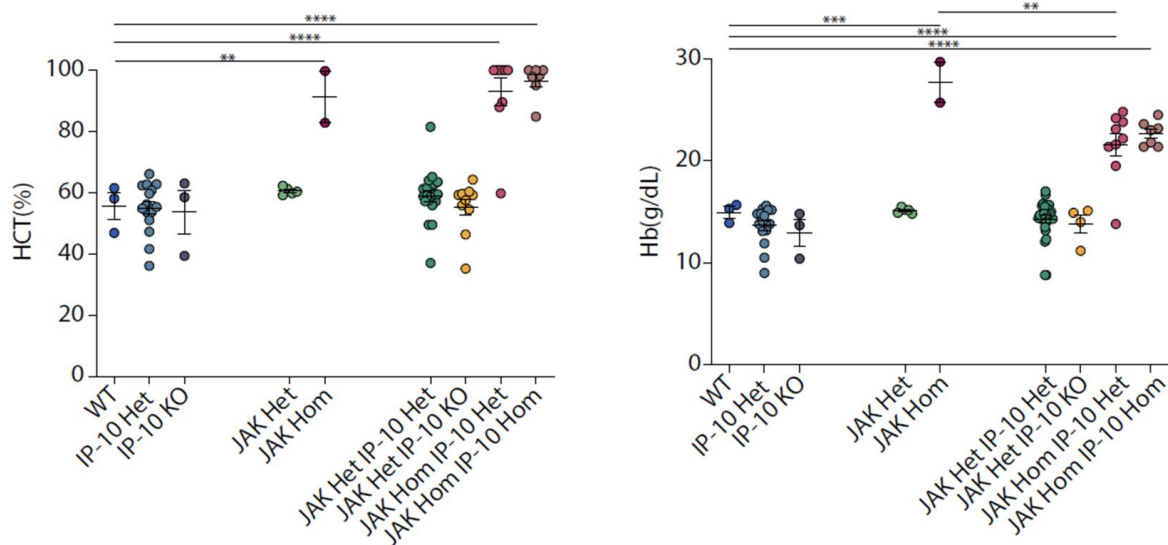


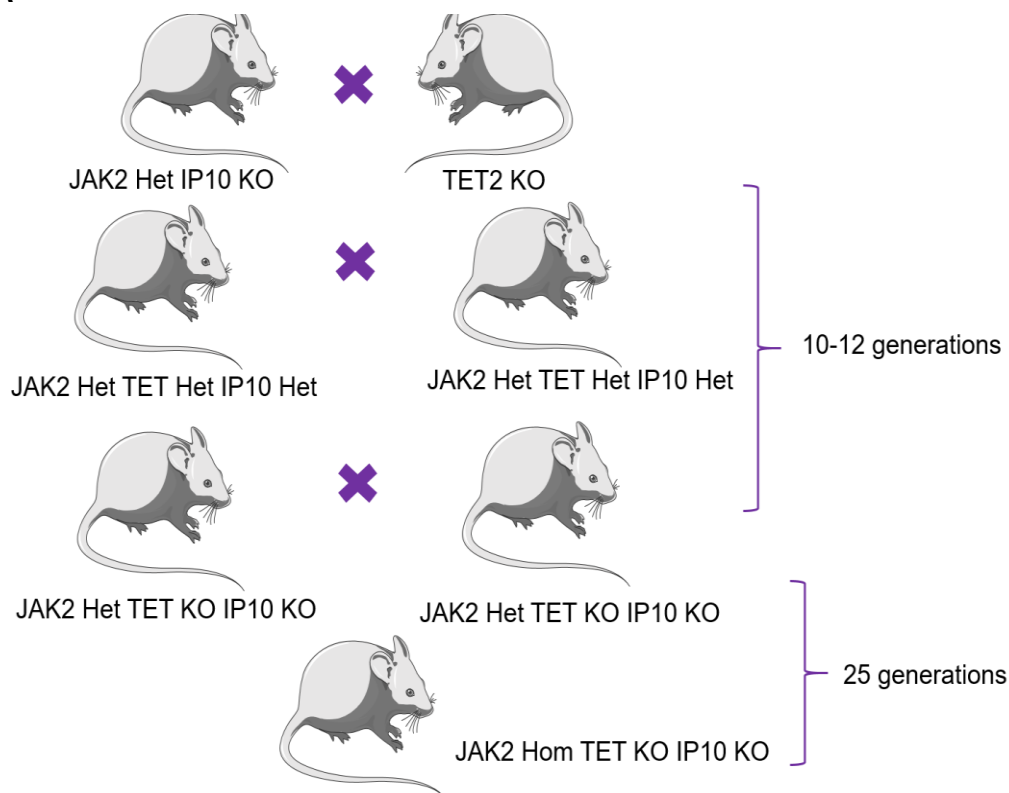
Figure 5.4 Phenotypic characterisation of WT, JAK-2-, IP-10-, and JAK2/IP10 mutant mouse models. Disease monitoring was conducted on wild-type and single- or double-mutant mouse models to assess the impact of JAK2 homozygosity and IP-10 loss on MPN disease phenotype. A slight reduction in haemoglobin of JAK2^{V617F/V617F} IP10^{-/-} mice was observed in comparison to JAK2^{V617F/V617F} mice. Haematocrit levels did not change significantly for JAK2^{V617F/V617F} IP10^{-/-} mice, thereby implying that IP-10 loss in a JAK2 homozygous context did not contribute to the restoration of the PV-like phenotype in mice. One-way ANOVA was performed to calculate statistical significance between groups. * p<0.05; ** p<0.01; ***p<0.001; ****p<0.0001. Figure adapted from Belmonte et al, 2024.

5.2 Generation of an allelic series of a triple-mutant model

Even though the depletion of IP-10 in JAK2 homozygous mice could not completely dampen the red blood cell phenotype, it remained relevant that IP-10 might still play a role in disease development. Moreover, our previous cytokine profiling experiments showed that the presence of homozygous JAK2 and TET2 mutations correlated most strongly with dysregulated expression levels of IP-10. Therefore, we next moved to mutate a third allele - TET2 - in the JAK2/IP10-double mutant cross to generate a triple-mutant cross which carried homozygous mutations of the three genes to fully comprehend how IP-10 was involved in MPNs with the most severe phenotype. A simplified, representative schematic of the breeding strategy is shown on Figure 5.5A. Throughout all breeding rounds, the presence or absence of JAK2, TET2 and IP-10 wild-type, heterozygous and homozygous alleles was validated through genomic PCR (Figure 5.3), which served two main purposes: 1) Selection of mice for breeding 2) Selection of mice for disease monitoring and haematopoietic tissue profiling. Once

JAK2^{V617F/+} TET2^{-/-} IP-10^{-/-} mice were consistently being bred, they were set up as parents of the upcoming generations. The F1 generation offspring from every new breeding pair or trio was genotyped for all three genes to confirm that the allelic combination of the parents was in fact correct (Figure 5.5B). Genotyping results would show that all mice in the progeny carried TET2 and IP-10 homozygous mutations. On the other hand, JAK2 allelic combinations varied due to the fact that, for breeding purposes, male breeders must only carry a JAK2 heterozygous mutation, since male JAK2 homozygous mice are at a higher risk of dying from a spontaneous haematological event during breeding (Li et al, 2014). Death rate in male JAK2^{V617F} homozygous mice is higher than in females. The heaviest rounds of breeding continued for approximately 25 generations until a consistent number of mice of the triple-homozygous genotype were obtained in the progeny.

A



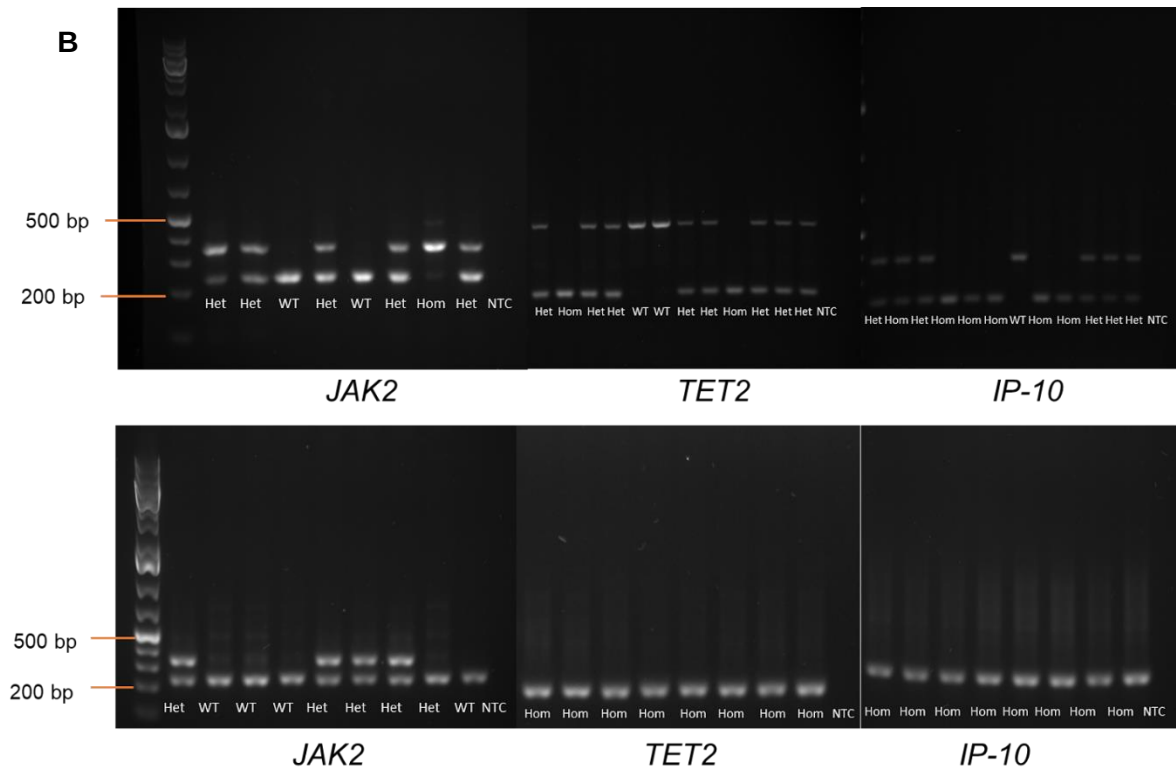
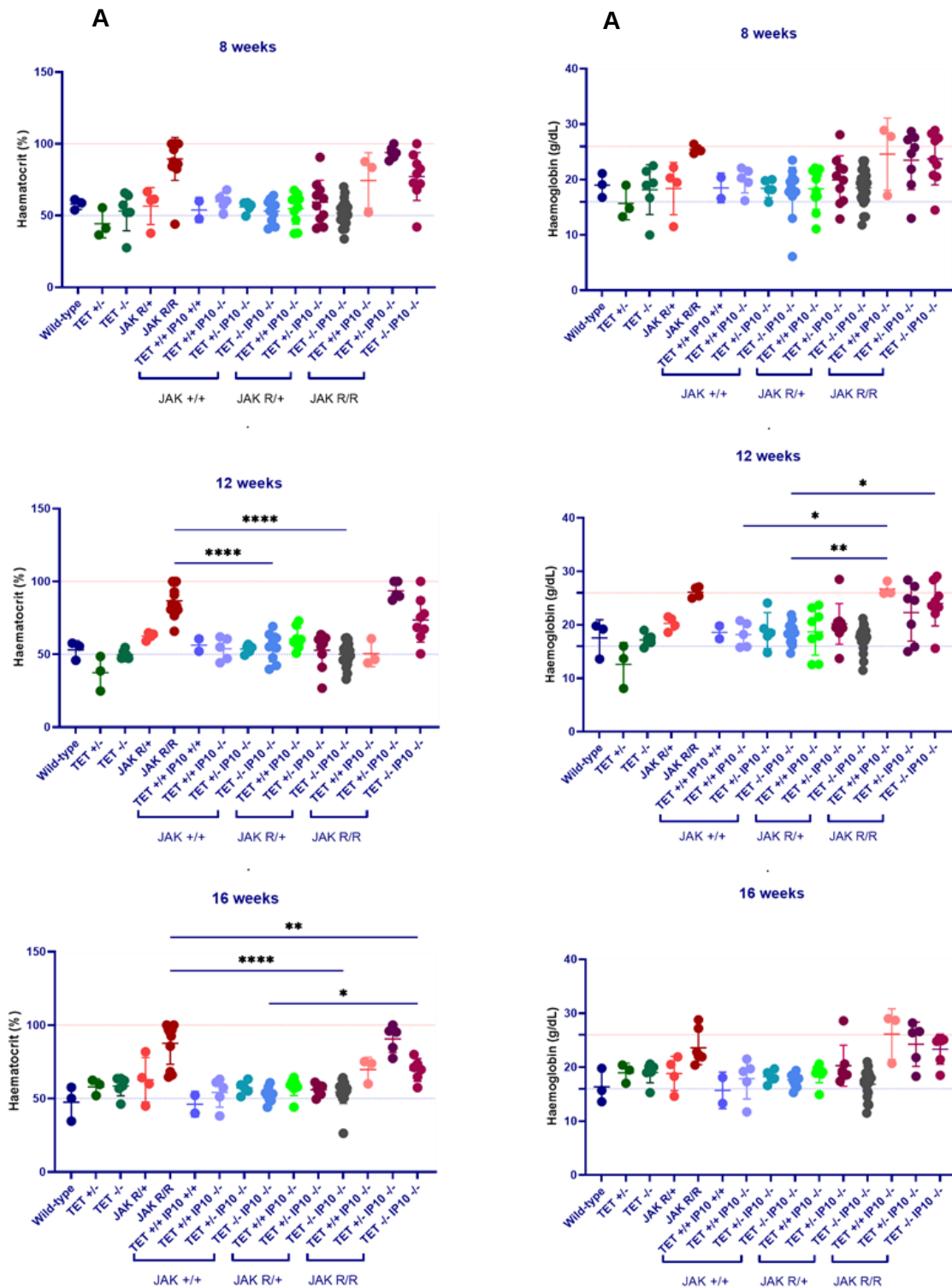


Figure 5.5. Breeding and monitoring of genotypes for the generation of an allelic series of MPN mouse models. A) Breeding strategy. For the initial phase of allelic crosses, we focused on generating a JAK2 heterozygous $TET2^{-/-}$ $IP10^{-/-}$ genotype, which undertook 10-12 rounds of breeding. Mice of this genotype were crossed in order to obtain the $JAK2^{V617F/V617F}$ $TET2^{-/-}$ $IP10^{-/-}$ genotype (this genotype cannot be bred due to the lethality inflicted by JAK2V617F homozygous mutations on males during copulation). B) Genotyping experiments during generation of triple-mutant allelic series mouse model showing wild-type (WT), heterozygous (Het) and homozygous (Hom) allele bands for the JAK2, TET2 and IP-10 genes. Top, genotyping experiment from the earliest generations of individual mice to show various genotypes to illustrate each mutant allele. Bottom, genotyping experiment from the F0 generation of a $JAK2^{R/+}$ $TET2^{-/-}$ $IP10^{-/-}$, where all of the progeny descended from the TET2 and IP-10 homozygous genotype, with JAK2 being maintained as heterozygotes and showing all possible genotypic combinations. To the left of each panel are shown bands of 500-bp and 200-bp size for reference.

5.2.1 Disease monitoring revealed a decrease in haematocrit levels toward late stages of the disease in triple-homozygous mice

As the triple-cross colony was being generated, the impact of IP-10 on the development and maintenance of MPN disease was investigated in mice of the pre-existent JAK2- and TET2- single-mutant mouse models, and JAK/TET/IP10 triple-mutant mice, including a number of wild-type mice as controls. For these disease monitoring experiments, mice were bled every 4 weeks starting at 8 weeks of age through to 16 weeks (Figure 5.6) As reported previously, the $JAK2^{V617F/V617F}$ mutation drives the development of a strong red blood cell phenotype in mice, which resembles the PV phenotype observed in human patients. Such phenotype is

characterised by abnormally high haematocrit, red blood cell and haemoglobin counts, which is prevalent in all JAK2 homozygous mice (Figure 5.6A). Consistently, we observed this same phenotype displayed throughout time in our in-house bred JAK2 mouse model. Disease evolution appeared to be quite similar in the triple-homozygous mouse model as in the JAK2 homozygous mice in the earlier stages, i.e., at 8 and 12 weeks of age specifically when focusing on two of the main informative parameters for MPN evolution - Haematocrit (defined as the ratio between red blood cells and total blood volume) and Haemoglobin (measured in g/dL) (Figure 5.6B). Notably, at 12 weeks, haematocrit in JAK2^{+/+} TET2^{-/-} IP-10^{-/-} and in JAK2^{V617F/+} TET2^{-/-} IP-10^{-/-} mice were significantly lower ($p < 0.0001$) than in JAK2 homozygous mice only. However, at 16 weeks, haematocrit levels were significantly reduced in the triple-homozygous mice when compared with JAK2 homozygous mice ($p < 0.01$) (Figure 5.6A), almost approaching the normal expected range of haematocrit in wild-type mice (approximately 50%), implying that the absence of IP-10 initiates the restoration of the normal phenotype from the one caused by JAK2 homozygous mutations. On the other hand, mice of the JAK2^{+/+} TET2^{-/-} IP10^{-/-} genotype showed lower haematocrit at 16 weeks than triple-homozygous animals, probably indicating that it is mainly the JAK2-driven phenotype the IP-10 depletion is attempting to rescue. Despite the fact that there were no significant reductions in haemoglobin counts throughout time between JAK2-mutant and triple-homozygous mice, there was a traceable trend of reduction towards the latest time point, which concurs with the decrease of the haematocrit around the same stage of disease evolution (Figure 5.6B).



average haematocrit and haemoglobin values in wild-type mice. Bars show mean with standard deviation. One-way ordinary ANOVA analysis with multiple comparisons: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

5.2.1.1 White blood cells and platelets remained unaffected by the loss of IP-10 in a JAK2/TET2 homozygous background

Platelet and white blood cell counts (WBC) were not majorly altered by the absence of IP-10 in the triple-mutant mouse model. Nonetheless, WBC counts were significantly increased in JAK2^{+/+} TET2^{-/-} IP10^{-/-} mice in relation to TET2^{-/-} ($p < 0.01$) and JAK2^{V617F/+} TET2^{-/-} IP-10^{-/-} ($p < 0.05$) mice at 8 weeks of age (Figure 5.7A). Furthermore, WBC counts at 8 and 12 weeks were modestly, although not significantly, increased in the triple-homozygous mice (Figure 5.7A). Notwithstanding, at 16 weeks, WBC counts decreased, very similarly to what was observed in wild-type mice.

Platelet counts varied throughout the different timepoints for mice which were monitored (Figure 5.7B). For most of the genotypes, platelet counts mildly decreased toward the latest timepoint, with the exception of the JAK2^{V617F/V617F} TET2^{+/+} IP-10^{-/-} and JAK2^{V617F/V617F} TET2^{+/-} IP-10^{-/-} mice, in which values were maintained consistently across timepoints. Although not statistically significant, platelet counts in triple-homozygous mice were similar to those from JAK2 homozygous mice at 8 and 16 weeks particularly. At 12 weeks, nevertheless, platelet counts were slightly decreased in the triple-homozygous mice when compared to JAK2 homozygous mice.

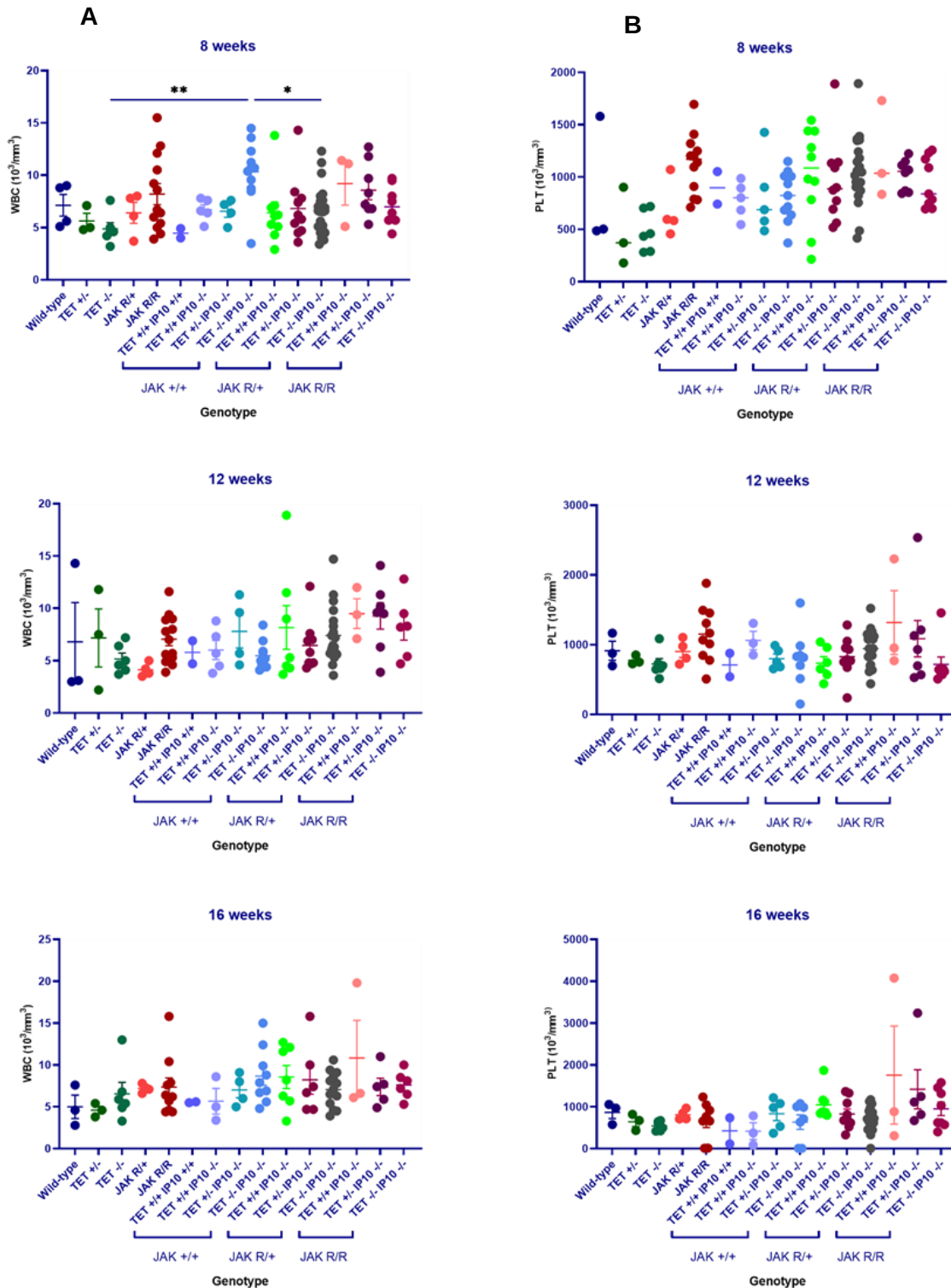


Figure 5.7. Disease monitoring in MPN mouse models by blood phenotyping. A) White blood cell and b) Platelet counts at 8, 12 and 16 weeks across all genotypes monitored. Elevated WBC counts were observed in JAK^{+/+} TET^{-/-} IP10^{-/-} (TET Hom IP10 Hom) mice at 8 weeks of age, compared to TET²^{-/-} and JAK2 Het TET²^{-/-} IP10^{-/-} mice. Throughout the rest of the monitoring, blood counts remained at comparable levels across the myriad of genotypes. Bars show mean with standard deviation. One-way ANOVA analysis with multiple comparisons: * = p<0.05, ** = p<0.01.

5.3 Analysis of cell populations in bone marrow and spleen of wild-type, single- and triple-mutant mouse models associated depletion of IP-10 with potential cellular composition changes

To understand the effects of IP-10 loss in the cellular composition of haematopoietic tissues, bone marrow and spleen of all monitored animals were collected at the end point of the study, namely, between 16 and 24 weeks of age. Tissues were analysed in their intact form (i.e., no magnetic separation of cellular fractions) and were analysed to search two kinds of populations:

1) Mature cell populations of the erythroid lineage (CD71/TER119 populations) (Figure 5.8), the myeloid lineage (Ly6G+ CD11b+ (GM) and CD11b+ monocytes) and the lymphoid lineage (B220+ B cells and CD3+ T cells) (Figure 5.9). The rationale for choosing these population is as follows: In mouse models of the JAK2 V617F mutation, one of the main features of the PV phenotype is the increase of CD71+ Ter119+ and CD71- Ter119+ progenitors in the bone marrow, in addition to elevated frequency of CD11b+ Gr-1+ cells in the bone marrow and spleen (Li et al, 2010). Furthermore, in CFU assays, the number of BFU-E colonies formed by JAK2 V617F mutant progenitors is equally elevated. Additionally, JAK2 V617F is not commonly detected in the lymphocyte fraction (Choi et al, 2024) due to the myeloid skewage of JAK2 V617F progenitors (Jamieson et al, 2006), also implying impaired lymphocyte differentiation (Li et al, 2010), which could then inform about the severity of disease in mice.

2) Stem/progenitor cells, which included lineage negative cells (Lin-), Lin- Sca-1+ c-Kit+ cells (LSK) and EPCR+ CD45+ CD150+ CD48- cells (ESLAM). The rationale for selecting such populations is as follows: The earliest studies of JAK2 V617F mutation in MPN patients revealed that the mutation is acquired in the HSC and HSPC compartment (Jamieson et al, 2006), which is why the different MPN types are considered to be diseases initiated at the single-cell level. While JAK2 V617F impairs the self-renewal potential of HSC and HSPC populations (Li et al, 2010; Kent et al, 2013), it does not affect the expansion potential of committed progenitors (Kent et al, 2013). In addition, extramedullary haematopoiesis is a phenomenon observed in MPN patients and mouse models, whereby the spleen size increases due to recruitment of HSCs/ HSPCs, which – in junction with other reasons – results in splenomegaly (Prakash et al, 2013). Therefore, to conduct a comprehensive characterisation of the triple-mutant cross, two panels were designed in order to observe the frequency of all these populations in bone marrow and spleen (see Tables 2.11 and 2.12).

5.3.1 Absence of IP-10 could potentially contribute to the restoration of erythrocyte development in the bone marrow and spleen of triple homozygous mice

The differential expression levels of CD71 and TER119 were analysed to understand the changes in erythropoiesis across genotypes, and especially in the novel triple-mutant mouse model. In the bone marrow particularly, there was a larger accumulation of stage I erythroblasts (CD71⁺ TER119⁻) in wild-type ($p < 0.05$), JAK2^{+/+} TET2^{-/-} IP-10^{-/-} ($p < 0.0001$) and triple homozygous ($p < 0.05$) mice than in JAK2 homozygous mice (Figure 5.8A). CD71⁺ TER119⁺ stage III erythroblasts were significantly more abundant in the bone marrow of JAK2^{+/+} TET2^{-/-} IP-10^{-/-} mice than in triple-homozygous ($p < 0.05$) and JAK2^{V617F/+} TET2^{-/-} IP-10^{-/-} ($p < 0.001$) mice. Toward later stages of erythroid development, as well as in stage II (CD71⁺ TER119^{low}) there were no major changes across genotypes.

In the spleen, JAK2 homozygous mice had a lower accumulation of proerythroblasts than JAK2^{+/+} TET2^{-/-} IP-10^{-/-} mice, whereas in triple homozygous mice this early-stage cells were less abundant than in JAK2^{V617F/+} TET2^{-/-} IP-10^{-/-} animals (Figure 5.8B). Stage III erythroblasts, on the other hand, were significantly more abundant in JAK2^{V617F/V617F} TET2^{+/-} IP-10^{-/-} and JAK2 homozygous mice in comparison to JAK2^{V617F/+} TET2^{-/-} IP-10^{-/-} mice, suggesting that a heterozygous mutation in JAK2 accompanied by TET2 LOF and IP-10 absence decreases the proportion of stages I and III erythroblasts. Moreover, splenic stage V erythroblasts were accumulated in a higher proportion in triple homozygous ($p < 0.05$) and JAK2^{V617F/+} TET2^{-/-} IP-10^{-/-} mice in relation to JAK2 homozygous animals.

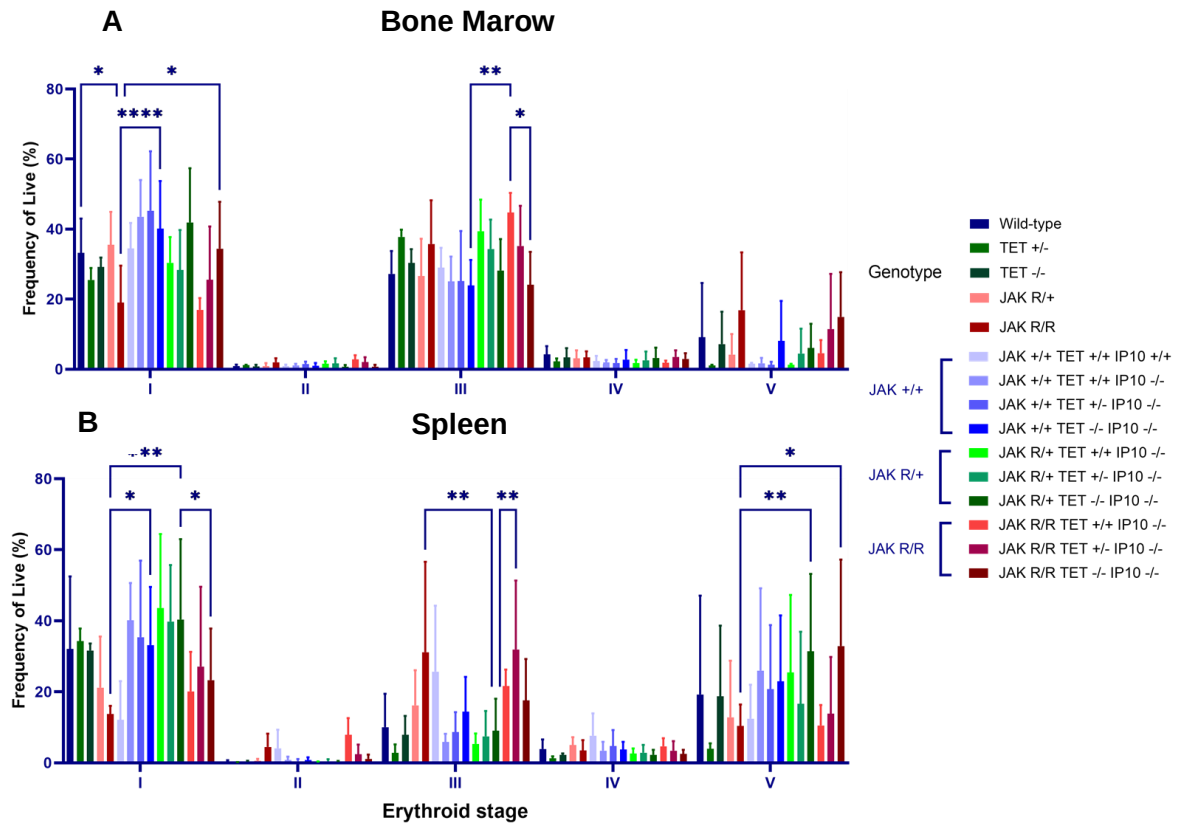


Figure 5.8. Erythroid stages in A) Bone Marrow and B) Spleen of MPN mouse models. Analysis of CD71 and TER119 expression allowed for the determination of five stages of erythroid differentiation: I = CD71⁻ TER119⁻, II = CD71⁺ TER119^{low}, III = CD71⁺ TER119⁺, IV = CD71^{low} TER119⁺, V = CD71⁻ TER119⁺. Bars represent mean with standard deviation. Two-way ANOVA to calculate statistical significance between multiple groups: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

5.3.2 The granulo-myeloid compartment was not considerably affected by homozygous mutations in JAK2, TET2 and IP-10

The granulo-myeloid (GM) compartment, defined as double Ly6G/CD11b positive cells as well as CD11b⁺ monocytes were likewise profiled in the bone marrow and spleen. GM proportions were generally higher in the bone marrow across genotypes (Figure 5.9A), although there was a slight increase in GM frequency in JAK2^{+/+} TET2^{-/-} IP-10^{-/-} and triple homozygous mice - which displayed similar proportions to wild-type specimens - than in JAK2 homozygous animals. CD11b⁺ monocyte frequencies did not vary significantly in the bone marrow, yet the frequency was modestly lower in JAK2 homozygous than for the rest of the genotypes.

As shown in Figure 5.9B, the GM proportions in the spleen were uniform across genotypes, in contrast to CD11b⁺ monocytes, which were decreased in JAK2 homozygous, JAK2^{+/+} TET2^{-/-} IP-10^{-/-} and JAK2^{V617F/V617F} TET2^{+/+} IP-10^{-/-} specimens, although these changes were not statistically significant.

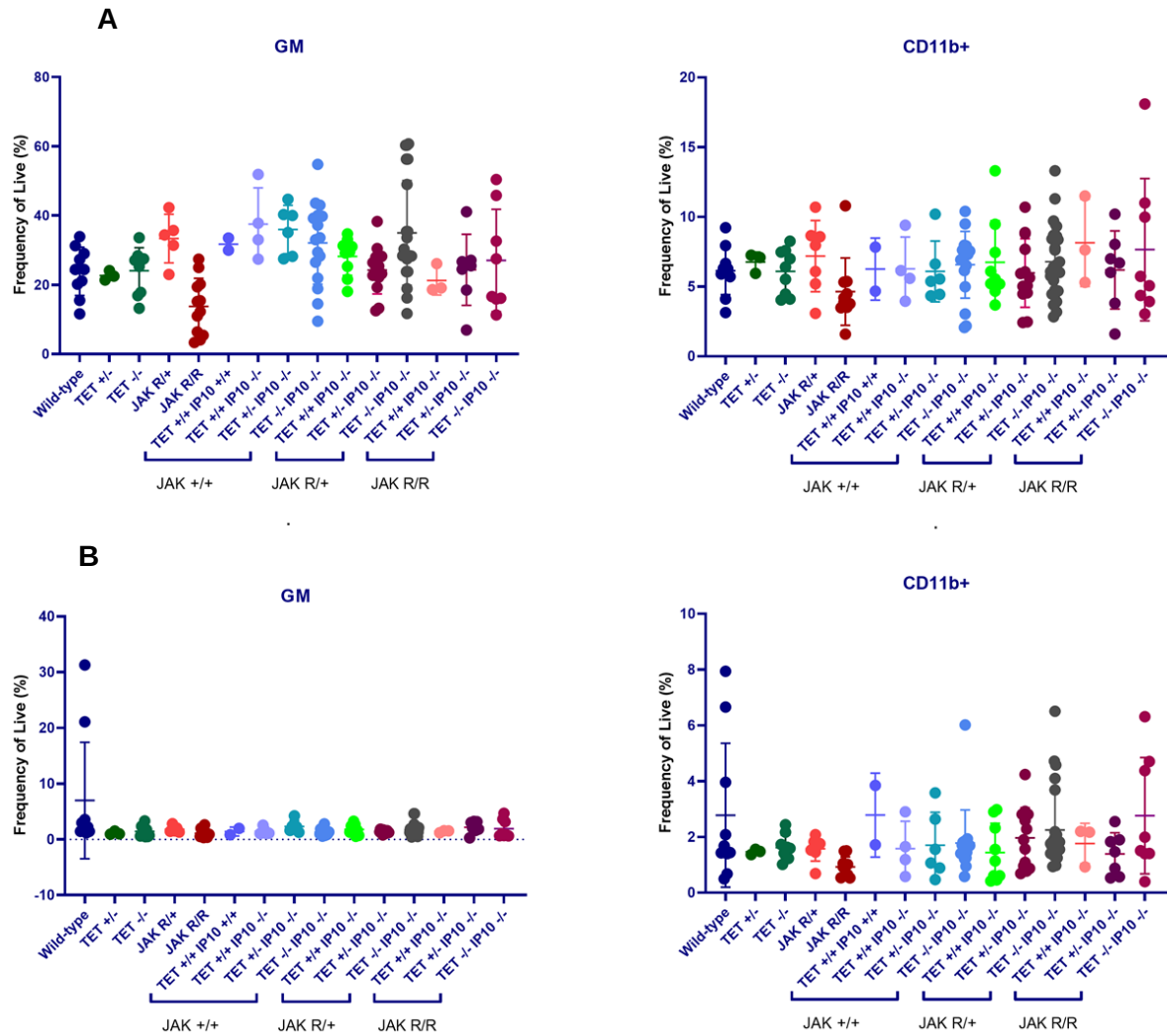


Figure 5.9. Granulo-myeloid (GM) and Monocyte (CD11b+) frequencies in: A) Bone Marrow and B) Spleen. In both tissues, the frequencies of the GM and monocyte (CD11b+) fractions were kept at comparable levels across the different genotypes, regardless of JAK2 mutational status. Frequencies were calculated out of Live cells. Bars represent mean with SEM. One-way ANOVA analysis did not identify any statistically significant differences.

5.3.2.1 B-lymphocytes in the bone marrow were reduced in triple homozygous mice, while remaining unaffected in the spleen

When analysing the B- and T-cell compartments, which in this piece of work are defined as B220+ and CD3+ cells, respectively, there were some significant differences both in bone marrow and spleen tissue. B-cell frequency in the bone marrow of triple homozygous mice was significantly lower ($p < 0.01$) than in wild-type mice (Figure 5.10A). This was equally observed in JAK2V617F/+ TET2-/- IP-10-/- mice ($p < 0.01$) in relation to wild-type specimens. The CD3+ compartment did not undergo any major changes in the bone marrow across

genotypes. Notwithstanding, CD3⁺ frequencies were quite mildly increased in JAK2 homozygous and JAK2^{V16F/+} TET2^{-/-} IP-10^{-/-} in comparison to the rest of the genotypes. Additionally, B-cell proportions in the spleen were significantly higher in JAK2V617F/+ TET^{+/+} IP-10^{-/-} animals than in JAK2 homozygous mice (Figure 5.10B). Triple homozygous likewise displayed a lower frequency of B-cells. As expected, the frequency of CD3⁺ T-cells in the spleen was lower in JAK2 homozygous animals, and was shown to be significantly lower than in JAK2/TET2 heterozygous and IP-10 homozygous mice.

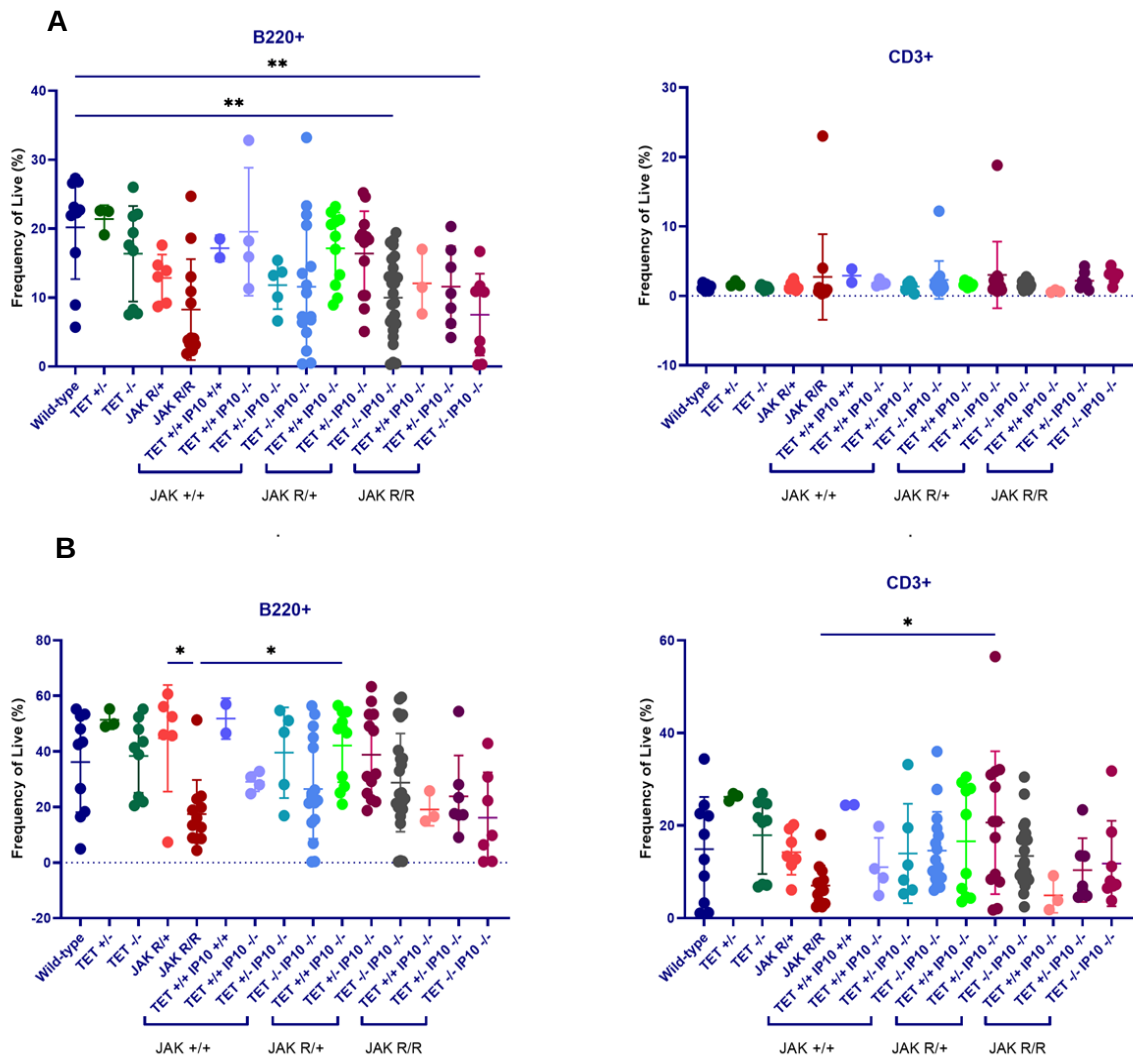


Figure 5.10. B- (B220⁺) and T- (CD3⁺) cell frequencies in: A) Bone Marrow and B) Spleen. B-cell frequencies were significantly reduced in the triple homozygous mice, in comparison to wild-type controls. On the other hand, the T-cell fraction remained largely unchanged across genotypes in bone marrow and spleen, with significant increases in mice with JAK2 heterozygous mutations, which implied that JAK2 homozygosity in conjunction with TET2 and IP-10 loss did not impact the frequencies of B and T cells in haematopoietic tissues. Bars represent mean with standard deviation. One-way ordinary ANOVA analysis: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

5.3.2.2 Lineage-negative and LSK frequencies in the bone marrow were lower by IP-10 loss in a JAK2/TET2 homozygous background

To further our understanding of the changes caused by the absence of IP-10 in the newly developed triple-cross, stem and progenitor cell populations were surveyed in bone marrow and spleen (Figure 5.10). The lineage-negative (Lin⁻) population in the bone marrow (Figure 5.10A) was largely intact across genotypes, however, in JAK2^{V617F/+} TET2^{-/-} IP10^{-/-} animals, the Lin⁻ frequency was significantly higher than in wild-type ($p < 0.0001$) and triple-homozygous mice ($p < 0.001$). Interestingly, LSK frequency in mice of the JAK2^{+/+} TET2^{-/-} IP-10^{-/-} genotype had a pronounced increase in relation to TET2 homozygous ($p < 0.01$), JAK2 homozygous ($p < 0.0001$) and triple homozygous mice ($p < 0.05$).

Contrastingly, in the spleen, Lin⁻ frequencies did not undergo any significant changes (Figure 5.10B). The ESLAM compartment, however, was more abundant in the triple homozygous mice than for the rest of the genotypes surveyed, although this could also be due to a larger standard deviation error. IP-10 depletion did not induce any significant changes over the LSK population in the presence or absence of JAK2/TET2 homozygosity.

extramedullary haematopoiesis. Bars represent mean with standard deviation. One-way ordinary ANOVA analysis: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

5.4 Serum cytokine and chemokine screen across wild-type, single- and triple-mutant mouse models

To elucidate cytokine expression patterns in the newly developed triple-cross line compared to our historic MPN mouse model data, a serum cytokine/chemokine screen was conducted in 6 different genotypes which had been regularly monitored for disease parameters and haematopoietic tissue composition: wild-type, JAK2 homozygous, TET2 homozygous, JAK2^{+/+} TET2^{-/-} IP-10^{-/-}, JAK2^{V617F/+} TET2^{-/-} IP-10^{-/-} and JAK2^{V617F/V617F} TET2^{+/+} IP-10^{-/-}. The genotypes were selected based on the following criteria: 1) Data from interval blood monitoring 2) Data from tissue cellular composition analysis and 3) Relevance of IP-10 absence for the present study, for which IP-10 knockout genotypes were included preferentially over IP-10 heterozygous or IP-10 wild-type genotypes. Due to the lack of availability of at least three independent replicates from the triple-homozygous line at the time this experiment was performed, it was not possible to include such genotype in this analysis. Nevertheless, it was included in the validation of this experiment, which will be described in the next section.

5.4.1 Dimensionality reduction analysis identified IL-12(p40), RANTES, IL-2 and MCP-1 as main contributors to differences in the data

The first multi-dimensional data analysis approach applied to the cytokine/chemokine screen dataset was the cosine similarity method (Figure 5.12A), which calculates the distance between vectors in a high-parameter dataset to determine the extent to which they contribute to differences within the data (Canessa et al, 2020; Han, Kamber & Pei, 2012). Parallel to this method, a loading plot of a principal component analysis (PCA) of variables only was obtained to visualise cosine similarity (Figure 5.12B). From both methods, it was inferred that the vectors represented by IL-12(p40), CCL5 (RANTES), M-CSF, IL-2 and MCP-1 (CCL2) were the most distanced from the point of origin in the dataset space, implying that these molecules could be strongly correlated with each other or else, with a specific genotype or group of genotypes. However, since IP-10 was absent in three of the six genotypes analysed, it was possible that it was driving part of the differences in the data. Therefore, IP-10 was removed from further analysis, and I continued with the remaining 31 cytokines in the panel.

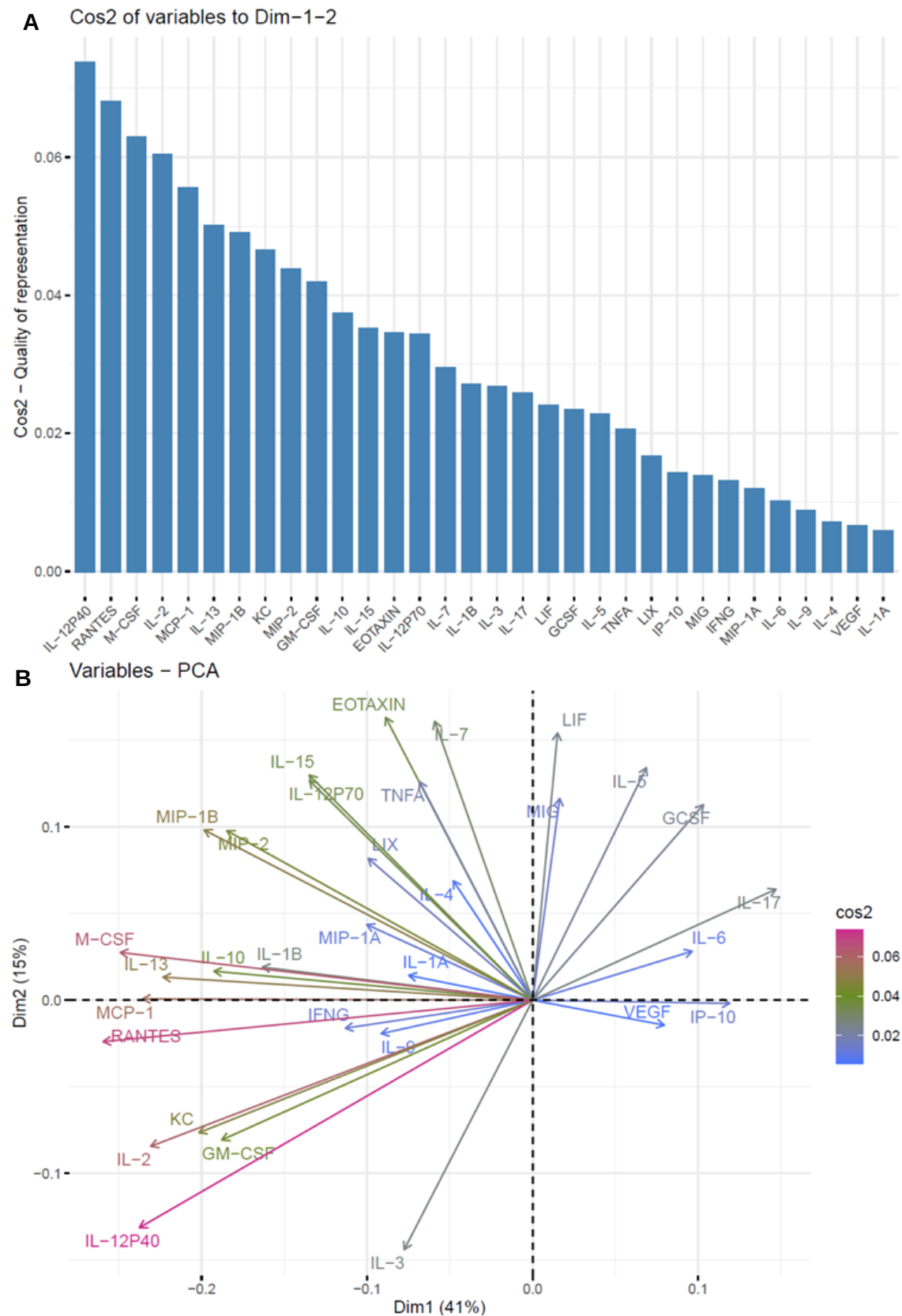
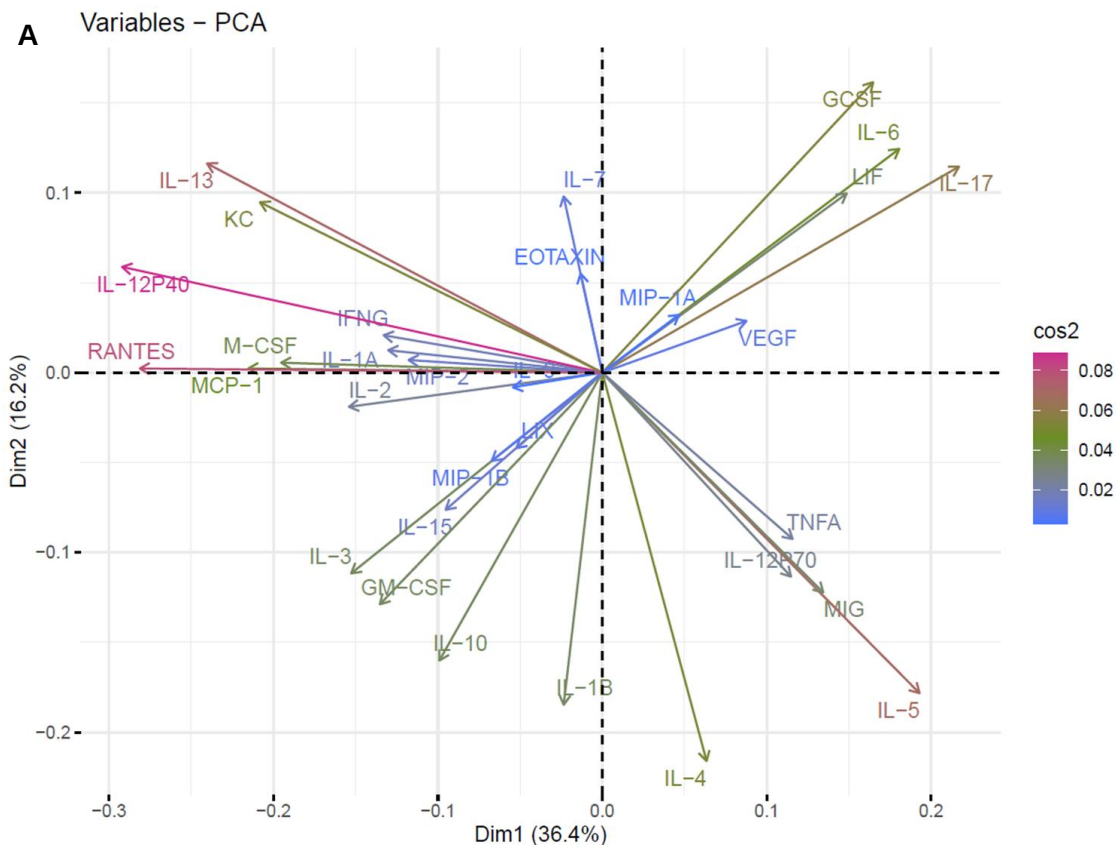


Figure 5.12. Dimensionality reduction analysis of the cytokine/chemokine screen analysis by two methods.
A) Cosine similarity A high Cos2 (quality of representation) indicates a good correlation of variable within the

principal components, here referred to as Dimensions 1 and 2. A lower Cos2 indicates lower representation of the variable in the principal components. B) Loading plot from principal component analysis. Colour scale bar at the right of the panel represents the Cos2 quality of representation assigned to each vector or variable in the analysis.

5.4.1.1 Principal component analysis of genotype groups identified data clustering mainly associated with IL-12(p40), RANTES and G-CSF

Once IP-10 had been removed from the analysis, dimensionality reduction analysis was performed again and a second loading plot was produced to visualise the data (Figure 5.13A). IL-12(p40), CCL5, M-CSF, IL-2 and CCL2 were still observed to potentially drive differences in the data. However, the vectors corresponding to Eotaxin and CXCL1 were likewise distanced from the axis, therefore implying that these could also contribute to data variation. To understand what the implications of the dimensionality analysis results were for data clustering, a PCA analysis was performed introducing the 6 different genotypes as another variable (Figure 5.13B). JAK2 homozygous, JAK2^{V617F/V617F} TET2^{+/-} IP10^{-/-}, JAK2^{V617F/+} TET2^{-/-} IP10^{-/-} samples were positioned separately from the majority of the genotypes, specifically from wild-type controls and mice without loss of IP-10 (IP-10 wild-type). Visualisation of the loading plot onto data clusters allowed determination of the molecules which strongly contributed to sample clustering, thus confirming the results obtained from the cosine similarity analysis (Figure 5.13B).



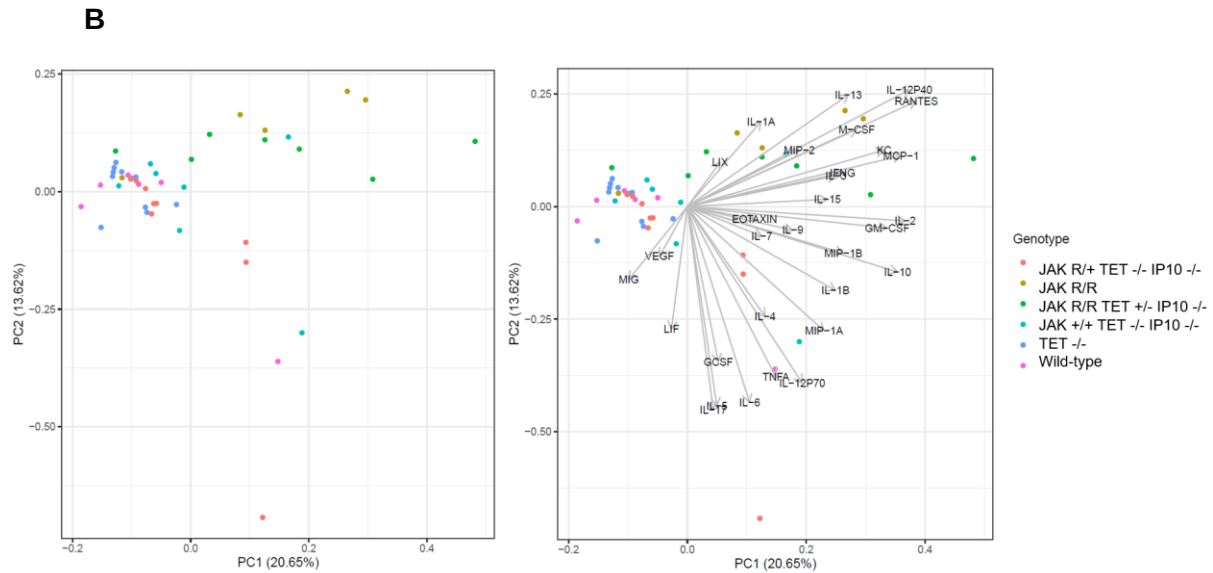


Figure 5.13. PCA of mouse serum samples from the multiplex magnetic bead-based multiplexed ELISA experiment. A) Loading plot from principal component analysis with IP-10 removed from the analysis, displaying data from 31 cytokines. Colour scale bar at the right of the panel represents the Cos2 quality of representation assigned to each vector or variable in the analysis. B) PCA (PC1 = 13.62%, PC2 = 20.65%) plot with loadings, showing the molecules identified from the cosine similarity analysis contributing to sample clustering by genotype.

5.4.1.2 Activation of JAK2 in combination with IP-10 loss drives a distinct cytokine expression pattern

Considering the results from the PCA analysis and data clustering by genotype, expression levels of cytokines and chemokines as individual genotypic entities was assessed (Figure 5.14). A correlation coefficient analysis was executed to investigate the potential interplay between different molecules in the microenvironment. Correlations between CXCL1 and IL-12(p40), M-CSF and IL-13, IL-6 and IL-17, were among the most prominent (Figure 5.14A), primarily due to what the PCA analysis had shown, in addition to the results shown by correlation analysis of cytokine expression levels across the different genotypes. Although these correlations have not been specifically described in myeloproliferative disorders, for instance, previous studies have implicated IL-13 signalling in the activation of the JAK/STAT pathway, mainly through the phosphorylation of JAK2 and STAT6 in endothelial cell lines (Palmer-Crocker, 1996), with oncogenic consequences in colon carcinoma cell lines (Murata et al, 1996). Aberrant IL-13 signalling in macrophages has been shown to induce breast cancer invasion (Little et al, 2019), attributing oncogenic roles to IL-13. Importantly, CXCL1 (equivalent to GRO- α in humans) along with its receptor, CXCR2, has previously been implicated in AML (Tang et al, 2020; Korbecki et al, 2024), as well as MPNs (Collinson et al, 2020). In the MPN serum cytokine profiling described in section 5.1, overexpression of GRO-

α was likewise associated with increased risk of disease transformation (Obro et al, 2020). In CML, TNF- α activates the CXCR2 receptor, and therefore supports expansion of the LSC compartment, while impairing normal HSC functions (Agarwal et al, 2021). Furthermore, given the crucial regulatory role of IL-6 on the JAK/STAT pathway, its aberrant expression and activity has been implicated in many types of cancer (Huan et al, 2022). In MPN patients, abnormal levels IL-6 secreted in the stromal compartment within the niche and in peripheral blood cells result in cytotoxicity to the immune system (Torres et al, 2022). In addition, the synergistic role between IL-6 and IL-17 in the regulation inflammation and immune cell recruitment through activation of the NF- κ B pathway (Camporeale & Poli, 2012), also accounts for the correlation observed between these two molecules in our dataset. Therefore, to further explore these correlations, the serum levels of all 31 cytokines were analysed across the different MPN mouse models.

5.4.1.3 Cytokine expression signatures indicated a potential stromal contribution in compensation for IP-10 loss

When performing an individual cytokine expression survey, it was particularly evident that the loss of IP-10 in combination with JAK2 homozygous mutations allowed the elucidation of cytokine expression patterns (Figure 5.14B). Equally as interesting, combinatorial IP-10 heterozygosity with JAK2/TET2 homozygosity displayed quite a unique pattern, where the majority of serum cytokines profiled were highly expressed. However, since JAK2/TET2 homozygous and IP-10 heterozygous mice do not present a phenotype, this might be attributed to the JAK2/TET2 homozygous mutations. TET2 homozygous and heterozygous and JAK2 homozygous showed an elevated expression of IP-10, being particularly higher in the TET2 homozygous mice, which agrees with initial cytokine profiling experiment which identified IP-10 dysregulation in MPN mouse models. As expected, there was no IP-10 expression detected in the triple-mutant model, except for the JAK2^{V617F/V617F} TET2^{-/-} IP-10^{+/-} genotype group, confirming the absence of the molecule in the cross. Notably, in the JAK2^{V617F/V627F} TET2^{+/-} IP-10^{-/-} group, GM-CSF, CXCL1, IL-3, MCP-1, LIF (leukaemia inhibitory factor) were highly expressed and constituted the expression pattern in this particular genotype, which was consistent with previous analysis results.

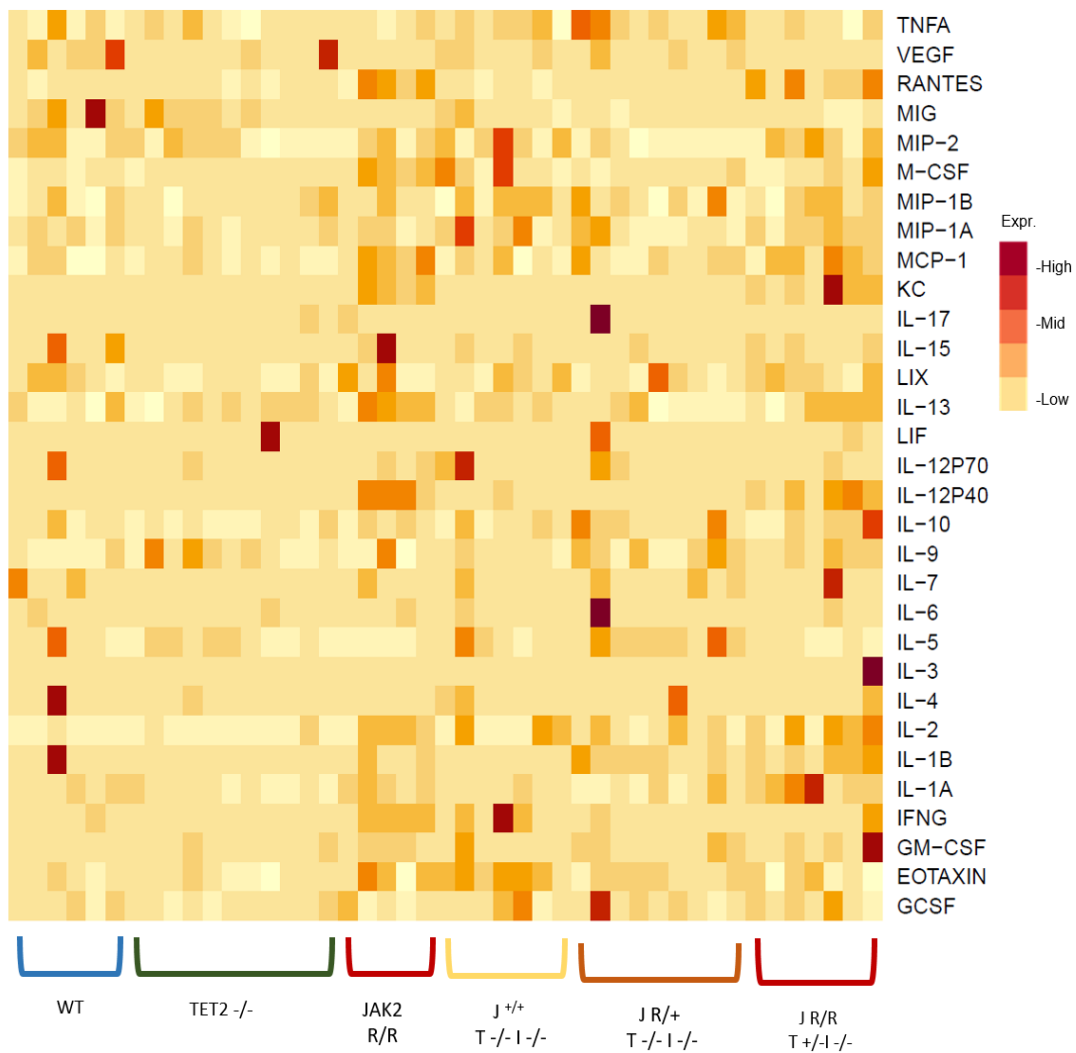
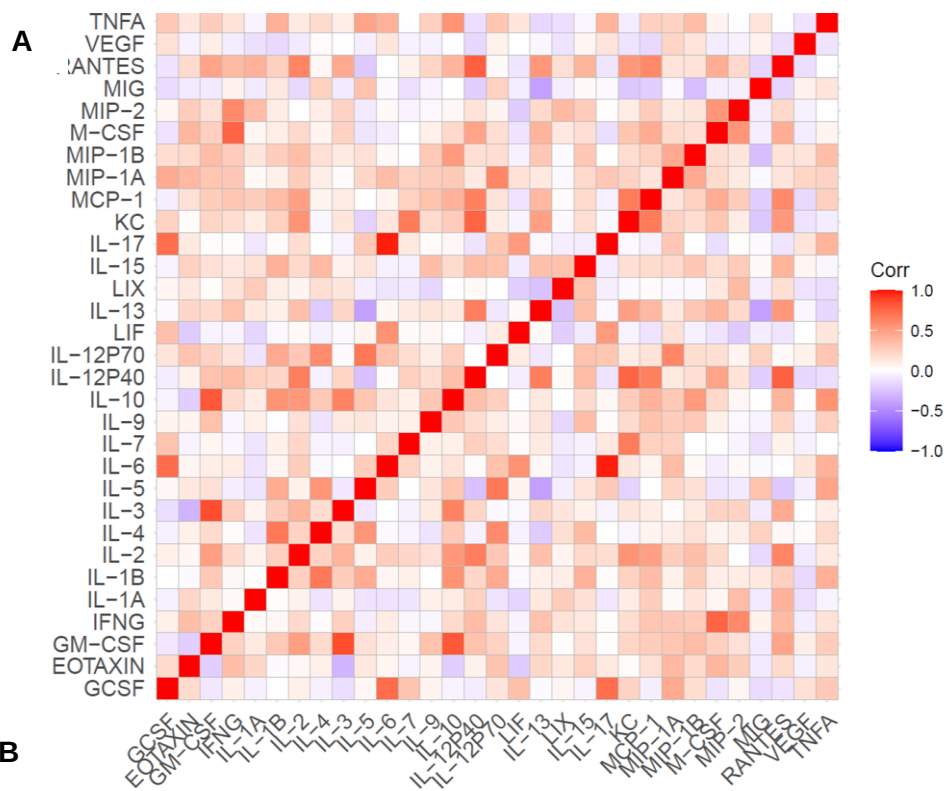


Figure 5.14. Cytokine expression profiling across MPN mouse models and wild-type controls. A), Correlation matrix showing relationships between the 31 different cytokines profiled in the multiplexed ELISA experiment. Correlation strength colour scale shown on the right side of the panel (+1 = highest to -1 = lowest). B) Heatmap showing individual cytokine expression by genotype. Expression colour scale bar to the right of the panel shows higher to lower expression by red colour tone.

5.4.1.4 The expression of IL-12P40, CCL5, and Eotaxin was significantly elevated in the serum of JAK2^{V617F/V617F} and JAK2^{V617F/V617F} TET2^{-/-} IP10^{-/-} mice

Single molecule expression values were plotted for six selected genotypes: wild-type controls, TET2 homozygous, JAK2 homozygous, JAK2^{V617F/+} TET2^{-/-} IP-10^{-/-}, JAK2^{+/+} TET2^{-/-} IP10^{-/-}, JAK2^{V617F/V617F} TET2^{+/-} IP-10^{-/-} to compared between these (Figure 5.15). Of note, IL-12(p40) and RANTES (CCL5) levels was significantly higher in JAK2^{V617F/V617F} and JAK2^{V617F/V617F} TET2^{+/-} IP-10^{-/-} mice than in wild-type ($p < 0.01$ and $p < 0.001$, respectively) and TET2^{-/-} animals ($p < 0.001$ and $p < 0.01$, respectively). Eotaxin was highly expressed in TET2/IP-10 homozygous mice in relation to wild-type and TET2 homozygous mice ($p < 0.01$), whereas expression in other triple-mutant genotypes did not show any significant changes. Furthermore, although M-CSF had been identified as a variable contributing to differences within the data, it was not significantly higher in any of the genotypes. However, it showed strong correlation with data variability and clustering.

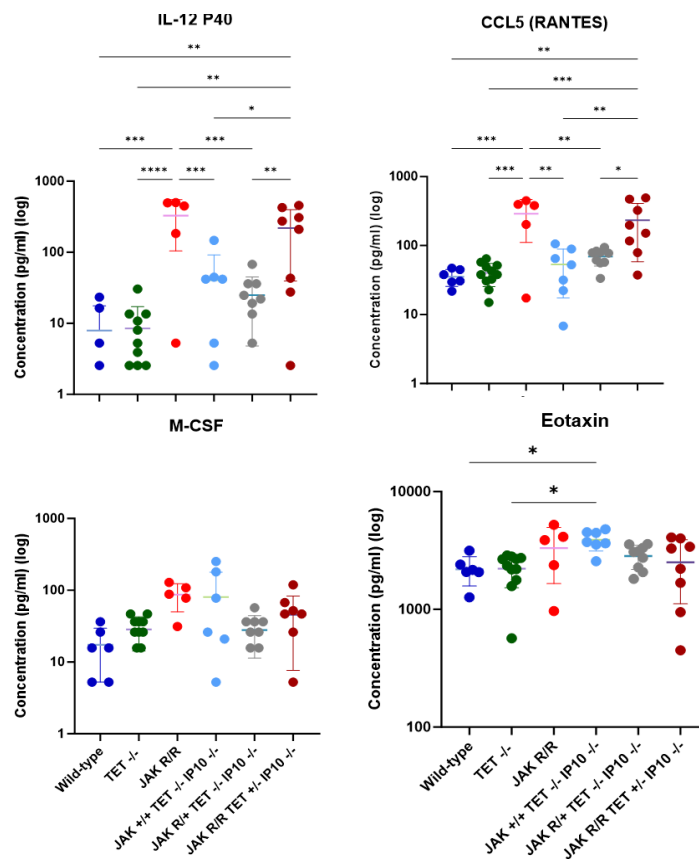
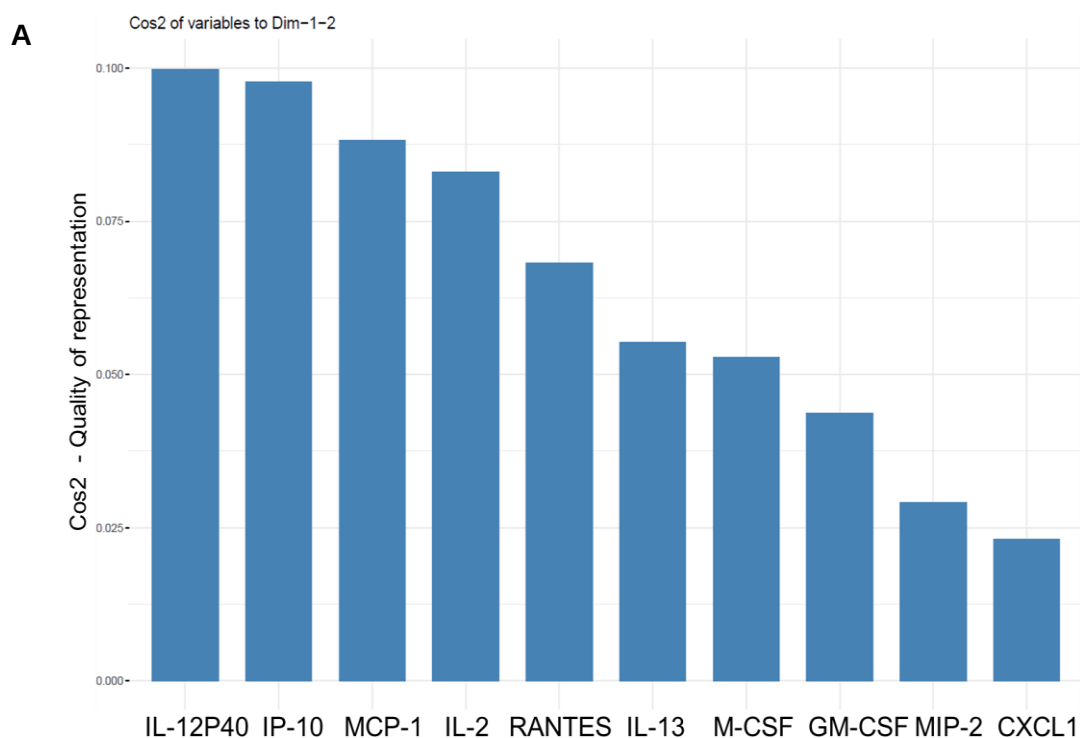


Figure 5.15. Expression levels of individual cytokines in serum of MPN mouse models. IL-12_{p40}, CCL5 (RANTES), M-CSF and Eotaxin cytokine levels are expressed as picograms/mL in logarithmic scale. IL-12_{p40} and CCL5 were expressed at significant elevated levels in JAK2^{V617F/V617F} and triple-homozygous mice. Eotaxin and M-CSF serum concentrations were comparable across genotypes, with the exception of TET2/IP10 homozygous mice, whereby Eotaxin levels were significantly increased in relation to TET2^{-/-} and wild-type mice. Bars represent mean with standard deviation. One-way ordinary ANOVA analysis: * = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001.

5.5 Analysis of selected 10 serum cytokines revealed subtle but noticeable differences across genotypes

In order to validate the results from the 32-plex experiment, the molecules that produced the most relevant and statistically significant data for all genotypes analysed were selected to design a new 10-cytokine panel. The 10 selected cytokines were IL-12_{p40}, IL-13, IL-2, GM-CSF, IP-10, CXCL1, MCP-1, M-CSF, MIP-2 and RANTES (CCL5) (IP-10 was selected mainly to verify its low to null expression in the IP-10 knockout genotypes) (Figure 5.16). Similarly as for the 32-plex experiment, serum samples from mice of 10 genotypes were included: wild-type, TET2 homozygous, JAK2 homozygous, JAK2^{+/+} TET2^{-/-} IP10^{-/-}, JAK2^{+/+} TET2^{+/-} IP10^{-/-}, JAK2^{V617F/+} TET2^{-/-} IP10^{-/-}, JAK2^{V617F/+} TET2^{+/-}, JAK2^{V617F/V617F} TET2^{-/-} IP10^{-/-}, JAK2^{V617F/V617F} TET2^{+/-} IP10^{-/-} and JAK2^{V617F/V617F} TET2^{-/-} IP10^{+/+}. Samples from the latter genotype were not available at the time when the first experiment was performed, which is the reason why it was not included. Likewise, for this second experiment, it was possible to successfully collect 10 serum samples from triple-homozygous mice, which added grater statistical power to the dataset.



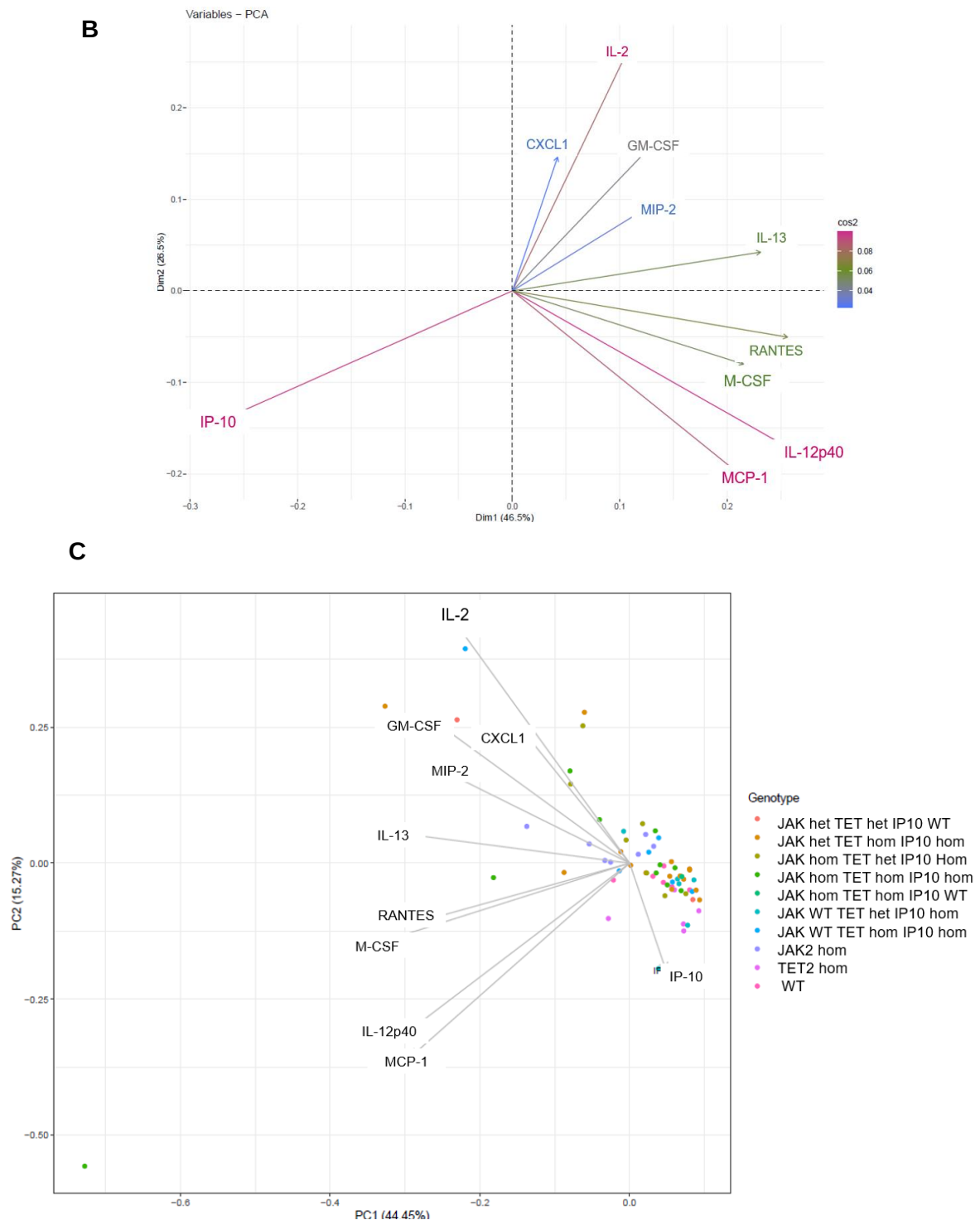


Figure 5.16 Dimensionality reduction of 10-plex data. A) Cosine similarity analysis of 10-plex data identified IL-12p40, IP-10, MCP-1, IL-2 and RANTES as the five major drivers of data variability. B) Loading plot showing the results from principal component analysis. Colour scale bar represents the Cos2 quality of representation assigned to each vector or variable in the analysis, agreeing with the previous analysis results. C) PCA plot from 10-plex data with loadings. Each genotype is represented by an unique colour, and each variable (cytokine) is represented by lines distributing across all the plot. Despite the lack of specific clustering by genotype, the results were consistent with cosine similarity analysis.

5.5.1 IL-12P40, MCP-1, IL-2, RANTES and IP-10 were identified as the major drivers of variability within the 10-plex dataset

By applying the cosine similarity method for dimensional analysis of the data (Figure 5.16A), the five molecules with the highest contribution to differences in the data were: IL-12_{p40}, IP-10, MCP-1, IL-2 and RANTES (Figure 5.16B), which were equally identified as the main drivers of data variability in the 32-plex experiment. It is important to consider that IP-10 expression was lacking in five of the ten profiled genotypes, which might account for it being identified as a potential driver of data variability. Moreover, no IP-10 heterozygous genotypes were part of the sample panel of this experiment, unlike the first one, which may also produce a more pronounced difference in IP-10 expression.

Furthermore, PCA analysis revealed no specific clustering of the data by genotype (Figure 5.16C), with the exception of a number of samples which individually separated from the majority, primarily driven by the expression of IL-2, CXCL1, GM-CSF, MIP-2 and IL-13. These samples belonged to the JAK2 homozygous, TET2 homozygous, JAK2^{V617F/+} TET2^{-/-} IP10^{-/-}, JAK2^{V617F/V617F} TET2^{+/-} IP10^{-/-}, JAK2^{V617F/+} TET2^{+/-} IP10^{+/-}, and the triple-homozygous genotypes.

Notably, not all the molecules which were producing the individual separation of the samples were identified as significant in the Cos2 similarity analysis (Figure 5.16A), correlation analysis of the ten molecules in the dataset revealed three pairs of molecules with the highest correlation score: IL-2 and GM-CSF, IL-12p40 and MCP-1, and IL-13 and RANTES, which supported the results from the dimensionality reduction analysis. Accordingly, IP-10 showed the lowest correlation scores with any of the molecules, which helped confirm that the mice deemed as IP-10 knockouts were in fact lacking the expression of IP-10.

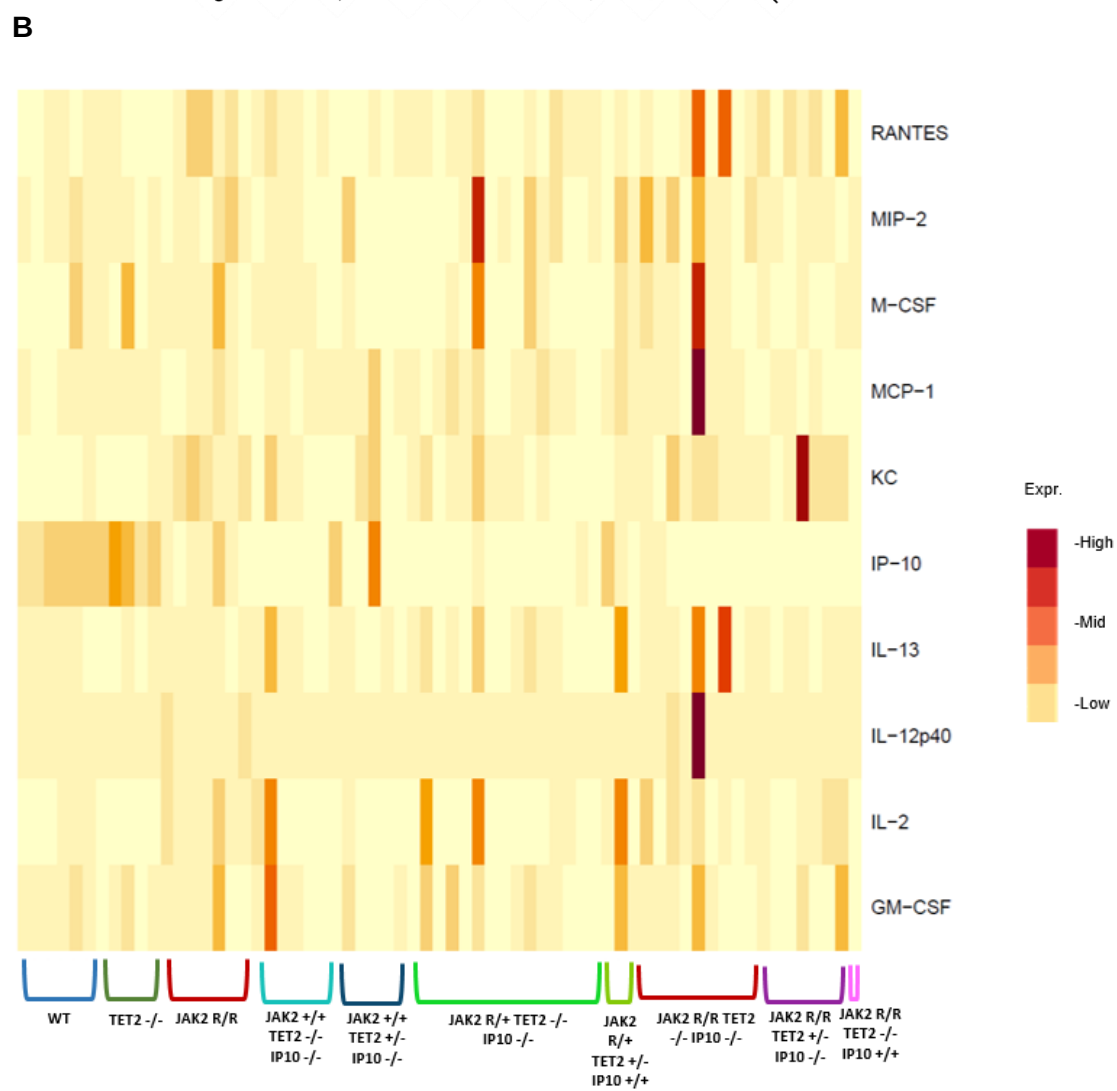
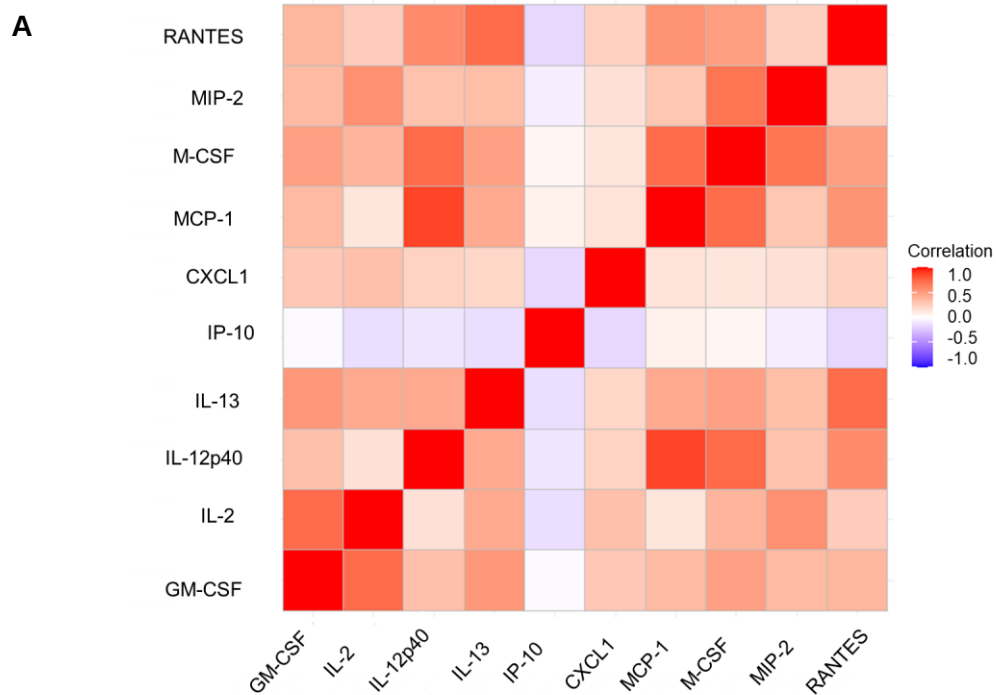
5.5.1.1 Cytokine expression patterns elicited by JAK2/TET2 concomitant mutations and IP-10 loss

In order to further validate the observed cytokine signatures from the 32-plex analysis, I profiled the expression of each of the 10 cytokines across the 10 genotypes (Figure 5.17). Indeed, CCL5, M-CSF, MCP-1, IL-12^{p40} and IL-13 were the most highly expressed molecules in JAK2^{V617F/V617F} TET2^{+/-} IP10^{-/-} and triple-homozygous mice, reinforcing the possibility of a compensatory mechanism mediated by cells of the bone marrow stroma elicited by the depletion of IP-10 (Figure 5.17A). This pattern was quite distinct from those exhibited by other genotypes. For instance, in serum of JAK2^{V617F/+} TET2^{-/-} IP10^{-/-} mice, IL-2, MIP-2 and M-CSF were upregulated, which - together with an increase of IL-2 in TET2^{-/-} IP10^{-/-} mice - indicated

that TET2 homozygous mutations induced higher expression of such molecules in the absence of IP-10, independent of JAK2 V617F mutations. Agreeing with findings from section 5.1, IP-10 expression was elevated in the serum of TET2^{-/-} mice. Nonetheless, it was not possible to determine cytokine expression in JAK2/TET2 homozygous mice, which could be profiled in future multiplex experiments when more serum samples corresponding to this genotype become available.

Correlation analysis (Figure 5.17B) identified associations between molecules which were similar to those inferred from the 32-plex dataset. IL-12_{p40} was observed to strongly correlate with MCP-1, M-CSF and CCL5, while IL-13 was also shown to correlate with CCL5, as well as IL-13. However, CXCL1 did not show the same level of correlation with any of the molecules, which differed from our previous results. This might be explained by the introduction of the triple-homozygous serum samples for this 10-plex cytokine profiling, consequently resulting in a different association between CXCL1 and the rest of the molecules. Therefore, to validate these findings, I analysed the expression of the six most highly expressed and correlated molecules: IL-12_{p40}, CCL5, IL-13, IL-2, MCP-1, and GM-CSF (Figure 5.17C). Serum cytokine concentrations remained at comparable levels across all genotypes, however, in the triple homozygous mice, IL-12p40, CCL5, IL-13 and IL-2 were elevated, although not significantly. However, the relevance of these results relies on previous associations between IP-10 and some of these molecules. Particularly, abnormally high CCL5 and CXCL10 levels have been implicated in juvenile idiopathic (Pharoah et al, 2006) and rheumatoid arthritis (Yu et al, 2020), and especially in infectious diseases (Pranclova et al, 2024). Likewise, a synergistic role has been described for IL-12p40 and CXCL10 in infection (Vasquez et al, 2007; Saruhan-Direskeneli et al, 2005), possibly due to IFN- γ control of IL-12p40 expression in macrophages (Ma et al, 1996; Wang et al, 2000).

Collectively, these cytokine profiling data provide a framework for future research on the synergistic effects of concomitant mutations with secreted inflammatory cytokines in MPN disease onset, maintenance and evolution *in vivo*.



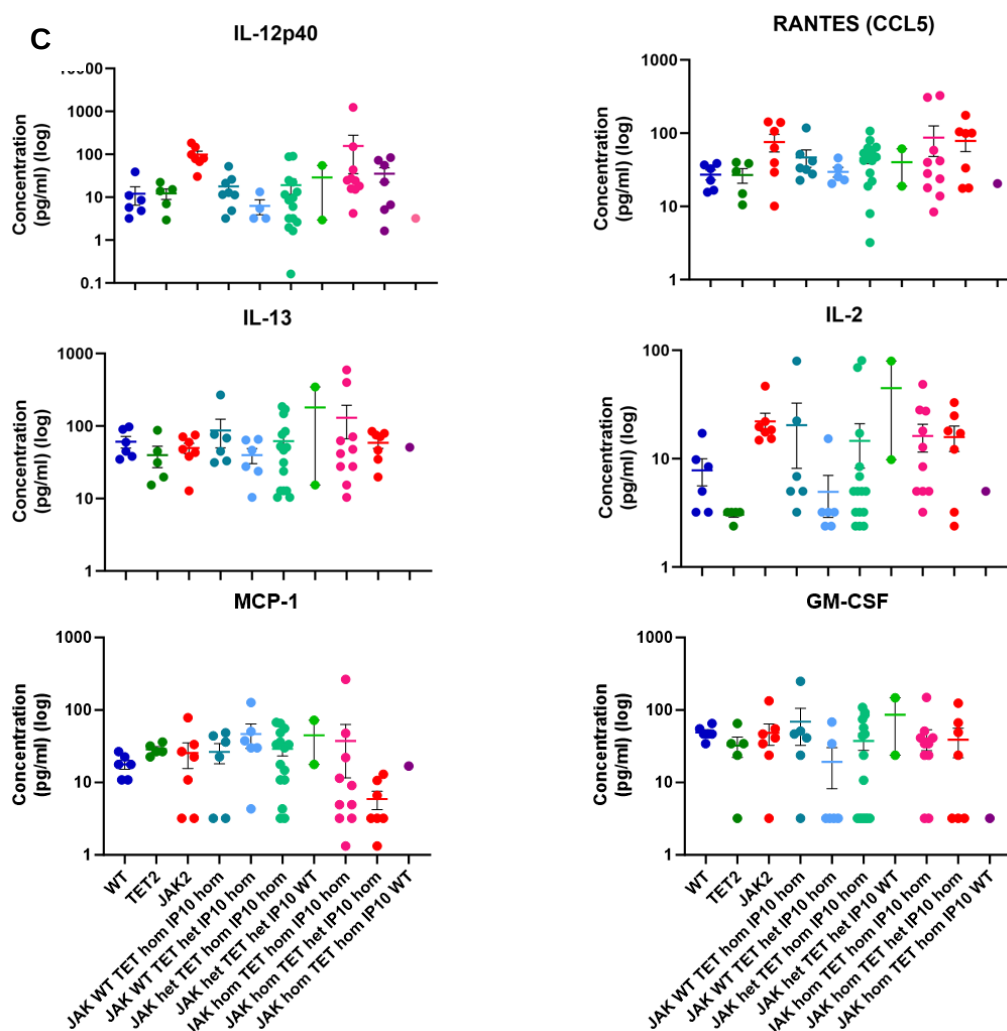


Figure 5.17. Validation of serum cytokine profiling in different MPN mouse models. A) Heatmap showing expression of the ten selected molecules in each of the profiled samples. Colour scale indicates expression level, from bottom to top, low (yellow) to high expression (red). B) Correlation plot of the ten selected molecules to show relationships between each cytokine across all genotypes. C) Concentration of individual cytokines selected based on the correlation and expression analyses. Concentration is expressed as log values in picograms per millilitre of serum. No significant differences were found.

5.6 Summary of findings

Through the work presented in this chapter, I developed a triple-mutant mouse model of MPN, an allelic combination with a gain-of-function mutation in JAK2 V617F, a loss-of-function mutation of TET2 and a mutation in IP-10, which depleted its expression in the organism. The thorough characterisation of disease evolution and impact, as well as serum cytokine profiling, led to the conclusion that, although deletion of IP-10 did not produce a phenotype where the disease was ‘rescued’, there was an important reduction of haematocrit levels in peripheral blood, as well as in the proportion of immature erythroid progenitors in haematopoietic tissues,

particularly in the spleen. Likewise, the complete loss of IP-10 resulted in higher levels of CCL5 and IL-12 p40 in the serum of triple-mutant mice, when compared to the other genotypes. These findings suggest that loss of TET2 in conjunction with IP-10 in a JAK2 homozygous background, produces a milder red blood cell phenotype in mouse models, and could help pave the way for further studies of the myriad of cytokines, chemokines and inflammatory molecules present in the BM microenvironment surrounding HSCs.

5.6.1 Summary of new contributions to the field

1. CXCL10 is significantly elevated in the serum of JAK2/TET2-double mutant MPN patients and mouse models
2. A triple-mutant mouse model revealed that CXCL10 loss partially rescues the severe PV phenotype driven by JAK2 V617F
3. Loss of CXCL10 induces the production of stromal cytokines, as demonstrated by multiplexed serum cytokine analysis

5.6.2 Future experiments

1. To identify the cell population in the bone marrow niche producing CXCL10. This was done in the published work by Obro et al (2020), whereby a subset of CD56+ monocytes were identified as the CXCL10 or IP-10-producing cells in the niche. Nevertheless, it would be informative to perform the same experiments in mouse models of distinct genotypes, particularly JAK2 homozygous and TET2 homozygous to compare against the double- and triple-mutant genotypes, i.e., JAK2/TET2 double t and the JAK2/TET2/IP10 triple homozygous mice.
2. To perform microscopy experiments of bone marrow sections from JAK2 V617F and JAK2/TET2/IP10 mice, as loss of IP10 might potentially drive a bone marrow niche remodelling. The serum cytokine profiling data suggested that molecules secreted in possible 'compensation' for the loss of IP-10 could be secreted by the stromal component of the bone marrow (Baraywno et al, 2021). This could have elicited a compensatory mechanism driven by a specific cell subtype, which will therefore need further scrutinisation.
3. To conduct in vitro experiments to treat stem and progenitor cells with different IP-10 concentrations and determine cell division kinetics, as well as clone size. This could be performed on wild-type cells in the first instance, to then treat JAK2 V617F, TET2 and JAK2/TET2 cells, especially since JAK2/TET2-double mutant patients and mouse models displayed the highest IP-10 serum levels.

5.6.3 Potential implications of new insights from this Chapter

Prevalent patient heterogeneity has puzzled scientist and clinicians for many decades, which raises the need to develop new diagnostic tools which can be coupled together with genomic profiling. Mutations in driver genes, such as JAK2, MPL and CALR, can result in different disease genotypes in patients, which complicates clinical management of the disease. By generating an allelic series of MPN mouse models, we have now elucidated the role of IP-10 in disease evolution in the context of homozygous JAK2 and TET2 mutations. Despite not observing a complete phenotype rescue, we have demonstrated the utility of combining genomic and serum cytokine screenings for MPN patient stratification. This could likewise be applied to other myeloid cancers, such as AML or CML, whereby the inflammatory microenvironment has been shown to sustain the malignant phenotype (Naji et al, 2024; Patterson & Copland, 2023)

Currently, the only curative treatment for high risk MPNs is allogeneic haematopoietic cell transplantation (allo-HCT) (Carturan et al, 2024), however, many times this option is not available for patients of advanced old age (Adekola et al, 2020). Alternative, inhibitors of JAK2 (Carturan et al, 2024) and bromodomain and extraterminal protein (BET) (Saenz et al, 2018), are alternatives which have shown to ameliorate the severe symptoms and consequences of the disease. Recently, interferon α (IFN- α) therapy (Vachhani et al, 2021; Carturan et al, 2024), together with combination therapies (i.e., combining JAK2 inhibitors with pegylated IFN- α or other targeted drugs) (Brkic & Meyer, 2024), are emerging as safer, more widely applicable therapies for patients across the MPN spectrum. Even though therapies targeting the inflammatory microenvironment - examples of which are agents against IL-1 β and NF- κ B (Jutzi & Mullaly, 2020) - the bone marrow niche is a highly complex milieu where an entire ecosystem of different cell types secrete a vast array of molecules, each exerting specific roles in the regulation, and in cancer, the dysregulation of HSC behaviour. Thus, more research should focus on the synergistic activity between altered inflammatory signalling and somatic mutations, in order to develop personalised therapies for MPN patients.

To conclude, the complex cellular crosstalk within the microenvironment governs HSC function and behaviour through signalling pathways, including the JAK/STAT pathway, which are disrupted in disease, resulting in the proliferation of malignant HSC clones and the potential remodelling of the niche, sustaining such aberrant cellular processes. In addition to JAK2 V617F, the acquisition of recurrent mutations, particularly in TET2 in this body of work, is necessary for the development of an aggressive MPN phenotype, which does not impair HSC function, as observed in JAK2 single-mutant mouse models. Nevertheless, since HSC biology is largely regulated by their surrounding microenvironment, it is crucial to explore the effects of secreted inflammatory molecules in a JAK2/TET2 homozygous context. Even though IP-10

serum levels were abnormally elevated in JAK2/TET2 mutant patients, as well as mouse models, loss of IP-10 mildly ameliorated aberrant erythrocytosis and produced a partial rescue of the PV-like phenotype in mice. It will therefore be necessary to profile different molecules which could be executing greater contributions to MPN disease biology.

Results from this Chapter, together with Chapters 3 and 4, will be discussed in the following section.

Chapter 6 - Discussion

HSCs are a remarkable type of somatic cell: they have served as the conceptual basis for understanding how other adult stem cell systems are organised, and subsequently, the process of tissue formation and replenishment in homeostasis and following stress. As any other cell type in the body, HSCs are prone to the natural acquisition of mutations, and this accumulation of unique mutations per cell lineage can be harnessed as a molecular barcode to study relationships between HSC-derived clones, while also inferring their relative level of contribution to overall haematopoiesis. For a subset of these somatic mutations, however, a functional change can occur that confers a growth advantage, potentially resulting in the leukaemic transformation of HSCs. This clonal “competition” underlies the pathophysiology of a wide range of haematological malignancies, ranging from growth deficiencies (BM failure) to growth advantages (leukaemia) with selective pressures driving the relative outgrowth of some clones versus others. The complex interplay of cell- intrinsic and extrinsic factors is known to contribute to disease biology, and particularly for haematological malignancies, the BM microenvironment has been shown to induce inflammation states which enhance the proliferation and expansion of mutant HSC clones. Moreover, very little is known regarding the different anatomical BM sites housing HSCs and a major outstanding question is whether residing within a particular microenvironment might also shape cell fate.

Throughout my doctoral research, I addressed fundamental questions on HSC clonal dynamics, HSC clonal competition and the relevance of the HSC niche in health and disease through the development and application of novel experimental approaches. Through this work, and utilising novel cellular barcoding and single cell approaches, I have provided the first evidence that single HSCs isolated from four different anatomical locations (femur, tibia, ilium and spine) differ at both the molecular and functional level and are embedded in their future microenvironmental location early in development. Secondly, I co-developed a clonal competition model *in vitro* which allowed the discovery of a new type of pre-leukaemic cell behaviour - the TET2 “super competitors” which actively restrict the growth of their wildtype counterparts. Lastly, I characterised a new triple-mutant mouse model of haematological malignancies in order to understand the synergy between JAK2 V617F and TET2 LOF mutations in the absence of IP-10, demonstrating that loss of IP-10 in the presence of concurrent somatic mutations partially ameliorates the severe red blood cell phenotype that is regularly observed in MPN patients. In the following sections, I will discuss the results obtained from my research in light of contributions made by others in the field.

6.1 The impact of anatomical location on fundamental HSC biology

The extensive genomic, transcriptomic and molecular profiling performed on HSCs from four distinct anatomical regions (Chapter 3) unveiled fundamental differences in HSCs. Immunophenotypic data showed that CD45 expression was generally lower on HSCs isolated from the spine compared to other bones, and consequently, the frequency of long-term HSCs (ESLAM) calculated from viable CD45⁺ cells was also reduced. One plausible explanation for this might lie on the three-dimensional microarchitecture of long bones and the vertebrae that comprise the spine as well as the diversity of cell types that occupy the local microenvironment. The vertebrae are mainly composed of dense trabecular bone networks, and, unlike most long and short bones, they are not covered by a true bone cortex formed by osteons (Cowan et al, 2024; Roux et al, 2024). The spine is surrounded by an abundant layer of connective tissue, including ligaments, tendons and skeletal muscle, and in addition, the spine serves as a protective passageway for the spinal cord (Harrison et al, 2013), which again might explain the overall reduction of CD45-expressing cells in the marrow. Likewise, the reduced expression of CD150 in spine HSCs results of interest since CD150 has been incorporated into mouse HSC purification strategies due to its high fidelity and precision to detect the most primitive cells in the haematopoietic hierarchy, in combination with other markers (Kiel et al, 2005; Weksberg et al, 2007; Kent et al, 2016). These data imply that HSCs across all bones might not be identical, but questions such as how closely related they are, how their progeny distribute across different BM anatomical regions remained unaddressed.

6.1.1 Niche-localised HSC clustering throughout life

The ability to isolate the most primitive haematopoietic cells in the blood formation hierarchy has now made way to investigate fundamental questions about HSC clonal dynamics which are not yet possible to address using patient-derived samples. Founding studies on native haematopoiesis relied on *Cre*-recombinase or transposon tagging systems to profile clonal dynamics and HSC contribution to overall blood cell production (Busch et al, 2015; Sun et al, 2014; Rodriguez-Fraticelli et al, 2018). We have now conducted the first analysis of clonal relatedness in unperturbed (healthy) haematopoiesis, using somatic mutations as endogenous barcodes, acquired in the cells of the most primitive haematopoietic compartment. In our study, somatic mutation burdens were similar to those reported by Cagan and colleagues (2022), in which they profiled mutation acquisition rates across 16 species of mammals, including mice and humans. We inferred 260 and 360 mutations were acquired in the first and second experimental animals, respectively, to the moment of tissue collection. Likewise, increased mutation acquisition followed the pattern of a clock-like SBS1 mutational

signature, which is classically observed in ageing (Cagan et al, 2022; Alexandrov et al, 2015). Furthermore, the approximate number of somatic substitutions per cell per year was slightly higher than that reported by Kapadia and colleagues (2025) - approximately 65.22 SBS acquired per cell by 29 weeks of age in our study, compared to 59.5 SBS at 3 months of age reported by Kapadia. Since single HSCs and MPPs were expanded in semisolid medium in Kapadia's study, this might have resulted in fewer mutational processes occurring *in vitro*, but also, importantly, the fact that these haematopoietic progenitors were isolated from a pool of BM from different bones, in contrast to our study where we profiled single HSCs from individually processed bones. Further and more refined computational analysis pipelines will have to be implemented in order to determine whether longer branches in the tree correspond to HSCs from specific bones which underwent higher mutation acquisition *in vivo*, thus resulting in a larger number of substitutions of molecular time. Nevertheless, our somatic mutation burden estimates in both experimental mice are still higher somatic mutational burden per murine HSC per year than in human haematopoietic progenitors (Mitchell et al, 2021).

The remarkable observation that HSCs cluster by anatomical region demonstrates that their HSC progeny preferentially reside - and remain within - the bone from which they originated early in life. This suggests that, in steady-state conditions, HSCs might not migrate to a different BM location in the adult organism, which could reframe the way HSC mobilisation and re-seeding is currently understood. During the developmental stage of definitive haematopoiesis, HSCs migrate from the foetal liver to foetal BM and spleen (late gestation - day E17.5 - E18.5) (Ciriza et al, 2013; Gao et al, 2018). HSC frequency increases exponentially in the BM after seeding, where they will take up residence and contribute to lifelong haematopoiesis (Gao et al, 2018). It has been suggested that HSCs which exit the BM and enter peripheral circulation do not return to the marrow, regardless of which type of bone, but might take up residence in skeletal muscle under homeostatic conditions (McKinney-Freeman & Goodell, 2004). However, there have not been more studies performed on homeostatic adult HSC BM migration, except on those consisting on imaging techniques that have been applied to the characterisation of HSC extramedullary upon transplantation (Rademakers et al, 2020) or myeloablation via 5-fluorouracil (5-FU) treatment (Johansson et al, 2024). From our findings, we inferred that HSCs give rise to progeny with a niche-localised 'preference' from the earlier (pre-conception) to the later stages in life, indicating that regardless of bone of origin or age of the mouse, HSCs will not go through evacuation mechanisms or receive external cues which would prompt them to move from their original BM location. This phenomenon is unlikely to be permanently imprinted on HSCs since, upon

transplantation into a new host, they spread across multiple anatomical sites and do not “remember” where they came from.

Nevertheless, it is possible that under non-physiological conditions, HSC migration across bones will occur. For instance, time-lapse imaging techniques have shown that, upon infection, HSCs distanced from the infection area remain within confined niches, while those closer to the site of infection migrate to different regions of the bone marrow (Rashidi et al, 2014). Notwithstanding, whether this same phenomenon will be observed in HSC migration across bones has not yet been thoroughly investigated. In order to test this concept further, a similar kind of phylogenetic approach could be applied to study the dynamics murine HSCs and their progeny upon different types of stress.

6.1.2 Distinctive molecular features in femur and tibia bone marrow derived single HSCs

The inference that HSC clonal relatedness was possibly underscored by their tissue of origin, denoted potential differences in terms of their molecular and functional features. Profiling HSCs at the single-cell level allows the accurate examination of the transcriptomic and differentiation programmes that each HSC follows as an individual entity, which then translate into the largely prevailing heterogeneity which characterises HSCs when studied at the population-level (Martin-Rufino & Sankaran, 2021; Monga et al, 2022; Zhang et al, 2022). Single-cell heterogeneity in the haematopoietic system has focused on characterising different stem and progenitor cell populations (Yamamoto et al, 2013; Paul et al, 2015; Velten et al, 2017; Karamitros et al, 2017; Alberti-Servera et al, 2017) or at different developmental (Gherzi et al, 2023) and adult life stages (Yamamoto et al, 2018; Yang et al, 2023). However, it is equally possible that this heterogeneity could rely on the location of origin of the different HSC subsets contributing to haematopoiesis.

In our data, global differential gene expression analysis uncovered expression of genes like *Ctdspl* (Su et al, 2015), *Npc1* (Groenen et al, 2022), and *Igsf6* (Zheng et al, 2022) was upregulated in femur and tibia only, which have been primarily described in cells of the myeloid lineage. These findings gave an early indication of the functional consequences of these upregulated genes, since the subtype of cells with higher serially transplantable potential are biased towards myeloid differentiation (Dykstra et al, 2007; Iwasaki & Akashi, 2007). However, these assumptions were validated through profiling of gene expression signatures across the four BM locations, strongly indicating that HSCs from the femur and tibia had prominently higher scores for the MoIO and RepopSig molecular signatures (Wilson et al, 2025; Che et al, 2022), which both correlate gene expression with functional performance in freshly isolated

(MoLO) and *ex vivo* expanded HSCs (RepopSig). Then, the molecular profile of HSCs from the femur shared greater similarity with those from the tibia, and spine and iliac were more similar to each other than to the other two bones. Therefore, one of the potential ways that HSC heterogeneity could be explained would be in context of their anatomical location, and since the molecular differences we observed in femur and tibia were made at the single-cell level, the likelihood of this statement was further supported by our *in vivo* findings.

6.1.3 Extensive functional *in vivo* characterisation proposes femur and tibia as niche locations harbouring better-performing HSCs

Functional profiling of HSC in single bones has been scarce in the history of the HSC field, particularly in the set of bones (femur, tibia, spine, ilium) which have been the most widely used in experimental work. A handful of studies have been conducted on the biology of HSCs residing in the mouse calvaria, due to its rich vascular network, and the fact that cerebral blood circulation is insulated from peripheral circulation. High-throughput imaging modalities on histological sections of pools of long bones have discovered fundamental differences across the three marrow compartments in murine long bones: epiphysis, diaphysis and metaphysis, and along with live imaging of calvaria, it was determined that the epiphysis and calvaria derived HSCs have higher long-term reconstitution potential (Lassailly et al, 2013). A recent report showed that the skull BM expands as a result of ageing, and can act as a reservoir of long-term reconstituting HSCs, thanks to the comparably intact status of skull BM to that found in bones of the axial skeleton (Koh et al, 2024). However, functional characterisation of FACS-sorted murine HSCs from different bone types was only recently reported earlier this year (Niizuma et al, 2025). The primary finding of this study was that alveolar BM (equivalent to human maxilla) harboured a larger number of immunophenotypic HSCs in comparison to eight bone types: pelvis, ramus (equivalent to human mandible), spine, scapula, sternum, calvarium, and femur and tibia pooled together (not processed individually); and in whole BM transplantations, alveolar BM cells displayed higher chimaerism than femur and tibia BM. Nonetheless, injection of 10 purified HSCs into primary recipients did not show any significant differences in functional potential between alveolar and femur/tibia HSCs. Thereof, higher content of putative HSCs in different BM compartments or bone types does not necessarily correlate with functional performance of such HSCs.

In my studies, I discovered that highly purified single HSCs from the femur and tibia were able to serially reconstitute transplant recipients, and that ilium and spine BM HSCs had a more finite reconstitution potential, which resulted in no detectable progeny in secondary transplantations. Moreover, femur-derived HSCs exhibited the highest engraftment frequency

among the four bones, i.e., femur HSCs reconstituted more recipients than did the rest of the other BM groups. Likewise, in accordance with previous findings (Dykstra et al, 2007; Benz et al, 2012), there were distinct haematopoietic differentiation patterns displayed by HSCs across the four bones. The femur and tibia contained HSCs from all four subtypes - α , β , γ , and δ -, of which β stem cells comprised the highest proportion, followed by α HSCs. Chimaerism observed in secondary transplant recipients confirmed that HSCs isolated from femur and tibia exhibited durable, serially transplantable functional properties that the iliac and spine HSCs failed to do. Finally, limiting dilution assays validated the serial transplantation data, demonstrating a higher frequency of functional HSCs in the femur and tibia in comparison to spine. These findings serve as the premise that functional properties in single HSCs are acquired in a specifically anatomical localisation fashion, although this could change upon different stimuli and stress. One key study has applied single-cell imaging techniques to grasp the spatial organisation of haematopoiesis across the skeleton, illustrating the differences in how tibia, skull and sternum BM HSCs respond to various forms of stress, including infection, inflammation and ageing. Even though this study does not demonstrate differences at the molecular or functional level, it is an excellent approach to study the localised HSC activity both in unperturbed and perturbed haematopoiesis (Wu et al, 2024).

6.1.4 Remaining questions in the anatomical mapping of HSC activity

What underscores the differences between HSCs across the skeleton?

There is a rather alluring proposal formulated by Gale and others as to why most HSCs reside in the BM, rather than other tissues, during adult life (Gale et al, 2023). The authors speculate that, as life forms transitioned from aquatic to terrestrial, animals were exposed to greater radiation, which resulted in DNA damage in cells from all tissues, including stem cells. Blood production was localised to the BM in order to protect HSCs from radiation exposure, and so ensuring balanced blood cell production. Although it is a theoretical proposal, it could be the case that these adaptive evolutionary responses might influence the functional and molecular properties of HSCs from different BM environments. Likewise, as discussed above, bone microarchitecture varies from one tissue to another, as each of them fulfills different anatomical functions in the body. This results in unique biomechanical properties in long bones, such as the femur, and the vertebrae (Aslam et al, 2016). It has likewise been shown that the pressure imposed on a bone by weight (known as mechanical bone loading or just loading) varies even within long bone types, such as the femur and tibia (Umemura et al, 1997), and can induce bone remodelling both in long bones and the vertebrae (Rockoff et al, 1969; Turner, 1998; Chow et al, 1993). Nevertheless, the spine in particular is subject to

greater mechanical loading when walking, which modifies the microarchitecture of cancellous bone (Theo et al, 1997), therefore potentially impacting the functional potential of resident HSCs.

Is bone marrow architecture anatomically or functionally defined by bone type?

All bones, including their BM, derive from the same germ layer - the mesoderm, same as blood - and their ossification processes are essential for the formation of BM niche - with the exception of flat bones like those of the skull - (Breeland et al, 2023), and alterations in this process result in bones without any marrow (Chan et al, 2008). Nevertheless, the BM in the skull and vertebrae of the spine are densely interconnected with components of the central nervous system (CNS); such connections are supported by bidirectional conduits which form channels that enter the BM cavity (Mazzitelli et al, 2023), which could explain the lower abundance of CD45-expressing cells in the spine than in the rest of the bones observed in our data.

High-resolution imaging data has demonstrated that HSCs and HSPCs locate preferentially closer to sinusoids and arterioles within the BM niche (Kunisaki et al, 2014), a phenomenon that has been highlighted in the trabecular-rich region of the metaphysis of long bones (Ellis et al, 2011). Therefore, the superior functional performance of HSCs derived from femur and tibia BM could be attributed to several factors: first, these tissues are composed of trabecular and cortical bone, enabling the marrow to distribute along three anatomical regions, therefore allowing HSCs to locate closer to blood vessels; second, time of embryological HSC seeding of BM might differ between bone types, and it is likely that - if femur and tibia are colonised first - then HSCs would be able to adapt in these bones ahead of the others; third, since spine likely undergoes more mechanical loading cycles compared to long bones, this might hinder HSC functionality, and therefore reduce their transplantable potential.

6.1.5 Limitations of the study

Historically, elementary concepts in HSC biology have been unveiled thanks to the use of laboratory mice (*Mus musculus*), which share 95% genetic similarity with human beings (*Homo sapiens*), together with rats (*Rattus norvegicus*), as all three species harbour around 3×10^4 genes in each respective genome (Bryda, 2013; Breschi et al, 2017). Although there are numerous advantages to using laboratory mice for biomedical research, and specifically in the HSC field (Sykes & Scadden, 2014), it is evident that there are important limitations when comparing human and murine biology.

In this study, I profiled HSCs from different locations in the mouse, which is not feasible to perform on human donors, and particularly since this was done at the end of the mouse's lifetime. There remains, therefore, an important technical challenge, which will require a minimally invasive method to sample bone marrow from locations other than the iliac crest, the most common site for bone marrow aspiration (Bierman, 1952; Byrd et al, 2023). Nevertheless, considering that towards adulthood, active bone marrow haematopoietic sites - those where haematopoiesis is active - are restricted to the axial skeleton (Rindy & Chambers, 2023), sampling of bone marrow would ideally come from these locations.

Likewise, phylogenetic analysis of HSC progeny revealed that HSCs have a preference for their bone marrow location of residence, from embryogenesis to adulthood. Although the sequencing depth of this experiment was high, and strict cutoffs were set in order to draw conclusions from the data with confidence, I still acknowledge that human HSCs might not display the same behaviour. This could be mainly due to the fact that every human being is completely different to another (Ou et al, 2020; Rydell-Tormanen & Johnson, 2019), while laboratory mice are kept under controlled conditions in the best way possible (Perlman, 2016). Therefore, differences in fundamental biological features, such as body size and lifespan, in addition to habitual features, specifically diet, travel, interactions with other individuals, as well as emotional and behavioural aspects of human life, complicates the translation of findings achieved from mouse studies to human beings.

Many new insights can be drawn from high-throughput sequencing studies in mice, as has been demonstrated throughout this study. However, in human beings, it is known that genetic changes can also be associated with age, sex and even environmental factors (Lynch, 2016), which is difficult to study in mice due to the reasons outlined above. Being therefore aware that the mutational landscape of a complex organism, such as a human being, shifts and changes as time advances (Martincorena & Campbell, 2015; Moore et al, 2021), it is of utter importance to conduct more of this in-depth studies in cohorts of individuals with different characteristics, e.g., age, sex, ethnicity, diet, occupation, exercise routine among others.

Future outlook and studies

More thorough studies will be needed in order to determine which of the non-haematopoietic cell types in the BM across different anatomical sites are more or less abundant. Likewise, levels of secreted molecules are also likely to be different among bone types, and this could likewise change the way HSCs respond to microenvironmental cues. Phylogenetic analysis of clonal relatedness between HSCs from different BM locations could be performed under non-physiological conditions, such as inflammation and infection, in order to understand if these conditions alter the number of acquired mutations, even extending to more committed

progenitors, such as CMPs or MEPs. This might also help understand the dynamics of HSC migration in response to environmental insults, which could result in HSCs abandoning their tissue of origin if such is the site where stress is being inflicted, despite their strong clonal relationship bonds.

6.2 Novel *ex vivo* HSC expansion methods leveraged to study the phenomenon of clonal competition

Since its discovery over 55 years ago (Bryant & Schneiderman, 1969; Garcia-Bellido & Meriam, 1971) - and then entering a paucity for more than two decades (Johnston et al, 1999) - the phenomenon of cell competition has been described as an important process for the removal of defective, or less fit, cells from tissues during development, regulating tissue size and preserving homeostasis (Maheden et al, 2022; Ramos & Martins, 2021; Nichols et al, 2022). Cell competition also maintains homeostasis in the adult organism by eliminating cells with lower fitness primarily through programmed cell death mechanisms (Zhu et al, 2025; Gradeci et al, 2021; Claveria & Torres, 2016). However, implications of cell competition in cancer (Vishwakarma & Pidini, 2020; Cong & Cagan, 2024), and in leukaemia (Akinduro et al, 2018; Ramos & Martins, 2021; Fujino et al, 2022), led us to design a new experimental model to study HSC clonal competition dynamics in pre-leukaemic disorders.

Our novel *in vitro* clonal competition model was birthed as an optimised single-cell derived split culture approach, based on previously paired daughter assays (Weinreb et al, 2016; Wehling et al, 2022) and HSPC split cultures (Naik et al, 2013; Lin et al, 2018; Tian et al, 2021). Given that HSCs are highly heterogeneous (Haas et al, 2018), it is expected that two single HSCs will not be identical to each other, however, the progeny derived of them could perpetuate the traits of their parent clone. By conducting a detailed description of single-HSC split culture phenotypic, molecular and functional properties, we confirmed that split clones preserved the original features of the clone from which they derived, with very little variation in the ELSK output in culture or levels of engraftment in transplant recipients. From previous studies (Che et al, 2022), our group has demonstrated that *ex vivo* expanded single-HSC clones could be defined by a combination of cell surface markers as ELSK (phenotypic HSC) cells. Furthermore, recent work from our group has demonstrated that $TET2^{-/-}$ *ex vivo* expanded HSCs produce higher proportions of ELSKs than wild-type HSCs (Jassinskaja et al, 2025; Juan Rubio-Lara and Daniel Bode, personal communication), which allowed us to establish our model by making wild-type and $TET2^{-/-}$ HSC clones compete against each other.

6.2.1 Development of a competition assay by integrating state-of-the art *ex vivo* expansion and split cultures

Clonal competition has classically been modelled *in vivo* in *Drosophila* (de la Cova et al, 2004; Tyler et al, 2007; Martin et al, 2009), mouse embryos (Lima et al, 2022; Penzo-Mendez & Stanger, 2014; Bowling et al, 2018) and mouse models of glioblastoma (Ceresa et al, 2023). *In vitro* clonal competition assays have primarily been executed as co-cultures of cell lines (Haertle et al, 2024) or primary cultures, applied to the study of neuroepithelial cell competition (Azura et al, 2020); organoid cancer models Krotenberg Garcai et al, 2024; Flanagan et al, 2021); and competition in reprogrammed iPSCs (Shakiba et al, 2019). In these assays, wild-type cells are mixed together with mutant cells at equal or unequal ratios, depending on the experimental conditions, and then subjected to different experimental conditions, such as drug treatment (Haertle et al, 2024). Our clonal competition model allowed us to create four competition matrices on an readily accessible 96-well format, each consisting of nine unique competition wells whereby three different single-HSC derived TET2^{-/-} clones competed against three different single-HSC derived wild-type clones, adding greater statistical power to our assay and allowing us to execute experiments at a larger scale, in which we profiled 900 unique competition wells, equating to 300 wild-type and 300 TET2^{-/-} clones. This would not have been experimentally feasible with the current state-of-the-art *in vivo* or *in vitro* competition models, due to technical impediments, such as sample availability, number of biological replicates required and reproducibility of the assay. In addition, we were likewise able to maintain a separate group of untouched, control clones within the competition matrices. These clones were the original clones from which the competitors derived, which in accordance with our initial data from the split cultures, preserved their original properties. This was experimentally relevant, as this allowed us to conduct functional, molecular and proteomic profiling on the untouched clones, thereby increasing efficiency and applicability of the assay, simultaneously maximising sample usage and significantly decreasing costs in transplantations, sequencing and mass spectrometry. Furthermore, cell sorting by FACS was dispensable thanks to the existence of untouched, control clones, exploiting CD45.1/CD45.2 congenic system (corresponding to wild-type and TET2^{-/-} clones, respectively), which allowed us to discriminate between the two different genotypes in competition cultures.

6.2.2 Definition and identification of the TET2-super competitor clones

The most prominent finding from our clonal competition assays was the identification of a new type of TET2 clone - the TET2-super competitor. In the field of cell competition, a super competitor is defined by two fundamental features: it has the ability to eliminate its normal

counterparts, while also allowing for the proliferation and expansion of its own cells (DiGregorio et al, 2016; Yusupova & Fuchs, 2023; Cong & Cagan, 2024). This definition came from previous findings on cells in *Drosophila* and mouse embryos which carried a mutation in the *Myc* gene (Moreno & Basler, 2004; Claveria et al, 2013), and it has been reported to be mainly mediated by several pathways, including c-Jun N-terminal kinase (JNK) (Kodra et al, 2024), JNK and JAK/STAT (Kucinski et al, 2017), Hippo (Ziosi et al, 2010). Even though super competition had not been described in haematopoietic stem cells until now, cell competition in HSCs and HSPCs has been attributed to somatic mutations in genes such as DNMT3A, TET2 and ASXL1 (Cooper & Young, 2017), which drive clonal evolution and expansion of mutated clones in haematological malignancies (Ferrando & Lopez-Otin, 2017; Watson et al, 2024). Moreover, competition assays between Moloney murine leukaemia virus (MoLV) infected cell lines of AML, demonstrated that aggressive cell lines were dominant over less aggressive cells *in vivo* and *in vitro* (Aflalo & Weinstein, 1990). Another study showed that concomitant mutations in TET2 with FLT3 or DNMT3A with FLT3 had divergent roles in HSC/HSPC expansion, rate of transformation into leukaemia and overall survival in mice (Ostrander et al, 2020). Double knockout mouse models have demonstrated that LOF mutations in DNMT3A and TET2 result in the expansion of the LSK compartment, compared to wild-type or single-mutant models (Zhang et al, 2017). Nevertheless, none of the cell types were deemed as dominant or as displaying any kind of supercompetitive behaviour over other clones.

Therefore, we propose a new definition for TET2 HSC competitor clones. This definition considers the proportion of viable cells from each genotype (wild-type and TET2^{-/-}) present in the competition wells - supercompetitors must predominate by more than 70% of total viable cells. Furthermore, we also considered the cell viability of the untouched control clones, as this helped confirm that the absence of wild-type clones was not due to poor viability of the original clones of which they originally derived. One more important hallmark of supercompetitors was the significant increase in number and proportion of the Lin⁻, LSK and, particularly, the ELSK populations in comparison to wild-type and TET2 competitors, a feature which categorically distinguishes between super and non-super competitor clones. All these populations should be equally elevated in the control clones, which is a reliable metric to identify supercompetitors following the principle of phenotypic, molecular and functional plasticity of HSC split cultures. TET2-super competitors are a rare phenotype (7% of total clones), but consistently present across independent experiments, which used independent, age-matched biological replicates, and the ability to reproduce these experiments at a large scale allowed us to formally demonstrate the superiority of these TET2 clones.

6.2.3 Upregulated genes implicated in cell adhesion as a potential mechanism for TET2-super competitive behaviour

Functional profiling of different competitor clones revealed that TET2-super and TET2 competitors were comparably engraftable and contributed to multilineage blood cell production, unlike wild-type competitor clones. As previously reported in an *in vivo* competition model, TET2 deficient LSKs exhibited higher donor chimaerism in primary transplant recipients in comparison to wild-type LSKs injected together (Quivoron et al, 2011). Although these experiments were performed from freshly isolated progenitors, in contrast to ours, which we conducted on *ex vivo* expanded clones, we found that wild-type competitor clones were less effective at repopulating transplant recipients. Moreover, the statistically significant correlation between number of ELSK cells present in competition wells and donor chimaerism both in TET2 and TET2-super competitors, implied that higher ELSK number was not the only determinant of supercompetitive behaviour; it had to be exerted through a specific mechanism that resulted in the elimination of wild-type rival clones.

The upregulation of genes involved in cytoskeleton organisation, extracellular matrix anchoring and cell adhesion inferred from our molecular profiling data, together with the high secretion of molecules implicated in these physiological processes, led us to test inhibitors of the Rho GTPase family. Rho GTPases have emerged as “master regulators” of cytoskeletal organisation in diverse cell types, including platelets (Aslan & McCarty, 2013), T lymphocytes (Rougerie & Delon, 2012), and HSCs (Cancelas & Williams, 2009; Nayak et al, 2013). Results from our *in vitro* target validation experiments revealed that treatment with NSC23766, a specific inhibitor of the Rac1/GEF interaction *in vitro* (Gao et al, 2024), diminished wild-type cell survival and ELSK output in F12-PVA expansion cultures, but TET2 HSCs were resilient to such cytotoxic effects, and maintained stable ELSK numbers upon treatment.

Rac1 is a member of the Rac subfamily of Rho GTPases (Cancelas, 2009), and the latter are part of the Ras superfamily of enzymes (Parri & Chiarugi, 2010). Rac enzymes are activated by guanine nucleotide exchange factors (GEFs), which displace guanosine diphosphate (GDP) and replace with guanosine triphosphate (GTP) (Haga & Ridley, 2016). Furthermore, Rac1 has been implicated in AML (Wang et al, 2013), as well as other disorders including ALL (Freret et al, 2011), CLL (Hofbauer et al, 2014) and MPNs (Martin et al, 2013). The Williams group were the first to demonstrate that Rac GTPases and Cdc42 regulate several processes in HSCs, including cell adhesion, migration and cytoskeletal organisation, which are essential for HSC homing and mobilisation (Yang et al, 2001). Data from the same group showed that NSC23766 treatment of Lin⁻ c-Kit⁺ (LK) suppressed activation of Rac1 and Rac2 upon

stimulation with CXCL12 (Cancelas et al, 2005). While we observed that NSC23766 treatment does not affect the proliferation of *ex vivo* expanding TET2^{-/-} HSCs, previous studies have reported that NSC23766 inhibition of Rac1 reduces cell migration and growth of patient-derived acute leukemia cells (Wang et al, 2009). In another study, NSC23766 treatment of Flt3+/ITD NHD13 mouse cells acted in junction with the cytarabine (ara-C) to induce apoptosis (Wu et al, 2019). Furthermore, in the transcriptomic profiling of TET2-super competitors, we found overexpression of GEF-H1 (ARHGEF2), a Rho GEF subfamily of Rho GTPases (Bretch et al, 2004). Although GEF-H1 has been mainly implicated in pancreatic cancer (Nakao et al, 2021; Cullis et al, 2014), it has likewise been found in haematological malignancies (Birkenfield et al, 2008; Chang et al, 2006). It is possible that the overexpression of another member of the RhoGTPase family, can confer greater resistance to NSC23766 treatment to TET2^{-/-} HSCs *in vitro*.

6.2.4 Potential mechanisms for wild-type elimination by TET2-super competitors

If upregulation of genes implicated in cell adhesion and structural organisation of the cytoskeleton are the primary mechanism underlying TET2-super competitive behaviour, could there be additional mechanisms for the elimination or growth suppression of wild-type cells? Proteomic profiling of conditioned media identified overexpression of the insulin-degrading enzyme - Ide - and death-associated protein 1 - DAP1. The former has been found across many types of human tissues, both healthy and malignant, including normal granulocytes and erythrocytes (Weirich et al, 2008; Yfanti et al, 2008; Shii et al, 1996). Historically considered essential to understand the pathophysiology of Alzheimer's disease and diabetes (Pivovarova et al, 2016), its role in cancer has recently been highlighted, as it might increase insulin uptake into malignant cells, thereby promoting their proliferation and growth (Lesire et al, 2022). It is possible that TET2-super competitors consume most of the available insulin while competing, depleting it from the media, and therefore resulting in an underconsumption of insulin by wild-type cells. This would follow the principle of natural selection, in which the cells with a selective advantage overtake nutrients in the media, leaving their rivals susceptible to undernourishment, and consequently, death.

On the other hand, DAP1 is an effector of IFN- γ mediated cell death (Deiss et al, 1995; Feinstein et al, 1995), overexpressed in pancreatic cancer (Sui et al, 2021). Since we found several genes implicated in interferon signalling, it is likely that TET2-supercompetitor overexpression of these genes in junction with DAP1, might elicit a programmed cell death mechanism in their wild-type rivals, thereby eliminating them from the culture. In our experiments, however, we found that IFN- γ treatment did not exert any significant reduction

on viability or phenotypic HSC output either in wild-type or TET2 HSCs *in vitro*, which might be explained by the fact that overexpression of DAP1 could only be observed in competition, and could then be tested directly in competition cultures.

6.2.5 Remaining questions on the determinants of TET2 supercompetitive behaviour

What is the clinical significance of supercompetition for myeloid malignancies?

Pre-leukaemic disorders, such as MDS and clonal haematopoiesis, and AML, are characterised by the serial acquisition of genetic alterations and chromosomal rearrangements, which promote the selection and expansion of mutant clones that supersede non-mutant clones (Grove & Vassiliou, 2014). Clonal evolution in myeloid malignancies is driven by fitness advantage of mutant clones, and by the time a clone reaches a suitable population size, the switch from stochastic to deterministic growth allows for expansion of such clone (Fabre et al, 2022; Fabre & Vassiliou, 2024; Hartman & Metzeler, 2019). Hence, there are detrimental consequences for blood cell production and overall homeostasis, as mutant clones gradually predominate and healthy clones succumb and are eliminated, with malignant cells following different clonal trajectories, potentially resulting in disease progression and transformation into clinically overt leukemia (Dong-He et al, 2023). Loss of TET2 confers enhanced self-renewal and promotes expansion of the haematopoietic stem cell pool, as well as a competitive advantage over healthy HSC/HSPCs. For the first time, we report a subset of TET2 competitor clones with the outstanding ability to outperform their rival clones *in vitro*, and this could be of relevance to the pathophysiology of BM failure, clonal haematopoiesis and AML, due to the high prevalence of TET2 LOF mutations in these disorders (Ferrando & Lopez-Otin, 2017).

How can the clonal competition assay be applied to other mutations?

TET2 haploinsufficiency is not enough to initiate disease, as the acquisition of mutations in different genes are required for disease onset and full-blown leukaemia (Kaasinen et al, 2019). Hence, other mutations should likewise be considered when modelling cell competition between malignant and healthy HSCs. For instance, both DNMT3A and TET2 have been reported as the highest prevalent mutations in clonal haematopoiesis (Buscarlet et al, 2017; Cobo et al, 2022). Moreover, they have been shown to have convergent and divergent roles in regulating HSC function, with implications for myeloid disease (Zhang et al, 2016; Ostrander et al, 2020; Yang et al, 2021; Hinze et al, 2023; Jakobsen et al, 2024). Competition assays between DNMT3A- and TET2-mutant HSCs could enable a detailed study of the diverse types

of cooperative and competitive mechanisms exerted by both mutations, and help deconvolute the complex dynamics of somatic evolution, and shed light on leukaemic transformation.

Equally, concomitant mutations in JAK2 V617F and TET2 yield a more aggressive MPN phenotype in double-mutant mouse models than in models with single mutations (Shepherd et al, 2018), without the classic impaired self-renewal characteristic of JAK2-homozygous HSCs (Kent et al, 2013; Akada et al, 2014). JAK2/TET2 double-mutant HSCs can be expanded *ex vivo* (Juan Rubio-Lara, personal communication), while JAK2-homozygous HSCs fail to do so (unpublished data from our lab). Therefore, JAK2/TET2 HSCs could be set in competition against wild-type or TET2 single-mutant cells, in order to determine the existence of JAK2/TET2 supercompetitors, and if these could behave in an even more potent way that TET2-super competitors do. This could be of high clinical relevance since JAK2 V617F is one of the major driver mutations in MPNs, and co-mutations with TET2 substantially impact prognosis, disease evolution and survival (Ortmann et al, 2015; Schaub et al, 2010). Other co-occurring mutations with TET2 could likewise be profiled in clonal competition assays, such as FLT3^{ITD} (Ramdas et al, 2022), IDH1 and IDH2 (Fortin et al, 2022). Although a technical challenge, the assay could be optimised to study more than two mutations at a time, which would be significant for AML, as it has been inferred from patient-derived xenograft models (Sanden et al, 2020; Zeller et al, 2022).

6.2.6 Limitations of the study

The clonal competition model presents numerous advantages for the study of cell competition at scale, which is reproducible and yields consistent results across time. However, the dynamics of the competition phenomenon are likely vastly different *in vivo* from the *in vitro* setting that we have developed. HSC competition was first modelled *in vivo* in a series of competitive transplantation studies which identified DNA damage to be causative for a higher competitive behaviour mediated by p53 mutations (Bondar & Medzhitov, 2010). Niche competition has been described in different cellular system, including gonads during spermatogenesis (Garcia-Tejera et al, 2025), cells of the germline in *Drosophila* (Zhang et al, 2023; Zhao & Xi, 2010), as well as HSC transplantation (Czechowicz et al, 2007), which indicates that the niche factors could significantly impact the competitive performance of clones of distinct genotypes.

Additionally, a major technical challenge in setting up competition assays is the exorbitant amount of clones that need to be split, which requires intensive pipetting during the split and competition setup. It has been demonstrated that constant loading of hand muscles, especially those of the thumb while pipetting can cause musculoskeletal disorders (Wu et al, 2016). For

this reason, it is recommended to have periods of rest or to have the help of another colleague, in order to ease the workload, increase productivity and experimental efficiency.

Whereas our competition model allows for the study of the dynamics between healthy and mutant cells cohabiting within the competition wells, it is known that the genomes of AML, MDS and clonal haematopoiesis contain more than one mutated gene (Welch et al, 2012; Tyner et al, 2018; Cook et al, 2021; Fabre et al, 2022). Hence, although studying the effects of a single mutation on competitive behaviour, it is necessary to conduct experiments and to therefore design a model which supports the profiling of cells with different mutations at the same time, without demanding profuse amounts of pipetting.

Likewise, this competition model was designed by leveraging the F12-PVA 28-day expansion system for mouse HSCs (Wilkinson et al, 2019), which limits its application to human HSC models. Nevertheless, with the novel, chemically-defined expansion system for human HSCs developed by Sakurai and colleagues (2023), it will be possible to adapt this method to the split culture technique, to eventually conduct cell competition assays (Sakurai et al, 2024).

Future experiments and outlook

The novel and innovative clonal competition model I have described and characterised throughout Chapter 4 of this thesis could help identify the subsets of clones which cooperate with each other - or in other instances, compete - to drive disease evolution and progression. It will be necessary to first determine the ability to expand stem and progenitor cells of different genotypes, as some mutations might diminish the feasibility of the assay due to the biological processes they disrupt. It might also be suitable to profile clones at different timepoints throughout the assay, i.e., 3, 7 and 14 days after splitting and mixing, for example, to help establish a timeline of competitive behaviour acquisition, thus bringing clarity to timing of events in clonal evolution.

These competition assays could readily be applied to profile TET2 competitor clones derived from human patient samples thanks to the recent development of a chemically defined media for human HSC expansion - also known as the 3A media expansion system - (Sakurai et al, 2023). Bulk cultures on this new expansion system can likewise be split and continue to proliferate without affecting viability or phenotypic HSC output (Juan Rubio-Lara, personal communication). Nevertheless, due to the very recent implementation of the technique, it is currently unclear whether this could also be feasible for single-cell cultures. It will be necessary to establish split clonal cultures on the 3A expansion system, in order to transition to clonal competition assays.

6.3 The impact of microenvironmental factors on disease evolution illustrated by a triple-mutant mouse model

The BM microenvironment crucially regulates HSC function and behaviour through complex interactions with niche cell populations, which secrete a plethora of cytokines, chemokines and other soluble factors that contribute to homeostasis, but also to disease. In Chapter 5 of this dissertation, I showed how the synergistic effect of JAK2 V617F gain-of-function and TET2 LOF with IP-10 impact disease evolution in an allelic series of MPN mouse models, which resulted in a mild, but considerable, rescue of the severe red blood cell phenotype characteristic of polycythemia vera.

6.3.1 The implications of niche soluble factors in MPN disease biology

The serum levels and signalling of many cytokines and chemokines have been shown to be dysregulated in healthy aged individuals, including the interferon- γ inducible protein 10 kDa, IP-10 (Fulop et al, 2006; Shurin et al, 2007; Mondet et al, 2015). Likewise, IP-10 has previously been implicated in diverse human diseases, including inflammatory and autoimmune disorders, infection and cancer (Liu et al, 2011). In addition, high levels of IP-10 have likewise been linked to renal (Rotondi et al, 2004), and lung allograft failure (Shino et al, 2022). Particularly relevant for cancer, IP-10 is overexpressed in metastatic melanoma (Reschke et al, 2021; Kim et al, 2024), invasive breast carcinoma (Kim et al, 2021), hepatocellular carcinoma (Brandt et al, 2022), pancreatic adenocarcinoma (Huang et al, 2021). Hence, the importance of studying the role of IP-10 in the pathogenesis of human cancer, and to understand the cellular cross-talk between cells of the BM niche and HSCs harbouring MPN driver mutations.

Inflammatory signatures have been described across all subtypes of myeloproliferative neoplasms, with the highest reported levels being found in PV patients (Gastaldi Cominal et al, 2021), together with PMF (Vaidya et al, 2012), as well as slight implications in ET (Tefferi et al, 2011). Furthermore, increased concentrations of IP-10 had previously been reported in serum of JAK2-mutant MPN patients and cell lines, linked to high risk of transformation into overt leukaemia (Schnoder et al, 2017). The central role of the JAK/STAT pathway in the onset and evolution of MPNs is sustained by the interplay of a plethora of soluble factors present in the BM microenvironment. JAK2, in particular, transduces signals from IL-3, IL-5, TPO, EPO cognate receptors, and is regulated by IFN- and G-CSF (Tefferi, 2010). In a seminal study, the Kent Lab showed that IP-10 correlated with disease severity and JAK2 V617F mutational burden in a large serum cytokine screen of more than 400 MPN patients (Obro et al, 2020).

Further experimental validation unveiled high levels of IP-10 in patients harbouring JAK2 V617F and TET2 LOF mutations, as well as in JAK2/TET2-double mutant mouse models, which prompted the development and phenotypic characterisation of our JAK2/TET2/IP10 mouse model.

6.3.2 The effect of IP-10 and concomitant mutations on MPN disease phenotype

In light of the findings by Obro et al, we reasoned that loss of IP-10 would reverse the PV-like phenotype resembled in JAK2 V617F mouse models (Li et al, 2010). Although we did observe a significant reduction in haemoglobin levels in the peripheral blood of JAK2/IP10 double-mutant mice in comparison to JAK2-homozygous mice, haematocrit levels were similar, both in JAK2 V617F IP-10 heterozygous and homozygous mice. Accordingly, previous studies have demonstrated that bone marrow stromal cells protect JAK2 V617F-positive cell lines from the apoptotic effect of atiprimod, a JAK2 inhibitor (Manshouri et al, 2011). In another study, treatment with the oral JAK2 inhibitor, CEP-701, did not produce any changes in the levels of inflammatory cytokines and chemokines, among them IP-10, either in responding or non-responding patients in relation to baseline cytokine levels (prior to treatment) (Santos et al, 2010). Treatment of PV mouse models with CYT387, another JAK2 inhibitor, did not inflict any significant amelioration of inflammatory cytokines; furthermore, levels of IP-10, GRO- α , IL-10, MCP-1, among others, remained elevated in comparison to untreated controls (Tyner et al, 2010). These data are suggestive of a non-autonomous cell mechanism by which IP-10 contributes to MPN pathogenesis. Furthermore, in our earlier findings, abnormal CXCL10 serum levels were detected in patients with JAK2 V617F and TET2 LOF mutations, as well as in mouse models, together with the fact that concomitant JAK2 and TET2 mutations produce the most aggressive disease phenotype in mouse models (Shepherd et al, 2018), we proceeded to profile the loss of IP-10 in a JAK2/TET2 homozygous context.

We observed a significant reduction in haematocrit levels in JAK2/TET2/IP10 triple-homozygous mice in comparison to JAK2 V617F single and JAK2/IP10 double-mutant mice, towards more advanced stages of the disease (16 weeks of age). There was also an important reduction in the haemoglobin levels - although not significant - in blood of triple-mutant than in JAK2-mutant mice. This was further supported by a partial restoration of the frequencies of stage I erythroblasts (CD71- TER119-) in the bone marrow and spleen of triple-mutant mice, accompanied by a slight decrease in stage V (CD71- TER119+) terminally differentiated erythroblasts. Splenomegaly was also partially ameliorated in the triple-mutants, in comparison to JAK2 V61F7 mice. These are prominent findings, given that loss of IP-10 and TET2 in a JAK2 homozygous context result in dampened erythrocytosis, which is one of the

hallmarks of the severe PV phenotype in JAK2 V617F mice (Li et al, 2014). Notably, in TET2/IP10 knockout mice, there were no significant changes observed to the frequencies of erythroblasts at any stage in bone marrow or spleen, neither were the haematocrit or haemoglobin levels elevated in the blood of these mice. In addition, spleen weights of the double knockout mice were significantly reduced in comparison to JAK2 V617F or triple-mutant mice. Although not a driver mutation, TET2 LOF can induce splenomegaly in mouse models (Li et al, 2011), but it is substantially more pronounced in JAK2 V617F mice (Li et al, 2014; Hasan et al, 2013) and patients (Barosi et al, 2007), which is a direct consequence of stem and progenitor cell recruitment to the spleen (Hsieh et al, 2007), and it is the more accentuated in myelofibrosis (Song et al, 2018).

Despite we observed a significant increase in LSK cells in the bone marrow of TET2/IP10 homozygous mice, along with elevated frequency of Lin⁻ cells in the spleen, this did not result in the development of a severe red cell phenotype. This indicates that, plausibly, an evolutionary selective pressure requires the acquisition of a JAK2 V617F mutation in the stem and progenitor cell compartment to initiate disease, which further supports the study of concomitant mutations in blood cancer mouse models. Fundamentally, sequential acquisition of mutations in JAK2 and TET2 shape different clinically relevant aspects of disease, as previously reported by our group (Ortmann et al, 2015; Kent & Green, 2017). Hence, future experiments could be performed on MPN patient samples with co-occurring JAK2 and TET2 mutations, in which the timing of acquisition has been determined, along with serum cytokine profiling, to infer which cytokines are elevated in JAK2- first or TET2-first patients, which are likely to differ given the impact of timing of mutations.

6.3.3 Remaining questions regarding the clinical significance of the microenvironment in MPNs

What cell types in the bone marrow niche could be contributing to IP-10 production?

Data from our lab (Belmonte et al, 2024), has previously demonstrated that CD14⁺ monocytes could be the major cellular source in the BM for IP-10 production. Notably, a subset of inflammatory monocytes (CD14⁺CD56⁺), yielded the largest secreted amounts of IP-10 in response to IFN- γ stimulation in PV patients with >95% JAK2 V617F allele burden in granulocytes. Likewise, T lymphocytes (CD3⁺CD56⁻) were observed to contribute to IP-10 production, mainly in myelofibrosis patients. These data agree with previous reports, whereby macrophages are an important source of secreted molecules, including IP-10 (Nathan, 1987). Crucially, monocytes and macrophages increase levels of IP-10 in JAK2 V617F-positive PV patients (Fan et al, 2023). Other cell types may include dendritic cells, given that their secretion

of IP-10 facilitates T cell maturation upon antigen recognition (Yoneyama et al, 2002). JAK2 V671F mutations in neutrophils lead to overproduction of CXCL10, although these cells are not the main drivers of disease (Haage et al, 2024). Therefore, a comprehensive profiling by flow cytometry or multiplexed assays, could be conducted to survey the expression of pro-inflammatory cytokines in these different cell types and subsets, in order to determine a therapeutic target in myeloid malignancies.

How to experimentally address the simultaneous effect of several cytokines on normal and malignant HSC biology?

Across the serum cytokine profiling I conducted on the multiple genotypes of our MPN mouse models, I identified a set of cytokines which could have been compensating for the loss of IP-10 in the triple-mutant mouse model. These molecules included cytokines which have been implicated in MPNs, such as CCL5 (Kong et al, 2025); CXCL1 - the equivalent of GRO- in humans - (Obro et al, 2020; Collinson et al, 2020); and IL-13, which contributes to fibrosis of the marrow (Melo-Cardenas et al, 2022). However, among our findings, there could be other molecules of potential interest such as the p40 monomer of IL-12 (IL-12P40), IL-2 and MCP-1 (CCL2). Since these were elevated in the serum of triple-mutant mice, indicating that the stromal component of the BM niche could be actively producing these cytokines (Vukotic et al, 2022), thus impeding the complete restoration of the phenotype. Profiling the types of responses elicited by the complex array of soluble factors in the microenvironment could shed new light on the role of the niche in MPN pathogenesis.

Although studying the synergistic effects of multiple cytokines is certainly an experimental challenge, it is now possible to address this question through novel methods. For instance, the recent publication of a *dictionary of immune responses*, has allowed to describe the response of 17 immune cell types to 86 different cytokines *in vivo* at the single-cell level, collected from scRNA-seq data, equating to more than 1400 cytokine combinations, delineating the cell-type specific response and mapping the production of cytokines (Cui et al, 2024). These types of experiments could likewise be performed on MPN mouse models, either single- or double-mutant, although it would be critical to determine the types of tissues to be profiled first, as this methodology was originally performed on mouse lymph nodes, and therefore its applicability across tissues remains to be validated.

Nevertheless, our main interest lies on the haematopoietic stem cell compartment, since myeloid malignancies are known to be the result of somatic mutations acquired at the stem cell levels, we must therefore explore the impact of cytokines on HSCs, in health or disease,

in a more succinct manner. To convey this idea, the Schroeder group reported a microfluidics platform which allowed to study the dynamics of ERK signalling and the responses elicited by single HSCs and MPPs derived from human samples (Wang et al, 2021). Here, the authors studied the combinatorial effects of different cytokines which are commonly used in defined media for HSC/HSPC culture through an innovative time-lapse imaging technique, unveiling how these cytokines influence cell fate. The applicability of this study could extend to understand JAK2 V617F or JAK2/TET2 mutant HSC response elicited upon treatment with single or multiple cytokines at a time *in vitro*.

6.3.4 Limitations of the study

Although the generation of an allelic series of a new mouse model has shed light on the contribution of niche factors to the onset and progression of MPNs, this study was confined to the role of only one molecule, CXCL10 or IP-10, which does not completely resemble what occurs naturally in the microenvironment. Considering the myriad of cell types which constitute the bone marrow - osteoblasts, osteocytes, osteoclasts, CAR cells, NG2+ cells, pericytes, endothelial cells, adipocytes, among others (Xiao et al, 2022; Reagan & Rosen, 2016), there is a great abundance of secreted factors circulating within the niche. These factors, of which are known SCF or CXCL12 to exert important regulatory roles over HSCs (Hassan & Zander, 1996; Greenbaum et al, 2013) under physiological conditions, others can be equally as important for the development of haematological malignancies. It would therefore be necessary to generate mouse models in which the potential effects caused by the loss of more than one soluble factor could be profiled across time.

Even though this study was dedicated to scrutinising the role of IP-10 in synergy with JAK2 and TET2 homozygous mutations in MPN disease biology, no experiments were conducted to identify the cellular source of IP-10 within the bone marrow of the newly generated triple-mutant mouse model. Although this was mainly due to the large amounts of breeding which needed to occur before obtaining the triple-homozygous genotype, consuming great amounts of time to be completed, this is one of the main experiments to be performed in the future. These type of experiments are essential in the characterisation of any new mouse model that has been generated with the purpose of studying the microenvironment surrounding HSCs, as it has been demonstrated previously (Ho et al, 2019; Gao et al, 2021).

Lastly, in our study, disease monitoring was conducted from 8 to 16 weeks of age. Notwithstanding, myeloid malignancies are more frequent in elderly patients (Almedia & Ramos, 2016; Klepin et al, 2014); and specifically in MPNs, median patient age is between 62-66 years (Mesa et al, 2016). Consequently, profiling the phenotype of MPN disease in aged mice would be informative regarding the role of IP-10 or a different inflammatory molecule, as

ageing contributes to the remodelling of the bone marrow landscape (Li et al, 2023), and can therefore be of interest to understand MPN disease evolution.

6.4 The bone marrow microenvironment and niche factors in normal and malignant haematopoiesis

One of the unifying concepts throughout this doctoral thesis work is the bone marrow niche. In Chapter 3, HSCs isolated from different bone marrow locations exhibited unique functional and molecular features, which might be associated with the composition of the niche in each of the bones profiled. Importantly, osteogenic differentiation has been observed to vary between marrow from femoral head, iliac crest and vertebral body in humans (Herrmann et al, 2019), as well as differential expression of mesenchymal stem cell markers, such as CD146 (Bikorimana et al, 2022) across locations. In addition, the stromal component of the bone marrow in the iliac crest and spine has been found to differ, also in regards to osteogenic differentiation (Fragkakis et al, 2018). Hence, analysis of cellular bone marrow populations within the mesenchymal, endothelial and neural compartments could help delineate the potential causes of heterogeneity among HSCs residing within different bones. In addition, given that HSCs and the various kinds of progenitors localise to specific regions within the bone marrow (Comazzetto et al, 2019) - and considering that these findings have been achieved by imaging experiments - it would be therefore beneficial to obtain imaging data from bone marrow sections where the location of HSCs in relation to different cellular populations in the niche could be identified.

In Chapter 4, the TET2-super competitor clones demonstrated to be superior to their wild-type counterparts, by outperforming them in a 70%:30% proportion, whereby in many of the competition matrices, there were no traces of wild-type cells remaining. Moreover, the elevated number of ELSK cells in comparison to both wild-type and TET2-super competitors, would suggest a higher repopulating capacity in vivo. Notwithstanding, since the TET2 competitor clones were those which showed the highest self-renewal potential in transplantation, it is possible that the in vivo setting could have impacted the super-competitive behaviour of the TET2-super competitors. For instance, a higher abundance of CXCL12+ cells has been observed in bone marrow of MDS patients, whereby CD34+ HSCs overexpress the antiapoptotic protein Bcl2, and consequently lower apoptotic rates than marrow samples with smaller CXCL12+ density (Abe-Suzuki et al, 2014). Moreover, as reviewed in Pontikoglou et al (2023), the MSC compartment exhibits morphological and cytogenetic abnormalities, which result in the normal regulation of HSC/HSPC maintenance, thereby promoting clonal expansion of malignant HSCs. In clonal haematopoiesis, where TET2 mutations are amongst the most frequent together with DNMT3A mutations, inflammatory molecules such as IL-6, IL-1 β and TNF- α are elevated both in patients and mouse models, whereby IL-1 β enhances

TET2-mutant HSC expansion, exacerbating myeloid bias and increasing numbers of monocyte populations (Clatchy et al, 2023). In addition to the stromal compartment of the bone marrow, TET2 deficiency in immune cells, such as macrophages, and T and B lymphocytes, results in elevated secretion of inflammatory cytokines, such as IL-1 β , which sustain the HSC malignant phenotype in myeloid disorders, including clonal haematopoiesis and AML (Tsiouplis et al, 2020; Ngo et al, 2024). Accordingly, the addition of a cytokine which elicits a strong inflammatory response, such as IL-6 or TNF- α would allow to infer if an inflammatory environment enhances or dampens the super-competitive behaviour of TET2 clones against wild-type clones. Alternatively, TET2 clones could be expanded in the presence of inflammatory cytokines prior to competition assays, in order to determine if they could still exhibit a super-competitive advantage over their normal counterparts when set in competition. In Chapter 5, I explored the synergistic role between the pro-inflammatory IP-10 and homozygous JAK2 and TET2 mutations, which are the most common co-occurring mutations in MPNs (Ortmann, Kent et al, 2015). Even though a subset of CD56⁺ monocytes was found to be a potential source of IP-10 in the bone marrow of MPN patients (Belmonte et al, 2024), these experiments were not conducted on the mouse models I characterised throughout this work. Considering the prevalence of JAK2 and TET2 concomitant mutations in MPNs, a careful characterisation of the cellular populations comprising the bone marrow in double- and triple-mutant mouse models would allow to determine the main source of IP-10 production. The JAK2 V617F-driven phenotype is accompanied by an upregulation of inflammatory cytokine axes, such as interleukins (IL-1 β , IL-4, IL-5), macrophage inflammatory proteins (MIPs), the monocyte chemoattractant CCL2 and TNF- α (Bellosillo et al, 2025), which highlights the role of the bone marrow in MPN disease biology. Since CCL2 and MIP-2 were found to be upregulated in the triple-mutant mouse model (Figure 5.14 of Chapter 5), it is reasonable to suggest that these molecules were secreted in order to compensate for the loss of IP-10, which at the same time would explain why the phenotype was not fully rescued as IP-10 was shown to be elevated in JAK2/TET2-double mutant patients and mouse models (Figure 5.2 of Chapter 5). Thence, targeted analysis of CCL2 and MIP-2 in MPN patients and mouse models could provide a clearer picture of the bone marrow remodelling upon the loss of key factors. Likewise, since IP-10 had initially been associated with fibrosis of the bone marrow and increased risk of evolving into AML (Obro et al, 2020), and considering that increased bone marrow vascularisation is one of the features of fibrosis (Korn & Mendez-Ferrer, 2017; Goulard et al, 2018), profiling the levels of VEGF in mouse models could address the question of how the endothelial component of the bone marrow response to the loss of IP-10, yet in the presence of JAK2/TET2 homozygous mutations. Although many other factors contribute to bone marrow fibrosis in MPN (Medinger & Passweg, 2014), VEGF would be a

suitable candidate to assess in order to delineate the mechanism by which disease is sustained.

6.5 Final remarks and future work

Throughout this doctoral research work, I explored fundamental questions of normal and malignant HSC biology through the establishment and application of novel experimental approaches. First, I demonstrated that HSCs preferentially reside within their geographical location of origin, without migrating to other BM sites, indicating that their phylogenetic relatedness determines where they remain for the entirety of a mouse's lifetime. I also showed how anatomical site of origin impacts the functionality of HSCs in a vast, comprehensive set of single-cell *in vivo* functional assays, posing the femur and tibia as the two sites which contain the long-term reconstituting cells, and the spine with the lowest frequency of truly functional HSCs. The careful examination of our scRNA-seq dataset, was validated by these assays, as femur and tibia-derived HSCs were the highest scoring groups for the MoLO and RepopSig signatures, two reliable computational metrics to predict functional *in vivo* performance. At the core of these findings, lies the extraordinary diversity of the stem cell pool, which has both fascinated and intrigued researchers for over six decades, and will continue to evoke more questions on how HSCs from different bones respond to distinct kinds of stimuli and stress, and how these can circumvent HSC heterogeneity.

For the second project, the novel *in vitro* model of clonal competition unveiled a previously undescribed type of competitor clone - the TET2 supercompetitor, which exhibited remarkable performance against its wild-type rival clones. These supercompetitors had a significantly elevated phenotypic HSC output in both competition and non-competition settings, and were characterised by the overexpression of genes implicated in cell adhesion, migration, cytoskeletal organisation and ECM interaction. This TET2 supercompetitive behaviour was consistently observed across hundreds of clones analysed, which supports the existence of supercompetitors in myeloid malignancies, and potentially also in leukaemia, which had only been reported in solid tumours. Following the principle of Darwinian evolution, TET2 supercompetitors could have developed ways in which they deprive their rivals from the available nutrients in the media, which could also translate into what is observed *in vivo*, as cancer cells have also been demonstrated to compete for niche space, in the same way that rival species - or even members of the same species - do in order to survive and advance. Since other mutations are required for the full onset of malignancy, it is entirely possible that there are supercompetitors of different genotypes, and these could collectively drive the extinction of healthy clones in leukaemia.

Lastly, I developed and fully characterised a triple-mutant mouse model of MPN, in order to understand the role of an important microenvironmental molecule, IP-10 or CXCL10, and the crucial cooperative effect of JAK2 gain-of-function and TET2 loss-of-function mutations. Paradoxically, even though our group had previously shown that JAK2/TET2 double-mutant mice exhibit the most aggressive MPN phenotype among other mouse models, it was the co-occurrence of these two mutations that, along with depletion of IP-10, resulted in a partial amelioration of the severe red blood cell phenotype. The reduction of haematocrit, as well as the restoration of terminally differentiated erythroblast frequencies in haematopoietic tissues of triple-mutant mice, provided evidence for the value in studying concomitant mutations in HSCs, and how the inflammatory landscape can shape the evolution of myeloproliferative disorders. It is likely that other molecules could be contributing to severe stages of MPN, particularly myelofibrosis and transformation into clinically overt leukaemia, which will require the development and application of experimental approaches that can help profile this phenomenon *in vivo* and *in vitro*. Likewise, the therapeutic target of cytokines, or their cellular source, could be a potential alternative in the treatment of MPNs, given that the complex patient heterogeneity has refrained the development of effective therapies. Thus, personalised medicine strategies could be implemented more widely across the different MPN subtypes.

In summary, further research would be necessary to gain deeper insight into the puzzling and fascinating biology of HSCs, derived from geographical sites, how somatic mutations can serve to understand how they relate, but also how they compete, and how the conjunction of the bone marrow niche and the malignant role of mutations can be leveraged to fulfill the mission to end cancer.

References

- 1000 Genomes Project Consortium, Abecasis, G. R., Altshuler, D., Auton, A., Brooks, L. D., Durbin, R. M., Gibbs, R. A., Hurles, M. E., & McVean, G. A. (2010). A map of human genome variation from population-scale sequencing. *Nature*, 467(7319), 1061–1073. <https://doi.org/10.1038/nature09534>
- Abe-Suzuki, S., Kurata, M., Abe, S., Onishi, I., Kirimura, S., Nashimoto, M., Murayama, T., Hidaka, M., & Kitagawa, M. (2014). CXCL12+ stromal cells as bone marrow niche for CD34+ hematopoietic cells and their association with disease progression in myelodysplastic syndromes. *Laboratory Investigation; a Journal of Technical Methods and Pathology*, 94(11), 1212–1223. <https://doi.org/10.1038/labinvest.2014.110>
- Abkowitz, J. L., Catlin, S. N., & Gutter, P. (1996). Evidence that hematopoiesis may be a stochastic process in vivo. *Nature Medicine*, 2(2), 190–197. <https://doi.org/10.1038/nm0296-190>
- Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F., George, R. A., Lewis, S. E., Richards, S., Ashburner, M., Henderson, S. N., Sutton, G. G., Wortman, J. R., Yandell, M. D., Zhang, Q., ... Venter, J. C. (2000). The genome sequence of *Drosophila melanogaster*. *Science (New York, N.Y.)*, 287(5461), 2185–2195. <https://doi.org/10.1126/science.287.5461.2185>
- Adekola, K., Popat, U., & Ciurea, S. O. (2014). An update on allogeneic hematopoietic progenitor cell transplantation for myeloproliferative neoplasms in the era of tyrosine kinase inhibitors. *Bone Marrow Transplantation*, 49(11), 1352–1359. <https://doi.org/10.1038/bmt.2014.176>
- Adolfsson, J., Månsson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C. T., Bryder, D., Yang, L., Borge, O.-J., Thoren, L. A. M., Anderson, K., Sitnicka, E., Sasaki, Y., Sigvardsson, M., & Jacobsen, S. E. W. (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell*, 121(2), 295–306. <https://doi.org/10.1016/j.cell.2005.02.013>
- Aflalo, E., & Weinstein, Y. (1990). Competition among leukemic cells. *Leukemia Research*, 14(11–12), 989–996. [https://doi.org/10.1016/0145-2126\(90\)90112-m](https://doi.org/10.1016/0145-2126(90)90112-m)
- Agarwal, P., Li, H., Choi, K., Hueneman, K., He, J., Welner, R. S., Starczynowski, D. T., & Bhatia, R. (2021). TNF- α -induced alterations in stromal progenitors enhance leukemic stem cell growth via CXCR2 signaling. *Cell Reports*, 36(2), 109386. <https://doi.org/10.1016/j.celrep.2021.109386>
- Akada, H., Akada, S., Hutchison, R. E., Sakamoto, K., Wagner, K.-U., & Mohi, G. (2014). Critical role of Jak2 in the maintenance and function of adult hematopoietic stem cells. *Stem Cells (Dayton, Ohio)*, 32(7), 1878–1889. <https://doi.org/10.1002/stem.1711>
- Akada, H., Yan, D., Zou, H., Fiering, S., Hutchison, R. E., & Mohi, M. G. (2010). Conditional expression of heterozygous or homozygous Jak2V617F from its endogenous promoter induces a polycythemia vera-like disease. *Blood*, 115(17), 3589–3597. <https://doi.org/10.1182/blood-2009-04-215848>
- Akinduro, O., Weber, T. S., Ang, H., Haltalli, M. L. R., Ruivo, N., Duarte, D., Rashidi, N. M., Hawkins, E. D., Duffy, K. R., & Lo Celso, C. (2018). Proliferation dynamics of acute myeloid

leukaemia and haematopoietic progenitors competing for bone marrow space. *Nature Communications*, 9(1), 519. <https://doi.org/10.1038/s41467-017-02376-5>

Alberti-Servera, L., von Muenchow, L., Tsapogas, P., Capoferri, G., Eschbach, K., Beisel, C., Ceredig, R., Ivanek, R., & Rolink, A. (2017). Single-cell RNA sequencing reveals developmental heterogeneity among early lymphoid progenitors. *The EMBO Journal*, 36(24), 3619–3633. <https://doi.org/10.15252/embj.201797105>

Alexandrov, L. B., Jones, P. H., Wedge, D. C., Sale, J. E., Campbell, P. J., Nik-Zainal, S., & Stratton, M. R. (2015). Clock-like mutational processes in human somatic cells. *Nature Genetics*, 47(12), 1402–1407. <https://doi.org/10.1038/ng.3441>

Alexandrov, L. B., Nik-Zainal, S., Wedge, D. C., Aparicio, S. A. J. R., Behjati, S., Biankin, A. V., Bignell, G. R., Bolli, N., Borg, A., Børresen-Dale, A.-L., Boyault, S., Burkhardt, B., Butler, A. P., Caldas, C., Davies, H. R., Desmedt, C., Eils, R., Eyfjörd, J. E., Foekens, J. A., ... Stratton, M. R. (2013). Signatures of mutational processes in human cancer. *Nature*, 500(7463), 415–421. <https://doi.org/10.1038/nature12477>

Allain-Maillet, S., Bosseboeuf, A., Mennesson, N., Bostoën, M., Dufeu, L., Choi, E. H., Cleyrat, C., Mansier, O., Lippert, E., Le Bris, Y., Gombert, J.-M., Girodon, F., Pettazzoni, M., Bigot-Corbel, E., & Hermouet, S. (2020). Anti-glucosylsphingosine autoimmunity, JAK2V617F-dependent interleukin-1 β and JAK2V617F-independent cytokines in myeloproliferative neoplasms. *Cancers*, 12(9), 2446. <https://doi.org/10.3390/cancers12092446>

Almeida, A. M., & Ramos, F. (2016). Acute myeloid leukemia in the older adults. *Leukemia Research Reports*, 6, 1–7. <https://doi.org/10.1016/j.lrr.2016.06.001>

An, J., Rao, A., & Ko, M. (2017). TET family dioxygenases and DNA demethylation in stem cells and cancers. *Experimental & Molecular Medicine*, 49(4), e323. <https://doi.org/10.1038/emm.2017.5>

Anand, S., Stedham, F., Beer, P., Gudgin, E., Ortmann, C. A., Bench, A., Erber, W., Green, A. R., & Huntly, B. J. P. (2011). Effects of the JAK2 mutation on the hematopoietic stem and progenitor compartment in human myeloproliferative neoplasms. *Blood*, 118(1), 177–181. <https://doi.org/10.1182/blood-2010-12-327593>

Andriole, V. T. (1959). Florence Rena Sabin—Teacher, Scientist, Citizen. *Journal of the History of Medicine and Allied Sciences*, 14(3), 320–350.

Appelbaum, F. R. (2020). Acute Leukemias in Adults. In *Abeloff's Clinical Oncology* (pp. 1783-1797.e1). Elsevier. <https://doi.org/10.1016/b978-0-323-47674-4.00095-5>

Arber, D. A., Orazi, A., Hasserjian, R. P., Borowitz, M. J., Calvo, K. R., Kvasnicka, H.-M., Wang, S. A., Bagg, A., Barbui, T., Branford, S., Bueso-Ramos, C. E., Cortes, J. E., Dal Cin, P., DiNardo, C. D., Dombret, H., Duncavage, E. J., Ebert, B. L., Estey, E. H., Facchetti, F., ... Tefferi, A. (2022). International Consensus Classification of Myeloid Neoplasms and Acute Leukemias: integrating morphologic, clinical, and genomic data. *Blood*, 140(11), 1200–1228. <https://doi.org/10.1182/blood.2022015850>

Arcilla, C. K., & Tadi, P. (2025). Neuroanatomy, unmyelinated nerve fibers. In *StatPearls*. StatPearls Publishing. <https://www.ncbi.nlm.nih.gov/books/NBK554461/>

Aslam, M. N., Jepsen, K. J., Khoury, B., Graf, K. H., & Varani, J. (2016). Bone structure and function in male C57BL/6 mice: Effects of a high-fat Western-style diet with or without trace minerals. *Bone Reports*, 5, 141–149. <https://doi.org/10.1016/j.bonr.2016.05.002>

Aslan, J. E., & McCarty, O. J. T. (2013). Rho GTPases in platelet function. *Journal of Thrombosis and Haemostasis: JTH*, 11(1), 35–46. <https://doi.org/10.1111/jth.12051>

Athanasiadis, E. I., Botthof, J. G., Andres, H., Ferreira, L., Lio, P., & Cvejic, A. (2017). Single-cell RNA-sequencing uncovers transcriptional states and fate decisions in haematopoiesis. *Nature Communications*, 8(1), 2045. <https://doi.org/10.1038/s41467-017-02305-6>

Bainbridge, L. J., & Daigaku, Y. (2024). Bulk synthesis and beyond: The roles of eukaryotic replicative DNA polymerases. *DNA Repair*, 141(103740), 103740. <https://doi.org/10.1016/j.dnarep.2024.103740>

Baker, N. E. (2020). Emerging mechanisms of cell competition. *Nature Reviews. Genetics*, 21(11), 683–697. <https://doi.org/10.1038/s41576-020-0262-8>

Balazs, A. B., Fabian, A. J., Esmon, C. T., & Mulligan, R. C. (2006). Endothelial protein C receptor (CD201) explicitly identifies hematopoietic stem cells in murine bone marrow. *Blood*, 107(6), 2317–2321. <https://doi.org/10.1182/blood-2005-06-2249>

Bamford, S., Dawson, E., Forbes, S., Clements, J., Pettett, R., Dogan, A., Flanagan, A., Teague, J., Futreal, P. A., Stratton, M. R., & Wooster, R. (2004). The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *British Journal of Cancer*, 91(2), 355–358. <https://doi.org/10.1038/sj.bjc.6601894>

Bandyopadhyay, S., Duffy, M. P., Ahn, K. J., Sussman, J. H., Pang, M., Smith, D., Duncan, G., Zhang, I., Huang, J., Lin, Y., Xiong, B., Imtiaz, T., Chen, C.-H., Thadi, A., Chen, C., Xu, J., Reichart, M., Martinez, Z., Diorio, C., ... Tan, K. (2024). Mapping the cellular biogeography of human bone marrow niches using single-cell transcriptomics and proteomic imaging. *Cell*, 187(12), 3120–3140.e29. <https://doi.org/10.1016/j.cell.2024.04.013>

Banjac, I., Maimets, M., & Jensen, K. B. (2023). Maintenance of high-turnover tissues during and beyond homeostasis. *Cell Stem Cell*, 30(4), 348–361. <https://doi.org/10.1016/j.stem.2023.03.008>

Bankaitis, E. D., Ha, A., Kuo, C. J., & Magness, S. T. (2018). Reserve stem cells in intestinal homeostasis and injury. *Gastroenterology*, 155(5), 1348–1361. <https://doi.org/10.1053/j.gastro.2018.08.016>

Bao, Z., Murray, J. I., Boyle, T., Ooi, S. L., Sandel, M. J., & Waterston, R. H. (2006). Automated cell lineage tracing in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America*, 103(8), 2707–2712. <https://doi.org/10.1073/pnas.0511111103>

Barisas, D. A. G., & Choi, K. (2024). Extramedullary hematopoiesis in cancer. *Experimental & Molecular Medicine*, 56(3), 549–558. <https://doi.org/10.1038/s12276-024-01192-4>

Barker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P. J., & Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene *Lgr5*. *Nature*, 449(7165), 1003–1007. <https://doi.org/10.1038/nature06196>

Baron, C. S., Kester, L., Klaus, A., Boisset, J.-C., Thambyrajah, R., Yvernogeu, L., Kouskoff, V., Lacaud, G., van Oudenaarden, A., & Robin, C. (2018). Single-cell transcriptomics reveal the dynamic of haematopoietic stem cell production in the aorta. *Nature Communications*, 9(1), 2517. <https://doi.org/10.1038/s41467-018-04893-3>

Barosi, G., Bergamaschi, G., Marchetti, M., Vannucchi, A. M., Guglielmelli, P., Antonioli, E., Massa, M., Rosti, V., Campanelli, R., Villani, L., Viarengo, G., Gattoni, E., Gerli, G., Specchia, G., Tinelli, C., Rambaldi, A., Barbui, T., & Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto (GIMEMA) Italian Registry of Myelofibrosis. (2007). JAK2 V617F mutational status predicts progression to large splenomegaly and leukemic transformation in primary myelofibrosis. *Blood*, 110(12), 4030–4036. <https://doi.org/10.1182/blood-2007-07-099184>

Baryawno, N., Przybylski, D., Kowalczyk, M. S., Kfoury, Y., Severe, N., Gustafsson, K., Kokkaliaris, K. D., Mercier, F., Tabaka, M., Hofree, M., Dionne, D., Papazian, A., Lee, D., Ashenberg, O., Subramanian, A., Vaishnav, E. D., Rozenblatt-Rosen, O., Regev, A., & Scadden, D. T. (2019). A cellular taxonomy of the bone marrow stroma in homeostasis and leukemia. *Cell*, 177(7), 1915–1932.e16. <https://doi.org/10.1016/j.cell.2019.04.040>

Bastani, S., Staal, F. J. T., & Canté-Barrett, K. (2023). The quest for the holy grail: overcoming challenges in expanding human hematopoietic stem cells for clinical use. *Stem Cell Investigation*, 10, 15. <https://doi.org/10.21037/sci-2023-016>

Baulies, A., Angelis, N., & Li, V. S. W. (2020). Hallmarks of intestinal stem cells. *Development (Cambridge, England)*, 147(15). <https://doi.org/10.1242/dev.182675>

Baxter, E. J., Scott, L. M., Campbell, P. J., East, C., Fourouclas, N., Swanton, S., Vassiliou, G. S., Bench, A. J., Boyd, E. M., Curtin, N., Scott, M. A., Erber, W. N., Green, A. R., & Cancer Genome Project. (2005). Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. *Lancet*, 365(9464), 1054–1061. [https://doi.org/10.1016/S0140-6736\(05\)71142-9](https://doi.org/10.1016/S0140-6736(05)71142-9)

Beaven, M. A. (2009). Our perception of the mast cell from Paul Ehrlich to now. *European Journal of Immunology*, 39(1), 11–25. <https://doi.org/10.1002/eji.200838899>

Becker, A. J., McCULLOCH, E. A., & Till, J. E. (1963). Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature*, 197(4866), 452–454. <https://doi.org/10.1038/197452a0>

Becker, H. J., Ishida, R., Wilkinson, A. C., Kimura, T., Lee, M. S. J., Coban, C., Ota, Y., Tanaka, Y., Roskamp, M., Sano, T., Tojo, A., Kent, D. G., & Yamazaki, S. (2023). Controlling genetic heterogeneity in gene-edited hematopoietic stem cells by single-cell expansion. *Cell Stem Cell*, 30(7), 987–1000.e8. <https://doi.org/10.1016/j.stem.2023.06.002>

Bellosillo, B., Doubek, M., Tomuleasa, C., Griesshammer, M., Marchetti, M., Sacha, T., & Gisslinger, H. (2024). JAK2 mutations in polycythemia vera: from molecular origins to inflammatory pathways and clinical implications. *Memo*. <https://doi.org/10.1007/s12254-024-01009-0>

Belmonte, M., Cabrera-Cosme, L., Øbro, N. F., Li, J., Grinfeld, J., Milek, J., Bennett, E., Irvine, M., Shepherd, M. S., Cull, A. H., Boyd, G., Riedel, L. M., Chi Che, J. L., Oedekoven, C. A., Baxter, E. J., Green, A. R., Barlow, J. L., & Kent, D. G. (2024). Increased CXCL10 (IP-10) is associated with advanced myeloproliferative neoplasms and its loss dampens erythrocytosis in mouse models. *Experimental Hematology*, 135(104246), 104246. <https://doi.org/10.1016/j.exphem.2024.104246>

Benveniste, P., Frelin, C., Janmohamed, S., Barbara, M., Herrington, R., Hyam, D., & Iscove, N. N. (2010). Intermediate-term hematopoietic stem cells with extended but time-limited reconstitution potential. *Cell Stem Cell*, 6(1), 48–58. <https://doi.org/10.1016/j.stem.2009.11.014>

Benz, C., Copley, M. R., Kent, D. G., Wohrer, S., Cortes, A., Aghaeepour, N., Ma, E., Mader, H., Rowe, K., Day, C., Treloar, D., Brinkman, R. R., & Eaves, C. J. (2012). Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. *Cell Stem Cell*, 10(3), 273–283. <https://doi.org/10.1016/j.stem.2012.02.007>

Beumer, J., & Clevers, H. (2024). Hallmarks of stemness in mammalian tissues. *Cell Stem Cell*, 31(1), 7–24. <https://doi.org/10.1016/j.stem.2023.12.006>

Bhatia, M., Bonnet, D., Kapp, U., Wang, J. C., Murdoch, B., & Dick, J. E. (1997). Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. *The Journal of Experimental Medicine*, 186(4), 619–624. <https://doi.org/10.1084/jem.186.4.619>

Biedzinski, S., Agsu, G., Vianay, B., Delord, M., Blanchoin, L., Larghero, J., Faivre, L., Théry, M., & Brunet, S. (2020). Microtubules control nuclear shape and gene expression during early stages of hematopoietic differentiation. *The EMBO Journal*, 39(23), e103957. <https://doi.org/10.15252/embj.2019103957>

Bierman, H. R. (1952). Bone marrow aspiration the posterior iliac crest, an additional safe site. *California Medicine*, 77(2), 138–139. <https://www.ncbi.nlm.nih.gov/pubmed/12978895>

Bikorimana, J.-P., Saad, W., Abusarah, J., Lahrichi, M., Talbot, S., Shammaa, R., & Rafei, M. (2022). CD146 defines a mesenchymal stromal cell subpopulation with enhanced suppressive properties. *Cells (Basel, Switzerland)*, 11(15), 2263. <https://doi.org/10.3390/cells11152263>

Billett, H. H. (1990). Hemoglobin and hematocrit. In *Clinical Methods: The History, Physical, and Laboratory Examinations*. Butterworths. <https://www.ncbi.nlm.nih.gov/books/NBK259/>

Birkenfeld, J., Nalbant, P., Yoon, S.-H., & Bokoch, G. M. (2008). Cellular functions of GEF-H1, a microtubule-regulated Rho-GEF: is altered GEF-H1 activity a crucial determinant of disease pathogenesis? *Trends in Cell Biology*, 18(5), 210–219. <https://doi.org/10.1016/j.tcb.2008.02.006>

Bittencourt, M. C. B., & Ciurea, S. O. (2020). Recent advances in allogeneic hematopoietic stem cell transplantation for acute myeloid leukemia. *Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation*, 26(9), e215–e221. <https://doi.org/10.1016/j.bbmt.2020.06.007>

Bjornson, C. R., Rietze, R. L., Reynolds, B. A., Magli, M. C., & Vescovi, A. L. (1999). Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells in vivo. *Science (New York, N.Y.)*, 283(5401), 534–537. <https://doi.org/10.1126/science.283.5401.534>

Blokzijl, F., de Ligt, J., Jager, M., Sasselli, V., Roerink, S., Sasaki, N., Huch, M., Boymans, S., Kuijk, E., Prins, P., Nijman, I. J., Martincorena, I., Mokry, M., Wiegerinck, C. L., Middendorp, S., Sato, T., Schwank, G., Nieuwenhuis, E. E. S., Verstegen, M. M. A., ... van Boxtel, R. (2016). Tissue-specific mutation accumulation in human adult stem cells during life. *Nature*, 538(7624), 260–264. <https://doi.org/10.1038/nature19768>

Boggs, D. R. (1984). The total marrow mass of the mouse: a simplified method of measurement. *American Journal of Hematology*, 16(3), 277–286. <https://doi.org/10.1002/ajh.2830160309>

Böhni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B. F., Beckingham, K., & Hafen, E. (1999). Autonomous control of cell and organ size by CHICO, a

Drosophila homolog of vertebrate IRS1-4. *Cell*, 97(7), 865–875. [https://doi.org/10.1016/s0092-8674\(00\)80799-0](https://doi.org/10.1016/s0092-8674(00)80799-0)

Boisset, J.-C., & Robin, C. (2012). On the origin of hematopoietic stem cells: progress and controversy. *Stem Cell Research*, 8(1), 1–13. <https://doi.org/10.1016/j.scr.2011.07.002>

Boitano, A. E., Wang, J., Romeo, R., Bouchez, L. C., Parker, A. E., Sutton, S. E., Walker, J. R., Flaveny, C. A., Perdew, G. H., Denison, M. S., Schultz, P. G., & Cooke, M. P. (2010). Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science (New York, N.Y.)*, 329(5997), 1345–1348. <https://doi.org/10.1126/science.1191536>

Bondar, T., & Medzhitov, R. (2010). P53-mediated hematopoietic stem and progenitor cell competition. *Cell Stem Cell*, 6(4), 309–322. <https://doi.org/10.1016/j.stem.2010.03.002>

Bonnet, D., & Dick, J. E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine*, 3(7), 730–737. <https://doi.org/10.1038/nm0797-730>

Bono, H., Kasukawa, T., Furuno, M., Hayashizaki, Y., & Okazaki, Y. (2002). FANTOM DB: Database of Functional Annotation of RIKEN Mouse cDNA Clones. *Nucleic Acids Research*, 30(1), 116–118. <https://doi.org/10.1093/nar/30.1.116>

Boueya, I. L., Sandhow, L., Albuquerque, J. R. P., Znaidi, R., & Passaro, D. (2025). Endothelial heterogeneity in bone marrow: insights across development, adult life and leukemia. *Leukemia*, 39(1), 8–24. <https://doi.org/10.1038/s41375-024-02453-x>

Boveri, T. (1892). Ueber die Entstehung des Gegensatzes zwischen den Geschlechtszellen und den somatischen Zellen bei *Ascaris megalocephala*, nebst Bemerkungen zur Entwicklungsgeschichte der Nematoden : Vorgetragen am 15. November 1892.

Bowie, M. B., Kent, D. G., Dykstra, B., McKnight, K. D., McCaffrey, L., Hoodless, P. A., & Eaves, C. J. (2007). Identification of a new intrinsically timed developmental checkpoint that reprograms key hematopoietic stem cell properties. *Proceedings of the National Academy of Sciences of the United States of America*, 104(14), 5878–5882. <https://doi.org/10.1073/pnas.0700460104>

Bowling, S., Di Gregorio, A., Sancho, M., Pozzi, S., Aarts, M., Signore, M., D Schneider, M., Martinez-Barbera, J. P., Gil, J., & Rodríguez, T. A. (2018). P53 and mTOR signalling determine fitness selection through cell competition during early mouse embryonic development. *Nature Communications*, 9(1), 1763. <https://doi.org/10.1038/s41467-018-04167-y>

Bowling, S., Sritharan, D., Osorio, F. G., Nguyen, M., Cheung, P., Rodriguez-Fraticelli, A., Patel, S., Yuan, W.-C., Fujiwara, Y., Li, B. E., Orkin, S. H., Hormoz, S., & Camargo, F. D. (2020). An engineered CRISPR-Cas9 mouse line for simultaneous readout of lineage histories and gene expression profiles in single cells. *Cell*, 181(6), 1410–1422.e27. <https://doi.org/10.1016/j.cell.2020.04.048>

Bowman, R. L., Busque, L., & Levine, R. L. (2018). Clonal hematopoiesis and evolution to hematopoietic malignancies. *Cell Stem Cell*, 22(2), 157–170. <https://doi.org/10.1016/j.stem.2018.01.011>

Bowman, R. L., & Levine, R. L. (2017). TET2 in normal and malignant hematopoiesis. *Cold Spring Harbor Perspectives in Medicine*, 7(8). <https://doi.org/10.1101/cshperspect.a026518>

Boysen, G., Alexandrov, L. B., Rahbari, R., Nookaew, I., Ussery, D., Chao, M.-R., Hu, C.-W., & Cooke, M. S. (2025). Investigating the origins of the mutational signatures in cancer. *Nucleic Acids Research*, 53(1). <https://doi.org/10.1093/nar/gkae1303>

Bozhilov, Y. K., Hsu, I., Brown, E. J., & Wilkinson, A. C. (2023). In vitro human haematopoietic stem cell expansion and differentiation. *Cells* (Basel, Switzerland), 12(6). <https://doi.org/10.3390/cells12060896>

Bradley, T. R., & Metcalf, D. (1966). The growth of mouse bone marrow cells in vitro. *The Australian Journal of Experimental Biology and Medical Science*, 44(3), 287–299. <https://doi.org/10.1038/icb.1966.28>

Brandt, E. F., Baues, M., Wirtz, T. H., May, J.-N., Fischer, P., Beckers, A., Schüre, B.-C., Sahin, H., Trautwein, C., Lammers, T., & Berres, M.-L. (2022). Chemokine CXCL10 modulates the tumor microenvironment of fibrosis-associated hepatocellular carcinoma. *International Journal of Molecular Sciences*, 23(15), 8112. <https://doi.org/10.3390/ijms23158112>

Breeland, G., Sinkler, M. A., & Menezes, R. G. (2025). Embryology, bone ossification. In *StatPearls*. StatPearls Publishing. <https://www.ncbi.nlm.nih.gov/books/NBK539718/>

Breschi, A., Gingeras, T. R., & Guigó, R. (2017). Comparative transcriptomics in human and mouse. *Nature Reviews. Genetics*, 18(7), 425–440. <https://doi.org/10.1038/nrg.2017.19>

Brkic, S., & Meyer, S. C. (2021). Challenges and perspectives for therapeutic targeting of myeloproliferative neoplasms. *HemaSphere*, 5(1), e516. <https://doi.org/10.1097/HS9.0000000000000516>

Brown, G., Tsapogas, P., & Ceredig, R. (2018). The changing face of hematopoiesis: a spectrum of options is available to stem cells. *Immunology and Cell Biology*, 96(9), 898–911. <https://doi.org/10.1111/imcb.12055>

Bryant, P. J., & Schneiderman, H. A. (1969). Cell lineage, growth, and determination in the imaginal leg discs of *Drosophila melanogaster*. *Developmental Biology*, 20(3), 263–290. [https://doi.org/10.1016/0012-1606\(69\)90015-3](https://doi.org/10.1016/0012-1606(69)90015-3)

Bryda, E. C. (2013). The Mighty Mouse: the impact of rodents on advances in biomedical research. *Missouri Medicine*, 110(3), 207–211. <https://www.ncbi.nlm.nih.gov/pubmed/23829104>

Bryder, D., Rossi, D. J., & Weissman, I. L. (2006). Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. *The American Journal of Pathology*, 169(2), 338–346. <https://doi.org/10.2353/ajpath.2006.060312>

Bumm, T. G. P., Elsea, C., Corbin, A. S., Loriaux, M., Sherbenou, D., Wood, L., Deininger, J., Silver, R. T., Druker, B. J., & Deininger, M. W. N. (2006). Characterization of murine JAK2V617F-positive myeloproliferative disease. *Cancer Research*, 66(23), 11156–11165. <https://doi.org/10.1158/0008-5472.CAN-06-2210>

Buscarlet, M., Provost, S., Zada, Y. F., Barhdadi, A., Bourgoïn, V., Lépine, G., Mollica, L., Szuber, N., Dubé, M.-P., & Busque, L. (2017). DNMT3A and TET2 dominate clonal hematopoiesis and demonstrate benign phenotypes and different genetic predispositions. *Blood*, 130(6), 753–762. <https://doi.org/10.1182/blood-2017-04-777029>

Busch, K., Klapproth, K., Barile, M., Flossdorf, M., Holland-Letz, T., Schlenner, S. M., Reth, M., Höfer, T., & Rodewald, H.-R. (2015). Fundamental properties of unperturbed

haematopoiesis from stem cells in vivo. *Nature*, 518(7540), 542–546. <https://doi.org/10.1038/nature14242>

Butler, A., Hoffman, P., Smibert, P., Papalexi, E., & Satija, R. (2018). Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nature Biotechnology*, 36(5), 411–420. <https://doi.org/10.1038/nbt.4096>

Byrd, J. J., Snow, E. L., & McCumber, T. L. (2023). Iliac bone biopsy and analysis: A clinical, translational, and cadaveric review. *Translational Research in Anatomy*, 31(100245), 100245. <https://doi.org/10.1016/j.tria.2023.100245>

Cabagnols, X., Favale, F., Pasquier, F., Messaoudi, K., Defour, J. P., Ianotto, J. C., Marzac, C., Le Couédic, J. P., Droin, N., Chachoua, I., Favier, R., Diop, M. K., Ugo, V., Casadevall, N., Debili, N., Raslova, H., Bellanné-Chantelot, C., Constantinescu, S. N., Bluteau, O., ... Vainchenker, W. (2016). Presence of atypical thrombopoietin receptor (MPL) mutations in triple-negative essential thrombocythemia patients. *Blood*, 127(3), 333–342. <https://doi.org/10.1182/blood-2015-07-661983>

Cagan, A., Baez-Ortega, A., Brzozowska, N., Abascal, F., Coorens, T. H. H., Sanders, M. A., Lawson, A. R. J., Harvey, L. M. R., Bhosle, S., Jones, D., Alcantara, R. E., Butler, T. M., Hooks, Y., Roberts, K., Anderson, E., Lunn, S., Flach, E., Spiro, S., Januszczak, I., ... Martincorena, I. (2022). Somatic mutation rates scale with lifespan across mammals. *Nature*, 604(7906), 517–524. <https://doi.org/10.1038/s41586-022-04618-z>

Caiado, F., Pietras, E. M., & Manz, M. G. (2021). Inflammation as a regulator of hematopoietic stem cell function in disease, aging, and clonal selection. *The Journal of Experimental Medicine*, 218(7). <https://doi.org/10.1084/jem.20201541>

Calvanese, V., Capellera-Garcia, S., Ma, F., Fares, I., Liebscher, S., Ng, E. S., Ekstrand, S., Aguadé-Gorgorió, J., Vavilina, A., Lefaudeaux, D., Nadel, B., Li, J. Y., Wang, Y., Lee, L. K., Ardehali, R., Iruela-Arispe, M. L., Pellegrini, M., Stanley, E. G., Elefanty, A. G., ... Mikkola, H. K. A. (2022). Mapping human haematopoietic stem cells from haemogenic endothelium to birth. *Nature*, 604(7906), 534–540. <https://doi.org/10.1038/s41586-022-04571-x>

Calvanese, V., & Mikkola, H. K. A. (2023). The genesis of human hematopoietic stem cells. *Blood*, 142(6), 519–532. <https://doi.org/10.1182/blood.2022017934>

Calvo, W. (1968). The innervation of the bone marrow in laboratory animals. *The American Journal of Anatomy*, 123(2), 315–328. <https://doi.org/10.1002/aja.1001230206>

Camporeale, A., & Poli, V. (2012). IL-6, IL-17 and STAT3: a holy trinity in auto-immunity? *Frontiers in Bioscience (Landmark Edition)*, 17(6), 2306–2326. <https://doi.org/10.2741/4054>

Cancelas, J. A. (2011). On how Rac controls hematopoietic stem cell activity. *Transfusion*, 51 Suppl 4, 153S–159S. <https://doi.org/10.1111/j.1537-2995.2011.03378.x>

Cancelas, Jose A., Lee, A. W., Prabhakar, R., Stringer, K. F., Zheng, Y., & Williams, D. A. (2005). Rac GTPases differentially integrate signals regulating hematopoietic stem cell localization. *Nature Medicine*, 11(8), 886–891. <https://doi.org/10.1038/nm1274>

Cancelas, Jose A., & Williams, D. A. (2009). Rho GTPases in hematopoietic stem cell functions. *Current Opinion in Hematology*, 16(4), 249–254. <https://doi.org/10.1097/MOH.0b013e32832c4b80>

Cancer Genome Atlas Research Network, Ley, T. J., Miller, C., Ding, L., Raphael, B. J., Mungall, A. J., Robertson, A. G., Hoadley, K., Triche, T. J., Jr, Laird, P. W., Baty, J. D., Fulton, L. L., Fulton, R., Heath, S. E., Kalicki-Veizer, J., Kandoth, C., Klco, J. M., Koboldt, D. C., Kanchi, K.-L., ... Eley, G. (2013). Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *The New England Journal of Medicine*, 368(22), 2059–2074. <https://doi.org/10.1056/NEJMoa1301689>

Cancer Today. (n.d.). Retrieved 29 May 2025, from <https://gco.iarc.who.int/today/en>

Cao, R., Thatavarty, A., & King, K. Y. (2024). Forged in the fire: Lasting impacts of inflammation on hematopoietic progenitors. *Experimental Hematology*, 134(104215), 104215. <https://doi.org/10.1016/j.exphem.2024.104215>

Carrelha, J., Meng, Y., Kettyle, L. M., Luis, T. C., Norfo, R., Alcolea, V., Boukarabila, H., Grasso, F., Gambardella, A., Grover, A., Höglstrand, K., Lord, A. M., Sanjuan-Pla, A., Woll, P. S., Nerlov, C., & Jacobsen, S. E. W. (2018). Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. *Nature*, 554(7690), 106–111. <https://doi.org/10.1038/nature25455>

Carturan, A., Morè, S., Poloni, A., Rupoli, S., & Morsia, E. (2024). Shaping the future of myeloproliferative neoplasm therapy: Immune-based strategies and targeted innovations. *Cancers*, 16(23), 4113. <https://doi.org/10.3390/cancers16234113>

Casado-Díaz, A. (2022). Stem cells in regenerative medicine. *Journal of Clinical Medicine*, 11(18), 5460. <https://doi.org/10.3390/jcm11185460>

Castillo-Azofeifa, D., Fazio, E. N., Nattiv, R., Good, H. J., Wald, T., Pest, M. A., de Sauvage, F. J., Klein, O. D., & Asfaha, S. (2019). Atoh1+ secretory progenitors possess renewal capacity independent of Lgr5+ cells during colonic regeneration. *The EMBO Journal*, 38(4), e99984. <https://doi.org/10.15252/embj.201899984>

Cavalli-Sforza, L. L., & Edwards, A. W. F. (1967). Phylogenetic analysis: Models and estimation procedures. *Evolution; International Journal of Organic Evolution*, 21(3), 550. <https://doi.org/10.2307/2406616>

Ceredig, R., Rolink, A. G., & Brown, G. (2009). Models of haematopoiesis: seeing the wood for the trees. *Nature Reviews. Immunology*, 9(4), 293–300. <https://doi.org/10.1038/nri2525>

Ceresa, D., Alessandrini, F., Lucchini, S., Marubbi, D., Piaggio, F., Mena Vera, J. M., Ceccherini, I., Reverberi, D., Appolloni, I., & Malatesta, P. (2023). Early clonal extinction in glioblastoma progression revealed by genetic barcoding. *Cancer Cell*, 41(8), 1466-1479.e9. <https://doi.org/10.1016/j.ccell.2023.07.001>

Chaligné, R., James, C., Tonetti, C., Besancenot, R., Le Couédic, J. P., Fava, F., Mazurier, F., Godin, I., Maloum, K., Larbret, F., Lécluse, Y., Vainchenker, W., & Giraudier, S. (2007). Evidence for MPL W515L/K mutations in hematopoietic stem cells in primitive myelofibrosis. *Blood*, 110(10), 3735–3743. <https://doi.org/10.1182/blood-2007-05-089003>

Challa, S., & Neelapu, N. R. R. (2019). Phylogenetic trees: Applications, construction, and assessment. In *Essentials of Bioinformatics, Volume III* (pp. 167–192). Springer International Publishing. https://doi.org/10.1007/978-3-030-19318-8_10

Challen, G. A., Boles, N., Lin, K. K.-Y., & Goodell, M. A. (2009). Mouse hematopoietic stem cell identification and analysis. *Cytometry. Part A: The Journal of the International Society for Analytical Cytology*, 75(1), 14–24. <https://doi.org/10.1002/cyto.a.20674>

Challen, G. A., Sun, D., Jeong, M., Luo, M., Jelinek, J., Berg, J. S., Bock, C., Vasanthakumar, A., Gu, H., Xi, Y., Liang, S., Lu, Y., Darlington, G. J., Meissner, A., Issa, J.-P. J., Godley, L. A., Li, W., & Goodell, M. A. (2011). Dnmt3a is essential for hematopoietic stem cell differentiation. *Nature Genetics*, 44(1), 23–31. <https://doi.org/10.1038/ng.1009>

Champlaud, M. F., Burgeson, R. E., Jin, W., Baden, H. P., & Olson, P. F. (1998). cDNA cloning and characterization of sciellin, a LIM domain protein of the keratinocyte cornified envelope. *The Journal of Biological Chemistry*, 273(47), 31547–31554. <https://doi.org/10.1074/jbc.273.47.31547>

Chan, C. K. F., Chen, C.-C., Luppen, C. A., Kim, J.-B., DeBoer, A. T., Wei, K., Helms, J. A., Kuo, C. J., Kraft, D. L., & Weissman, I. L. (2009). Endochondral ossification is required for haematopoietic stem-cell niche formation. *Nature*, 457(7228), 490–494. <https://doi.org/10.1038/nature07547>

Chan, S. M., & Majeti, R. (2013). Role of DNMT3A, TET2, and IDH1/2 mutations in pre-leukemic stem cells in acute myeloid leukemia. *International Journal of Hematology*, 98(6), 648–657. <https://doi.org/10.1007/s12185-013-1407-8>

Chang, Y.-C., Lee, H.-H., Chen, Y.-J., Bokoch, G. M., & Chang, Z.-F. (2006). Contribution of guanine exchange factor H1 in phorbol ester-induced apoptosis. *Cell Death and Differentiation*, 13(12), 2023–2032. <https://doi.org/10.1038/sj.cdd.4401901>

Charitos, I. A., Ballini, A., Cantore, S., Boccellino, M., Di Domenico, M., Borsani, E., Nocini, R., Di Cosola, M., Santacroce, L., & Bottalico, L. (2021). Stem cells: A historical review about biological, religious, and ethical issues. *Stem Cells International*, 2021, 9978837. <https://doi.org/10.1155/2021/9978837>

Chatzeli, L., & Simons, B. D. (2020). Tracing the dynamics of stem cell fate. *Cold Spring Harbor Perspectives in Biology*, 12(6), a036202. <https://doi.org/10.1101/cshperspect.a036202>

Chen, C., Liao, Y., & Peng, G. (2022). Connecting past and present: single-cell lineage tracing. *Protein & Cell*, 13(11), 790–807. <https://doi.org/10.1007/s13238-022-00913-7>

Chen, E., Schneider, R. K., Breyfogle, L. J., Rosen, E. A., Poveromo, L., Elf, S., Ko, A., Brumme, K., Levine, R., Ebert, B. L., & Mullally, A. (2015). Distinct effects of concomitant Jak2V617F expression and Tet2 loss in mice promote disease progression in myeloproliferative neoplasms. *Blood*, 125(2), 327–335. <https://doi.org/10.1182/blood-2014-04-567024>

Chen, K., Wallis, J. W., McLellan, M. D., Larson, D. E., Kalicki, J. M., Pohl, C. S., McGrath, S. D., Wendl, M. C., Zhang, Q., Locke, D. P., Shi, X., Fulton, R. S., Ley, T. J., Wilson, R. K., Ding, L., & Mardis, E. R. (2009). BreakDancer: an algorithm for high-resolution mapping of genomic structural variation. *Nature Methods*, 6(9), 677–681. <https://doi.org/10.1038/nmeth.1363>

Chen, P., Wu, B., Ji, L., Zhan, Y., Li, F., Cheng, L., Cao, J., Chen, H., Ke, Y., Min, Z., Sun, L., Hua, F., Chen, H., & Cheng, Y. (2021). Cytokine consistency between bone marrow and peripheral blood in patients with Philadelphia-negative myeloproliferative neoplasms. *Frontiers in Medicine*, 8, 598182. <https://doi.org/10.3389/fmed.2021.598182>

Chen, Y., Li, J., Xu, L., Găman, M.-A., & Zou, Z. (2022). The genesis and evolution of acute myeloid leukemia stem cells in the microenvironment: From biology to therapeutic targeting. *Cell Death Discovery*, 8(1), 397. <https://doi.org/10.1038/s41420-022-01193-0>

Cheng, H., Zheng, Z., & Cheng, T. (2020). New paradigms on hematopoietic stem cell differentiation. *Protein & Cell*, 11(1), 34–44. <https://doi.org/10.1007/s13238-019-0633-0>

Chennamadhavuni, A., Iyengar, V., Mukkamalla, S. K. R., & Shimanovsky, A. (2025). Leukemia. In *StatPearls*. StatPearls Publishing. <https://www.ncbi.nlm.nih.gov/books/NBK560490/>

Chervenick, P. A., Boggs, D. R., Marsh, J. C., Cartwright, G. E., & Wintrobe, M. M. (1968). Quantitative studies of blood and bone marrow neutrophils in normal mice. *The American Journal of Physiology*, 215(2), 353–360. <https://doi.org/10.1152/ajplegacy.1968.215.2.353>

Chi, A. W. S., Chavez, A., Xu, L., Weber, B. N., Shestova, O., Schaffer, A., Wertheim, G., Pear, W. S., Izon, D., & Bhandoola, A. (2011). Identification of Flt3+CD150[−] myeloid progenitors in adult mouse bone marrow that harbor T lymphoid developmental potential. *Blood*, 118(10), 2723–2732. <https://doi.org/10.1182/blood-2010-09-309989>

Chia, Y. C., Islam, M. A., Hider, P., Woon, P. Y., Johan, M. F., Hassan, R., & Ramli, M. (2021). The prevalence of TET2 gene mutations in patients with BCR-ABL-negative myeloproliferative neoplasms (MPN): A systematic review and meta-analysis. *Cancers*, 13(12), 3078. <https://doi.org/10.3390/cancers13123078>

Child, C. M. (1906). The organization and cell-lineage of the ascidian egg . By professor E. g. conklin, journal of the academy of natural sciences of Philadelphia, second series, volume XIII., part I., 1905. *Science* (New York, N.Y.), 23(583), 340–344. <https://doi.org/10.1126/science.23.583.340>

Cho, I. J., Lui, P. P., Obajdin, J., Riccio, F., Stroukov, W., Willis, T. L., Spagnoli, F., & Watt, F. M. (2019). Mechanisms, hallmarks, and implications of stem cell quiescence. *Stem Cell Reports*, 12(6), 1190–1200. <https://doi.org/10.1016/j.stemcr.2019.05.012>

Choi, D. C., Messali, N., Uda, N. R., Abu-Zeinah, G., Kermani, P., Yabut, M. M., Lischer, H. E. L., Castillo Tokumori, F., Erdos, K., Lehmann, T., Sobas, M., Rao, T. N., & Scandura, J. M. (2024). JAK2V617F impairs lymphoid differentiation in myeloproliferative neoplasms. *Leukemia*, 38(11), 2487–2491. <https://doi.org/10.1038/s41375-024-02388-3>

Choi, J., & Augenlicht, L. H. (2024). Intestinal stem cells: guardians of homeostasis in health and aging amid environmental challenges. *Experimental & Molecular Medicine*, 56(3), 495–500. <https://doi.org/10.1038/s12276-024-01179-1>

Chow, J. W., Jagger, C. J., & Chambers, T. J. (1993). Characterization of osteogenic response to mechanical stimulation in cancellous bone of rat caudal vertebrae. *The American Journal of Physiology*, 265(2 Pt 1), E340-7. <https://doi.org/10.1152/ajpendo.1993.265.2.E340>

Christiansen, F. B., & Loeschcke, V. (1990). Evolution and Competition. In *Population Biology* (pp. 367–394). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-74474-7_13

Ciriza, J., Thompson, H., Petrosian, R., Manilay, J. O., & García-Ojeda, M. E. (2013). The migration of hematopoietic progenitors from the fetal liver to the fetal bone marrow: lessons learned and possible clinical applications. *Experimental Hematology*, 41(5), 411–423. <https://doi.org/10.1016/j.exphem.2013.01.009>

Clavería, C., Giovinazzo, G., Sierra, R., & Torres, M. (2013). Myc-driven endogenous cell competition in the early mammalian embryo. *Nature*, 500(7460), 39–44. <https://doi.org/10.1038/nature12389>

Clavería, C., & Torres, M. (2016). Cell competition: Mechanisms and physiological roles. *Annual Review of Cell and Developmental Biology*, 32, 411–439. <https://doi.org/10.1146/annurev-cellbio-111315-125142>

Cobo, I., Tanaka, T., Glass, C. K., & Yeang, C. (2022). Clonal hematopoiesis driven by DNMT3A and TET2 mutations: role in monocyte and macrophage biology and atherosclerotic cardiovascular disease. *Current Opinion in Hematology*, 29(1), 1–7. <https://doi.org/10.1097/MOH.0000000000000688>

Cohen, S., Roy, J., Lachance, S., Marinier, A., Barabé, F., Delisle, J.-S., Roy, D.-C., Busque, L., Ahmad, I., Bambace, N. M., Bernard, L., Kiss, T., Caudrelier, P., Larochelle, F., Bouchard, P., Lemieux, S., Zandstra, P., & Sauvageau, G. (2018). Single UM171 expanded cord blood transplant is feasible, safe, and permits transplantation of better HLA matched cords with very low transplant related mortality. *Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation*, 24(3), S190–S191. <https://doi.org/10.1016/j.bbmt.2017.12.301>

Collinson, R. J., Mazza-Parton, A., Fuller, K. A., Linden, M. D., Erber, W. N., & Guo, B. B. (2020). Gene expression of CXCL1 (GRO- α) and EGF by platelets in myeloproliferative neoplasms. *HemaSphere*, 4(6), e490. <https://doi.org/10.1097/HS9.0000000000000490>

Colvin, G. A., Lambert, J.-F., Abedi, M., Hsieh, C.-C., Carlson, J. E., Stewart, F. M., & Quesenberry, P. J. (2004). Murine marrow cellularity and the concept of stem cell competition: geographic and quantitative determinants in stem cell biology. *Leukemia*, 18(3), 575–583. <https://doi.org/10.1038/sj.leu.2403268>

Cominal, J. G., Cacemiro, M. da C., Berzoti-Coelho, M. G., Pereira, I. E. G., Frantz, F. G., Souto, E. X., Covas, D. T., de Figueiredo-Pontes, L. L., Oliveira, M. C., Malmegrim, K. C. R., & de Castro, F. A. (2021). Bone marrow soluble mediator signatures of patients with Philadelphia chromosome-negative myeloproliferative neoplasms. *Frontiers in Oncology*, 11, 665037. <https://doi.org/10.3389/fonc.2021.665037>

Cong, B., & Cagan, R. L. (2024). Cell competition and cancer from *Drosophila* to mammals. *Oncogenesis*, 13(1), 1. <https://doi.org/10.1038/s41389-023-00505-y>

Cook, M. R., Karp, J. E., & Lai, C. (2022). The spectrum of genetic mutations in myelodysplastic syndrome: Should we update prognostication? *EJHaem*, 3(1), 301–313. <https://doi.org/10.1002/jha2.317>

Cooper, J. N., & Young, N. S. (2017). Clonality in context: hematopoietic clones in their marrow environment. *Blood*, 130(22), 2363–2372. <https://doi.org/10.1182/blood-2017-07-794362>

Copley, M. R., & Eaves, C. J. (2013). Developmental changes in hematopoietic stem cell properties. *Experimental & Molecular Medicine*, 45(11), e55. <https://doi.org/10.1038/emm.2013.98>

Cordeiro-Spinetti, E., Taichman, R. S., & Balduino, A. (2015). The bone marrow endosteal niche: how far from the surface? *Journal of Cellular Biochemistry*, 116(1), 6–11. <https://doi.org/10.1002/jcb.24952>

Cosgrove, J., Hustin, L. S. P., de Boer, R. J., & Perié, L. (2021). Hematopoiesis in numbers. *Trends in Immunology*, 42(12), 1100–1112. <https://doi.org/10.1016/j.it.2021.10.006>

Cowan, P. T., Launico, M. V., & Kahai, P. (2025). Anatomy, bones. In *StatPearls*. StatPearls Publishing. <https://www.ncbi.nlm.nih.gov/books/NBK537199/>

- Cui, A., Huang, T., Li, S., Ma, A., Pérez, J. L., Sander, C., Keskin, D. B., Wu, C. J., Fraenkel, E., & Hacohen, N. (2024). Dictionary of immune responses to cytokines at single-cell resolution. *Nature*, 625(7994), 377–384. <https://doi.org/10.1038/s41586-023-06816-9>
- Cui, X.-H., Peng, Q.-J., Li, R.-Z., Lyu, X.-J., Zhu, C.-F., & Qin, X.-H. (2021). Cell division cycle associated 8: A novel diagnostic and prognostic biomarker for hepatocellular carcinoma. *Journal of Cellular and Molecular Medicine*, 25(24), 11097–11112. <https://doi.org/10.1111/jcmm.17032>
- Cui, Z., Wei, H., Goding, C., & Cui, R. (2023). Stem cell heterogeneity, plasticity, and regulation. *Life Sciences*, 334(122240), 122240. <https://doi.org/10.1016/j.lfs.2023.122240>
- Cullis, J., Meiri, D., Sandi, M. J., Radulovich, N., Kent, O. A., Medrano, M., Mokady, D., Normand, J., Larose, J., Marcotte, R., Marshall, C. B., Ikura, M., Ketela, T., Moffat, J., Neel, B. G., Gingras, A.-C., Tsao, M.-S., & Rottapel, R. (2014). The RhoGEF GEF-H1 is required for oncogenic RAS signaling via KSR-1. *Cancer Cell*, 25(2), 181–195. <https://doi.org/10.1016/j.ccr.2014.01.025>
- Czechowicz, A., Kraft, D., Weissman, I. L., & Bhattacharya, D. (2007). Efficient transplantation via antibody-based clearance of hematopoietic stem cell niches. *Science (New York, N.Y.)*, 318(5854), 1296–1299. <https://doi.org/10.1126/science.1149726>
- Dainiak, N. (2002). Hematologic consequences of exposure to ionizing radiation. *Experimental Hematology*, 30(6), 513–528. [https://doi.org/10.1016/s0301-472x\(02\)00802-0](https://doi.org/10.1016/s0301-472x(02)00802-0)
- Daltveit, D. S., Morgan, E., Colombet, M., Steliarova-Foucher, E., Bendahhou, K., Marcos-Gragera, R., Rongshou, Z., Smith, A., Wei, H., & Soerjomataram, I. (2025). Global patterns of leukemia by subtype, age, and sex in 185 countries in 2022. *Leukemia*, 39(2), 412–419. <https://doi.org/10.1038/s41375-024-02452-y>
- Dameshek, W. (1951). Editorial: Some speculations on the myeloproliferative syndromes. *Blood*, 6(4), 372–375. <https://doi.org/10.1182/blood.v6.4.372.372>
- Dandamudi, A., Seibel, W., Tourdot, B., Cancelas, J. A., Akbar, H., & Zheng, Y. (2023). Structure-activity relationship analysis of Rhosin, a RhoA GTPase inhibitor, reveals a new class of antiplatelet agents. *International Journal of Molecular Sciences*, 24(4). <https://doi.org/10.3390/ijms24044167>
- Dang, C. V. (1999). c-Myc target genes involved in cell growth, apoptosis, and metabolism. *Molecular and Cellular Biology*, 19(1), 1–11. <https://doi.org/10.1128/MCB.19.1.1>
- Danovi, S. (2021). A sequencing revolution in cancer. *Nature Research*. <https://doi.org/10.1038/d42859-020-00106-4>
- Darlington, P. J., Jr. (1972). Competition, competitive repulsion, and coexistence. *Proceedings of the National Academy of Sciences of the United States of America*, 69(11), 3151–3155. <https://doi.org/10.1073/pnas.69.11.3151>
- de Bruin, A. M., Demirel, Ö., Hooibrink, B., Brandts, C. H., & Nolte, M. A. (2013). Interferon- γ impairs proliferation of hematopoietic stem cells in mice. *Blood*, 121(18), 3578–3585. <https://doi.org/10.1182/blood-2012-05-432906>

de la Cova, C., Abril, M., Bellosta, P., Gallant, P., & Johnston, L. A. (2004). *Drosophila myc* regulates organ size by inducing cell competition. *Cell*, 117(1), 107–116. [https://doi.org/10.1016/s0092-8674\(04\)00214-4](https://doi.org/10.1016/s0092-8674(04)00214-4)

de la Cova, C., Senoo-Matsuda, N., Ziosi, M., Wu, D. C., Bellosta, P., Quinzii, C. M., & Johnston, L. A. (2014). Supercompetitor status of *Drosophila Myc* cells requires p53 as a fitness sensor to reprogram metabolism and promote viability. *Cell Metabolism*, 19(3), 470–483. <https://doi.org/10.1016/j.cmet.2014.01.012>

de Queiroz, K. (2013). Nodes, branches, and phylogenetic definitions. *Systematic Biology*, 62(4), 625–632. <https://doi.org/10.1093/sysbio/syt027>

de Roo, J. J., Vloemans, S. A., Vrolijk, H., de Haas, E. F., & Staal, F. J. (2019). Development of an in vivo model to study clonal lineage relationships in hematopoietic cells using Brainbow2.1/Confetti mice. *Future Science OA*, 5(10), FSO427. <https://doi.org/10.2144/foa-2019-0083>

Deiss, L. P., Feinstein, E., Berissi, H., Cohen, O., & Kimchi, A. (1995). Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the gamma interferon-induced cell death. *Genes & Development*, 9(1), 15–30. <https://doi.org/10.1101/gad.9.1.15>

DeLaughter, D. M. (2018). The use of the Fluidigm C1 for RNA expression analyses of single cells. *Et al [Current Protocols in Molecular Biology]*, 122(1), e55. <https://doi.org/10.1002/cpmb.55>

Delhommeau, F., Dupont, S., Della Valle, V., James, C., Trannoy, S., Massé, A., Kosmider, O., Le Couedic, J.-P., Robert, F., Alberdi, A., Lécluse, Y., Plo, I., Dreyfus, F. J., Marzac, C., Casadevall, N., Lacombe, C., Romana, S. P., Dessen, P., Soulier, J., ... Bernard, O. A. (2009). Mutation in TET2 in myeloid cancers. *The New England Journal of Medicine*, 360(22), 2289–2301. <https://doi.org/10.1056/NEJMoa0810069>

Deng, L.-H., Li, M.-Z., Huang, X.-J., & Zhao, X.-Y. (2025). Single-cell lineage tracing techniques in hematology: unraveling the cellular narrative. *Journal of Translational Medicine*, 23(1), 270. <https://doi.org/10.1186/s12967-025-06318-4>

Dexter, T. M., Allen, T. D., & Lajtha, L. G. (1977). Conditions controlling the proliferation of haemopoietic stem cells in vitro. *Journal of Cellular Physiology*, 91(3), 335–344. <https://doi.org/10.1002/jcp.1040910303>

Deymier, S., Louvat, C., Fiorini, F., & Cimorelli, A. (2022). ISG20: an enigmatic antiviral RNase targeting multiple viruses. *FEBS Open Bio*, 12(6), 1096–1111. <https://doi.org/10.1002/2211-5463.13382>

Di Gregorio, A., Bowling, S., & Rodriguez, T. A. (2016). Cell competition and its role in the regulation of cell fitness from development to cancer. *Developmental Cell*, 38(6), 621–634. <https://doi.org/10.1016/j.devcel.2016.08.012>

Díaz-García, D., Filipová, A., Garza-Veloz, I., & Martinez-Fierro, M. L. (2021). A beginner's introduction to skin stem cells and wound healing. *International Journal of Molecular Sciences*, 22(20), 11030. <https://doi.org/10.3390/ijms222011030>

Dick, J. E., Magli, M. C., Huszar, D., Phillips, R. A., & Bernstein, A. (1985). Introduction of a selectable gene into primitive stem cells capable of long-term reconstitution of the hemopoietic system of W/W^v mice. *Cell*, 42(1), 71–79. [https://doi.org/10.1016/s0092-8674\(85\)80102-1](https://doi.org/10.1016/s0092-8674(85)80102-1)

Ding, L., Saunders, T. L., Enikolopov, G., & Morrison, S. J. (2012). Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*, 481(7382), 457–462. <https://doi.org/10.1038/nature10783>

Dolinska, M., Cai, H., Månsson, A., Shen, J., Xiao, P., Boudierlique, T., Li, X., Leonard, E., Chang, M., Gao, Y., Medina, J. P., Kondo, M., Sandhow, L., Johansson, A.-S., Deneberg, S., Söderlund, S., Jädersten, M., Ungerstedt, J., Tobiasson, M., ... Qian, H. (2023). Characterization of the bone marrow niche in patients with chronic myeloid leukemia identifies CXCL14 as a new therapeutic option. *Blood*, 142(1), 73–89. <https://doi.org/10.1182/blood.2022016896>

Dorshkind, K., Höfer, T., Montecino-Rodriguez, E., Pioli, P. D., & Rodewald, H.-R. (2020). Do haematopoietic stem cells age? *Nature Reviews. Immunology*, 20(3), 196–202. <https://doi.org/10.1038/s41577-019-0236-2>

Dröschner, A. (2014). Images of cell trees, cell lines, and cell fates: the legacy of Ernst Haeckel and August Weismann in stem cell research. *History and Philosophy of the Life Sciences*, 36(2), 157–186. <https://doi.org/10.1007/s40656-014-0028-8>

Duan, X., Perveen, R., Dandamudi, A., Adili, R., Johnson, J., Funk, K., Berryman, M., Davis, A. K., Holinstat, M., Zheng, Y., & Akbar, H. (2021). Pharmacologic targeting of Cdc42 GTPase by a small molecule Cdc42 activity-specific inhibitor prevents platelet activation and thrombosis. *Scientific Reports*, 11(1), 13170. <https://doi.org/10.1038/s41598-021-92654-6>

Dubrovsky, G., & Dunn, J. C. Y. (2018). Mechanisms for intestinal regeneration. *Current Opinion in Pediatrics*, 30(3), 424–429. <https://doi.org/10.1097/MOP.0000000000000630>

Dunbar, A. J., Rampal, R. K., & Levine, R. (2020). Leukemia secondary to myeloproliferative neoplasms. *Blood*, 136(1), 61–70. <https://doi.org/10.1182/blood.2019000943>

Dunbar, A., Nazir, A., & Levine, R. (2017). Overview of transgenic mouse models of myeloproliferative neoplasms (MPNs). *Et al [Current Protocols in Pharmacology]*, 77(1), 14.40.1-14.40.19. <https://doi.org/10.1002/cpph.23>

Dzierzak, E., & Speck, N. A. (2008). Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nature Immunology*, 9(2), 129–136. <https://doi.org/10.1038/ni1560>

Elf, S., Abdelfattah, N. S., Chen, E., Perales-Patón, J., Rosen, E. A., Ko, A., Peisker, F., Florescu, N., Giannini, S., Wolach, O., Morgan, E. A., Tothova, Z., Losman, J.-A., Schneider, R. K., Al-Shahrour, F., & Mullally, A. (2016). Mutant calreticulin requires both its mutant C-terminus and the thrombopoietin receptor for oncogenic transformation. *Cancer Discovery*, 6(4), 368–381. <https://doi.org/10.1158/2159-8290.CD-15-1434>

Elhamamsy, A. R. (2017). Role of DNA methylation in imprinting disorders: an updated review. *Journal of Assisted Reproduction and Genetics*, 34(5), 549–562. <https://doi.org/10.1007/s10815-017-0895-5>

Ellis, S. L., Grassinger, J., Jones, A., Borg, J., Camenisch, T., Haylock, D., Bertoncello, I., & Nilsson, S. K. (2011). The relationship between bone, hemopoietic stem cells, and vasculature. *Blood*, 118(6), 1516–1524. <https://doi.org/10.1182/blood-2010-08-303800>

Ema, H., Takano, H., Sudo, K., & Nakauchi, H. (2000). In vitro self-renewal division of hematopoietic stem cells. *The Journal of Experimental Medicine*, 192(9), 1281–1288. <https://doi.org/10.1084/jem.192.9.1281>

Espín-Palazón, R., Stachura, D. L., Campbell, C. A., García-Moreno, D., Del Cid, N., Kim, A. D., Candel, S., Meseguer, J., Mulero, V., & Traver, D. (2014). Proinflammatory signaling regulates hematopoietic stem cell emergence. *Cell*, 159(5), 1070–1085. <https://doi.org/10.1016/j.cell.2014.10.031>

Essers, M. A. G., Offner, S., Blanco-Bose, W. E., Waibler, Z., Kalinke, U., Duchosal, M. A., & Trumpp, A. (2009). IFN α activates dormant haematopoietic stem cells in vivo. *Nature*, 458(7240), 904–908. <https://doi.org/10.1038/nature07815>

Fabre, M. A., de Almeida, J. G., Fiorillo, E., Mitchell, E., Damaskou, A., Rak, J., Orrù, V., Marongiu, M., Chapman, M. S., Vijayabaskar, M. S., Baxter, J., Hardy, C., Abascal, F., Williams, N., Nangalia, J., Martincorena, I., Campbell, P. J., McKinney, E. F., Cucca, F., ... Vassiliou, G. S. (2022). The longitudinal dynamics and natural history of clonal haematopoiesis. *Nature*, 606(7913), 335–342. <https://doi.org/10.1038/s41586-022-04785-z>

Fabre, M. A., & Vassiliou, G. S. (2024). The lifelong natural history of clonal hematopoiesis and its links to myeloid neoplasia. *Blood*, 143(7), 573–581. <https://doi.org/10.1182/blood.2023019964>

Fan, W., Cao, W., Shi, J., Gao, F., Wang, M., Xu, L., Wang, F., Li, Y., Guo, R., Bian, Z., Li, W., Jiang, Z., & Ma, W. (2023). Contributions of bone marrow monocytes/macrophages in myeloproliferative neoplasms with JAK2V617F mutation. *Annals of Hematology*, 102(7), 1745–1759. <https://doi.org/10.1007/s00277-023-05284-5>

Farber, D. L. (2021). Tissues, not blood, are where immune cells function. *Nature*, 593(7860), 506–509. <https://doi.org/10.1038/d41586-021-01396-y>

Fares, I., Chagraoui, J., Gareau, Y., Gingras, S., Ruel, R., Mayotte, N., Csaszar, E., Knapp, D. J. H. F., Miller, P., Ngom, M., Imren, S., Roy, D.-C., Watts, K. L., Kiem, H.-P., Herrington, R., Iscove, N. N., Humphries, R. K., Eaves, C. J., Cohen, S., ... Sauvageau, G. (2014). Cord blood expansion. Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. *Science (New York, N.Y.)*, 345(6203), 1509–1512. <https://doi.org/10.1126/science.1256337>

Feinstein, E., Druck, T., Kastury, K., Berissi, H., Goodart, S. A., Overhauser, J., Kimchi, A., & Huebner, K. (1995). Assignment of DAP1 and DAPK--genes that positively mediate programmed cell death triggered by IFN- γ --to chromosome regions 5p12.2 and 9q34.1, respectively. *Genomics*, 29(1), 305–307. <https://doi.org/10.1006/geno.1995.1255>

Ferrando, A. A., & López-Otín, C. (2017). Clonal evolution in leukemia. *Nature Medicine*, 23(10), 1135–1145. <https://doi.org/10.1038/nm.4410>

Ferretti, E., & Hadjantonakis, A.-K. (2019). Mesoderm specification and diversification: from single cells to emergent tissues. *Current Opinion in Cell Biology*, 61, 110–116. <https://doi.org/10.1016/j.ceb.2019.07.012>

Flanagan, D. J., Pentimikko, N., Luopajarvi, K., Willis, N. J., Gilroy, K., Raven, A. P., McGarry, L., Englund, J. I., Webb, A. T., Scharaw, S., Nasreddin, N., Hodder, M. C., Ridgway, R. A., Minnee, E., Sphyris, N., Gilchrist, E., Najumudeen, A. K., Romagnolo, B., Perret, C., ... Sansom, O. J. (2021). NOTUM from Apc-mutant cells biases clonal competition to initiate cancer. *Nature*, 594(7863), 430–435. <https://doi.org/10.1038/s41586-021-03525-z>

Florian, M. C., Dörr, K., Niebel, A., Daria, D., Schrezenmeier, H., Rojewski, M., Filippi, M.-D., Hasenberg, A., Gunzer, M., Scharffetter-Kochanek, K., Zheng, Y., & Geiger, H. (2012). Cdc42

activity regulates hematopoietic stem cell aging and rejuvenation. *Cell Stem Cell*, 10(5), 520–530. <https://doi.org/10.1016/j.stem.2012.04.007>

Flurkey, K., Mcurrer, J., & Harrison, D. (2007). Mouse models in aging research. In *The Mouse in Biomedical Research* (pp. 637–672). Elsevier. <https://doi.org/10.1016/b978-012369454-6/50074-1>

Forsberg, E. C., Prohaska, S. S., Katzman, S., Heffner, G. C., Stuart, J. M., & Weissman, I. L. (2005). Differential expression of novel potential regulators in hematopoietic stem cells. *PLoS Genetics*, 1(3), e28. <https://doi.org/10.1371/journal.pgen.0010028>

Fortin, J., Chiang, M.-F., Meydan, C., Foox, J., Ramachandran, P., Leca, J., Lemonnier, F., Li, W. Y., Gams, M. S., Sakamoto, T., Chu, M., Tobin, C., Laugesen, E., Robinson, T. M., You-Ten, A., Butler, D. J., Berger, T., Minden, M. D., Levine, R. L., ... Mak, T. W. (2023). Distinct and opposite effects of leukemogenic *Idh* and *Tet2* mutations in hematopoietic stem and progenitor cells. *Proceedings of the National Academy of Sciences of the United States of America*, 120(4), e2208176120. <https://doi.org/10.1073/pnas.2208176120>

Fox, N., Priestley, G., Papayannopoulou, T., & Kaushansky, K. (2002). Thrombopoietin expands hematopoietic stem cells after transplantation. *The Journal of Clinical Investigation*, 110(3), 389–394. <https://doi.org/10.1172/JCI15430>

Fragkakis, E. M., El-Jawhari, J. J., Dunsmuir, R. A., Millner, P. A., Rao, A. S., Henshaw, K. T., Pountos, I., Jones, E., & Giannoudis, P. V. (2018). Vertebral body versus iliac crest bone marrow as a source of multipotential stromal cells: Comparison of processing techniques, tri-lineage differentiation and application on a scaffold for spine fusion. *PloS One*, 13(5), e0197969. <https://doi.org/10.1371/journal.pone.0197969>

Freret, M., Gouel, F., Buquet, C., Legrand, E., Vannier, J.-P., Vasse, M., & Dubus, I. (2011). Rac-1 GTPase controls the capacity of human leukaemic lymphoblasts to migrate on fibronectin in response to SDF-1 α (CXCL12). *Leukemia Research*, 35(7), 971–973. <https://doi.org/10.1016/j.leukres.2011.03.011>

Freysd'ottir, J. (2000). Production of monoclonal antibodies. *Methods in Molecular Medicine*, 40, 267–279. <https://doi.org/10.1385/1-59259-076-4:267>

Friedenstein, A. J., Chailakhyan, R. K., Latsinik, N. V., Panasyuk, A. F., & Keiliss-Borok, I. V. (1974). Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. *Transplantation*, 17(4), 331–340. <https://www.ncbi.nlm.nih.gov/pubmed/4150881>

Frisch, B. J., & Calvi, L. M. (2014). Hematopoietic stem cell cultures and assays. *Methods in Molecular Biology* (Clifton, N.J.), 1130, 315–324. https://doi.org/10.1007/978-1-62703-989-5_24

Fröbel, J., Landspersky, T., Percin, G., Schreck, C., Rahmig, S., Ori, A., Nowak, D., Essers, M., Waskow, C., & Oostendorp, R. A. J. (2021). The hematopoietic bone marrow niche ecosystem. *Frontiers in Cell and Developmental Biology*, 9, 705410. <https://doi.org/10.3389/fcell.2021.705410>

Frömel, R., Rühle, J., Bernal Martinez, A., Szu-Tu, C., Pacheco Pastor, F., Martinez-Corral, R., & Velten, L. (2025). Design principles of cell-state-specific enhancers in hematopoiesis. *Cell*. <https://doi.org/10.1016/j.cell.2025.04.017>

- Fruhman, G. J. (1964). Cell counts of mouse bone marrow. *Life Sciences* (1962), 3, 737–748. [https://doi.org/10.1016/0024-3205\(64\)90027-x](https://doi.org/10.1016/0024-3205(64)90027-x)
- Fucikova, J., Spisek, R., Kroemer, G., & Galluzzi, L. (2021). Calreticulin and cancer. *Cell Research*, 31(1), 5–16. <https://doi.org/10.1038/s41422-020-0383-9>
- Fujino, T., Asada, S., Goyama, S., & Kitamura, T. (2022). Mechanisms involved in hematopoietic stem cell aging. *Cellular and Molecular Life Sciences: CMLS*, 79(9), 473. <https://doi.org/10.1007/s00018-022-04356-5>
- Fulop, T., Larbi, A., Douziech, N., Levesque, I., Varin, A., & Herbein, G. (2006). Cytokine receptor signalling and aging. *Mechanisms of Ageing and Development*, 127(6), 526–537. <https://doi.org/10.1016/j.mad.2006.01.025>
- Gabbutt, C., Wright, N. A., Baker, A.-M., Shibata, D., & Graham, T. A. (2022). Lineage tracing in human tissues. *The Journal of Pathology*, 257(4), 501–512. <https://doi.org/10.1002/path.5911>
- Galán-Díez, M., & Kousteni, S. (2018). A bone marrow niche-derived molecular switch between osteogenesis and hematopoiesis. *Genes & Development*, 32(5–6), 324–326. <https://doi.org/10.1101/gad.314013.118>
- Gao, Q., Wang, L., Wang, S., Huang, B., Jing, Y., & Su, J. (2021). Bone marrow mesenchymal stromal cells: Identification, classification, and differentiation. *Frontiers in Cell and Developmental Biology*, 9, 787118. <https://doi.org/10.3389/fcell.2021.787118>
- Gao, Xiang, Murphy, M. M., Peyer, J. G., Ni, Y., Yang, M., Zhang, Y., Guo, J., Kara, N., Embree, C., Tasdogan, A., Ubellacker, J. M., Crane, G. M., Fang, S., Zhao, Z., Shen, B., & Morrison, S. J. (2023). Leptin receptor+ cells promote bone marrow innervation and regeneration by synthesizing nerve growth factor. *Nature Cell Biology*, 25(12), 1746–1757. <https://doi.org/10.1038/s41556-023-01284-9>
- Gao, Xin, Xu, C., Asada, N., & Frenette, P. S. (2018). The hematopoietic stem cell niche: from embryo to adult. *Development* (Cambridge, England), 145(2). <https://doi.org/10.1242/dev.139691>
- Gao, Xin, Zhang, D., Xu, C., Li, H., Caron, K. M., & Frenette, P. S. (2021). Nociceptive nerves regulate haematopoietic stem cell mobilization. *Nature*, 589(7843), 591–596. <https://doi.org/10.1038/s41586-020-03057-y>
- Gao, Y., Dickerson, J. B., Guo, F., Zheng, J., & Zheng, Y. (2004). Rational design and characterization of a Rac GTPase-specific small molecule inhibitor. *Proceedings of the National Academy of Sciences of the United States of America*, 101(20), 7618–7623. <https://doi.org/10.1073/pnas.0307512101>
- Garcia-Bellido, A., & Merriam, J. R. (1971). Parameters of the wing imaginal disc development of *Drosophila melanogaster*. *Developmental Biology*, 24(1), 61–87. [https://doi.org/10.1016/0012-1606\(71\)90047-9](https://doi.org/10.1016/0012-1606(71)90047-9)
- García-Tejera, R., Tian, J.-Y., Amoyel, M., Grima, R., & Schumacher, L. J. (2025). Licensing and niche competition in spermatogenesis: mathematical models suggest complementary regulation of tissue maintenance. *Development* (Cambridge, England), 152(1). <https://doi.org/10.1242/dev.202796>

Geetha, J. P., Arathi, C. A., Shalini, M., & Srinivasa Murthy, A. G. (2010). JAK2 negative polycythemia Vera. *Journal of Laboratory Physicians*, 2(2), 114–116. <https://doi.org/10.4103/0974-2727.72215>

Germani, F., Bergantinos, C., & Johnston, L. A. (2018). Mosaic analysis in *Drosophila*. *Genetics*, 208(2), 473–490. <https://doi.org/10.1534/genetics.117.300256>

Ghaedi, M., Steer, C. A., Martinez-Gonzalez, I., Halim, T. Y. F., Abraham, N., & Takei, F. (2016). Common-lymphoid-progenitor-independent pathways of innate and T lymphocyte development. *Cell Reports*, 15(3), 471–480. <https://doi.org/10.1016/j.celrep.2016.03.039>

Gherzi, J. J., Baldissera, G., Hintzen, J., Luff, S. A., Cheng, S., Xia, I. F., Sturgeon, C. M., & Nicoli, S. (2023). Haematopoietic stem and progenitor cell heterogeneity is inherited from the embryonic endothelium. *Nature Cell Biology*, 25(8), 1135–1145. <https://doi.org/10.1038/s41556-023-01187-9>

Ghiaur, G., Lee, A., Bailey, J., Cancelas, J. A., Zheng, Y., & Williams, D. A. (2006). Inhibition of RhoA GTPase activity enhances hematopoietic stem and progenitor cell proliferation and engraftment. *Blood*, 108(6), 2087–2094. <https://doi.org/10.1182/blood-2006-02-001560>

Gleitz, H. F. E., Benabid, A., & Schneider, R. K. (2021). Still a burning question: the interplay between inflammation and fibrosis in myeloproliferative neoplasms. *Current Opinion in Hematology*, 28(5), 364–371. <https://doi.org/10.1097/MOH.0000000000000669>

Gnanasambandan, K., & Sayeski, P. P. (2011). A structure-function perspective of Jak2 mutations and implications for alternate drug design strategies: the road not taken. *Current Medicinal Chemistry*, 18(30), 4659–4673. <https://doi.org/10.2174/092986711797379267>

Gonzales, K. A. U., & Fuchs, E. (2017). Skin and its regenerative powers: An alliance between stem cells and their niche. *Developmental Cell*, 43(4), 387–401. <https://doi.org/10.1016/j.devcel.2017.10.001>

Goodell, M. A., Brose, K., Paradis, G., Conner, A. S., & Mulligan, R. C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *The Journal of Experimental Medicine*, 183(4), 1797–1806. <https://doi.org/10.1084/jem.183.4.1797>

Goodell, Margaret A., Nguyen, H., & Shroyer, N. (2015). Somatic stem cell heterogeneity: diversity in the blood, skin and intestinal stem cell compartments. *Nature Reviews. Molecular Cell Biology*, 16(5), 299–309. <https://doi.org/10.1038/nrm3980>

Gordon Betts, J., Young, K. A., Wise, J. A., Johnson, E., Poe, B., Kruse, D. H., Korol, O., Johnson, J. E., Womble, M., & DeSaix, P. (2022, April 20). 18.1 an overview of blood. *Anatomy and Physiology 2e*; OpenStax. <https://openstax.org/books/anatomy-and-physiology-2e/pages/18-1-an-overview-of-blood>

Goulard, M., Dosquet, C., & Bonnet, D. (2018). Role of the microenvironment in myeloid malignancies. *Cellular and Molecular Life Sciences: CMLS*, 75(8), 1377–1391. <https://doi.org/10.1007/s00018-017-2725-4>

Grabek, J., Straube, J., Bywater, M., & Lane, S. W. (2020). MPN: The molecular drivers of disease initiation, progression and transformation and their effect on treatment. *Cells (Basel, Switzerland)*, 9(8), 1901. <https://doi.org/10.3390/cells9081901>

Gradeci, D., Bove, A., Vallardi, G., Lowe, A. R., Banerjee, S., & Charras, G. (2021). Cell-scale biophysical determinants of cell competition in epithelia. *eLife*, 10, e61011. <https://doi.org/10.7554/eLife.61011>

Graf, T., & Stadtfeld, M. (2008). Heterogeneity of embryonic and adult stem cells. *Cell Stem Cell*, 3(5), 480–483. <https://doi.org/10.1016/j.stem.2008.10.007>

Greenbaum, A., Hsu, Y.-M. S., Day, R. B., Schuettpelz, L. G., Christopher, M. J., Borgerding, J. N., Nagasawa, T., & Link, D. C. (2013). CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature*, 495(7440), 227–230. <https://doi.org/10.1038/nature11926>

Gregory, T. R. (2008). Understanding evolutionary trees. *Evolution*, 1(2), 121–137. <https://doi.org/10.1007/s12052-008-0035-x>

Grinenko, T., Eugster, A., Thielecke, L., Ramasz, B., Krüger, A., Dietz, S., Glauche, I., Gerbaulet, A., von Bonin, M., Basak, O., Clevers, H., Chavakis, T., & Wielockx, B. (2018). Hematopoietic stem cells can differentiate into restricted myeloid progenitors before cell division in mice. *Nature Communications*, 9(1), 1898. <https://doi.org/10.1038/s41467-018-04188-7>

Grinfeld, J., Nangalia, J., & Green, A. R. (2017). Molecular determinants of pathogenesis and clinical phenotype in myeloproliferative neoplasms. *Haematologica*, 102(1), 7–17. <https://doi.org/10.3324/haematol.2014.113845>

Grockowiak, E., Korn, C., Rak, J., Lysenko, V., Hallou, A., Panvini, F. M., Williams, M., Fielding, C., Fang, Z., Khatib-Massalha, E., García-García, A., Li, J., Khorshed, R. A., González-Antón, S., Baxter, E. J., Kusumbe, A., Wilkins, B. S., Green, A., Simons, B. D., ... Méndez-Ferrer, S. (2023). Different niches for stem cells carrying the same oncogenic driver affect pathogenesis and therapy response in myeloproliferative neoplasms. *Nature Cancer*, 4(8), 1193–1209. <https://doi.org/10.1038/s43018-023-00607-x>

Groenen, A. G., La Rose, A. M., Li, M., Bazioti, V., Svendsen, A. F., Kloosterhuis, N. J., Ausema, A., Pranger, A., Heiner-Fokkema, M. R., Niezen-Koning, K. E., Houben, T., Shiri-Sverdlov, R., & Westerterp, M. (2022). Elevated granulocyte-colony stimulating factor and hematopoietic stem cell mobilization in Niemann-Pick type C1 disease. *The Journal of Lipid Research*, 63(2), 100167. <https://doi.org/10.1016/j.jlr.2021.100167>

Haage, T. R., Charakopoulos, E., Bhuria, V., Baldauf, C. K., Korthals, M., Handschuh, J., Müller, P., Li, J., Harit, K., Nishanth, G., Frey, S., Böttcher, M., Fischer, K.-D., Dudeck, J., Dudeck, A., Lipka, D. B., Schraven, B., Green, A. R., Müller, A. J., ... Fischer, T. (2024). Neutrophil-specific expression of JAK2-V617F or CALRmut induces distinct inflammatory profiles in myeloproliferative neoplasia. *Journal of Hematology & Oncology*, 17(1), 43. <https://doi.org/10.1186/s13045-024-01562-5>

Haas, S., Trumpp, A., & Milsom, M. D. (2018). Causes and consequences of hematopoietic stem cell heterogeneity. *Cell Stem Cell*, 22(5), 627–638. <https://doi.org/10.1016/j.stem.2018.04.003>

Haeckel, E. H. P. A. (1866). *Generelle morphologie der organismen. Allgemeine grundzüge der organischen formen-wissenschaft, mechanisch begründet durch die von Charles Darwin reformirte descendenztheorie, von Ernst Haeckel.* G. Reimer,. <https://doi.org/10.5962/bhl.title.3953>

- Haertle, L., Munawar, U., Hernández, H. N. C., Arroyo-Barea, A., Heckel, T., Cuenca, I., Martin, L., Höschle, C., Müller, N., Vogt, C., Bischler, T., Del Campo, P. L., Han, S., Buenache, N., Zhou, X., Bassermann, F., Waldschmidt, J., Steinbrunn, T., Rasche, L., ... Barrio, S. (2024). Clonal competition assays identify fitness signatures in cancer progression and resistance in multiple myeloma. *HemaSphere*, 8(7), e110. <https://doi.org/10.1002/hem3.110>
- Hall, T., Gurbuxani, S., & Crispino, J. D. (2024). Malignant progression of preleukemic disorders. *Blood*, 143(22), 2245–2255. <https://doi.org/10.1182/blood.2023020817>
- Hamanaka, S., Ooehara, J., Morita, Y., Ema, H., Takahashi, S., Miyawaki, A., Otsu, M., Yamaguchi, T., Onodera, M., & Nakauchi, H. (2013). Generation of transgenic mouse line expressing Kusabira Orange throughout body, including erythrocytes, by random segregation of provirus method. *Biochemical and Biophysical Research Communications*, 435(4), 586–591. <https://doi.org/10.1016/j.bbrc.2013.05.017>
- Hamilton, W. D. (1964). The genetical evolution of social behaviour. I. *Journal of Theoretical Biology*, 7(1), 1–16. [https://doi.org/10.1016/0022-5193\(64\)90038-4](https://doi.org/10.1016/0022-5193(64)90038-4)
- Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, 100(1), 57–70. [https://doi.org/10.1016/s0092-8674\(00\)81683-9](https://doi.org/10.1016/s0092-8674(00)81683-9)
- Hanahan, Douglas. (2022). Hallmarks of cancer: New dimensions. *Cancer Discovery*, 12(1), 31–46. <https://doi.org/10.1158/2159-8290.CD-21-1059>
- Harrison, D. E. (1980). Competitive repopulation: a new assay for long-term stem cell functional capacity. *Blood*, 55(1), 77–81. <https://doi.org/10.1182/blood.v55.1.77.bloodjournal55177>
- Harrison, D. E., Jordan, C. T., Zhong, R. K., & Astle, C. M. (1993). Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculations. *Experimental Hematology*, 21(2), 206–219. <https://www.ncbi.nlm.nih.gov/pubmed/8425559>
- Harrison, M., O'Brien, A., Adams, L., Cowin, G., Ruitenberg, M. J., Sengul, G., & Watson, C. (2013). Vertebral landmarks for the identification of spinal cord segments in the mouse. *NeuroImage*, 68, 22–29. <https://doi.org/10.1016/j.neuroimage.2012.11.048>
- Harrison, W. J. (1962). The total cellularity of the bone marrow in man. *Journal of Clinical Pathology*, 15(3), 254–259. <https://doi.org/10.1136/jcp.15.3.254>
- Hartmann, L., & Metzeler, K. H. (2019). Clonal hematopoiesis and preleukemia-Genetics, biology, and clinical implications. *Genes, Chromosomes & Cancer*, 58(12), 828–838. <https://doi.org/10.1002/gcc.22756>
- Hasan, S., Lacout, C., Marty, C., Cuingnet, M., Solary, E., Vainchenker, W., & Villeval, J.-L. (2013). JAK2V617F expression in mice amplifies early hematopoietic cells and gives them a competitive advantage that is hampered by IFN α . *Blood*, 122(8), 1464–1477. <https://doi.org/10.1182/blood-2013-04-498956>
- Hassan, H. T., & Zander, A. (1996). Stem cell factor as a survival and growth factor in human normal and malignant hematopoiesis. *Acta Haematologica*, 95(3–4), 257–262. <https://doi.org/10.1159/000203893>

Hastings, J., Freedman, S., Rendon, O., Cooper, H. L., & Hirschhorn, K. (1961). Culture of human white cells using differential leucocyte separation. *Nature*, 192(4808), 1214–1215. <https://doi.org/10.1038/1921214a0>

Hatzimichael, E., & Tuthill, M. (2010). Hematopoietic stem cell transplantation. *Stem Cells and Cloning: Advances and Applications*, 3, 105–117. <https://doi.org/10.2147/SCCAA.S6815>

Haug, J. S., He, X. C., Grindley, J. C., Wunderlich, J. P., Gaudenz, K., Ross, J. T., Paulson, A., Wagner, K. P., Xie, Y., Zhu, R., Yin, T., Perry, J. M., Hembree, M. J., Redenbaugh, E. P., Radice, G. L., Seidel, C., & Li, L. (2008). N-cadherin expression level distinguishes reserved versus primed states of hematopoietic stem cells. *Cell Stem Cell*, 2(4), 367–379. <https://doi.org/10.1016/j.stem.2008.01.017>

Hayden, E. C. (2014). Technology: The \$1,000 genome. *Nature*, 507(7492), 294–295. <https://doi.org/10.1038/507294a>

He, X.-D., Xia, M.-F., Teng, J.-Y., Zhou, B.-B. S., & Wang, Q.-F. (2023). Clonal evolution in leukemia: preleukemia, evolutionary models, and clinical implications. *Genome Instability & Disease*, 4(4), 227–238. <https://doi.org/10.1007/s42764-023-00104-6>

Hermouet, S. (2023). Mutations, inflammation and phenotype of myeloproliferative neoplasms. *Frontiers in Oncology*, 13, 1196817. <https://doi.org/10.3389/fonc.2023.1196817>

Herms, A., & Jones, P. H. (2023). Somatic mutations in normal tissues: New perspectives on early carcinogenesis. *Annual Review of Cancer Biology*, 7(1). <https://doi.org/10.1146/annurev-cancerbio-061421-012447>

Herrmann, M., Hildebrand, M., Menzel, U., Fahy, N., Alini, M., Lang, S., Benneker, L., Verrier, S., Stoddart, M. J., & Bara, J. J. (2019). Phenotypic characterization of bone marrow mononuclear cells and derived stromal cell populations from human iliac crest, vertebral body and femoral head. *International Journal of Molecular Sciences*, 20(14), 3454. <https://doi.org/10.3390/ijms20143454>

Heyes, E., Wilhelmson, A. S., Wenzel, A., Manhart, G., Eder, T., Schuster, M. B., Rzepa, E., Pundhir, S., D'Altri, T., Frank, A.-K., Gentil, C., Woessmann, J., Schoof, E. M., Meggendorfer, M., Schwaller, J., Haferlach, T., Grebien, F., & Porse, B. T. (2023). TET2 lesions enhance the aggressiveness of CEBPA-mutant acute myeloid leukemia by rebalancing GATA2 expression. *Nature Communications*, 14(1), 6185. <https://doi.org/10.1038/s41467-023-41927-x>

Hillis, D. M. (1997). Phylogenetic analysis. *Current Biology: CB*, 7(3), R129–31. [https://doi.org/10.1016/s0960-9822\(97\)70070-8](https://doi.org/10.1016/s0960-9822(97)70070-8)

Hinze, A., Rinke, J., Crodel, C. C., Möbius, S., Schäfer, V., Heide, F. H., Hochhaus, A., & Ernst, T. (2023). Molecular-defined clonal evolution in patients with classical myeloproliferative neoplasms. *British Journal of Haematology*, 202(2), 308–317. <https://doi.org/10.1111/bjh.18834>

Hirao, A. (2014). TPO signal for stem cell genomic integrity. *Blood*, 123(4), 459–460. <https://doi.org/10.1182/blood-2013-11-537084>

Hitchcock, I. S., Hafer, M., Sangkhae, V., & Tucker, J. A. (2021). The thrombopoietin receptor: revisiting the master regulator of platelet production. *Platelets*, 32(6), 770–778. <https://doi.org/10.1080/09537104.2021.1925102>

Ho, Y.-H., Del Toro, R., Rivera-Torres, J., Rak, J., Korn, C., García-García, A., Macías, D., González-Gómez, C., Del Monte, A., Wittner, M., Waller, A. K., Foster, H. R., López-Otín, C., Johnson, R. S., Nerlov, C., Ghevaert, C., Vainchenker, W., Louache, F., Andrés, V., & Méndez-Ferrer, S. (2019). Remodeling of bone marrow hematopoietic stem cell niches promotes myeloid cell expansion during premature or physiological aging. *Cell Stem Cell*, 25(3), 407–418.e6. <https://doi.org/10.1016/j.stem.2019.06.007>

Hofbauer, S. W., Krenn, P. W., Ganghammer, S., Asslaber, D., Pichler, U., Oberascher, K., Henschler, R., Wallner, M., Kerschbaum, H., Greil, R., & Hartmann, T. N. (2014). Tiam1/Rac1 signals contribute to the proliferation and chemoresistance, but not motility, of chronic lymphocytic leukemia cells. *Blood*, 123(14), 2181–2188. <https://doi.org/10.1182/blood-2013-08-523563>

Hordyjewska, A., Popiolek, Ł., & Horecka, A. (2015). Characteristics of hematopoietic stem cells of umbilical cord blood. *Cytotechnology*, 67(3), 387–396. <https://doi.org/10.1007/s10616-014-9796-y>

Hotta, Y., & Benzer, S. (1972). Mapping of behaviour in *Drosophila* mosaics. *Nature*, 240(5383), 527–535. <https://doi.org/10.1038/240527a0>

Hu, T., Chong, Y., Lu, S., McGuinness, M., Williams, D. A., & Cowell, J. K. (2020). Rac1/2 activation promotes FGFR1 driven leukemogenesis in stem cell leukemia/lymphoma syndrome. *Haematologica*, 105(2), e68–e71. <https://doi.org/10.3324/haematol.2018.208058>

Hu, X., Li, J., Fu, M., Zhao, X., & Wang, W. (2021). The JAK/STAT signaling pathway: from bench to clinic. *Signal Transduction and Targeted Therapy*, 6(1), 402. <https://doi.org/10.1038/s41392-021-00791-1>

Hu, Y., & Smyth, G. K. (2009). ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. *Journal of Immunological Methods*, 347(1–2), 70–78. <https://doi.org/10.1016/j.jim.2009.06.008>

Huang, B., Lang, X., & Li, X. (2022). The role of IL-6/JAK2/STAT3 signaling pathway in cancers. *Frontiers in Oncology*, 12, 1023177. <https://doi.org/10.3389/fonc.2022.1023177>

Huang, H., Zhou, W., Chen, R., Xiang, B., Zhou, S., & Lan, L. (2021). CXCL10 is a tumor microenvironment and immune infiltration related prognostic biomarker in pancreatic adenocarcinoma. *Frontiers in Molecular Biosciences*, 8, 611508. <https://doi.org/10.3389/fmolb.2021.611508>

Huang, Y., & Rao, A. (2014). Connections between TET proteins and aberrant DNA modification in cancer. *Trends in Genetics: TIG*, 30(10), 464–474. <https://doi.org/10.1016/j.tig.2014.07.005>

Hulett, H. R., Bonner, W. A., Barrett, J., & Herzenberg, L. A. (1969). Cell sorting: automated separation of mammalian cells as a function of intracellular fluorescence. *Science (New York, N.Y.)*, 166(3906), 747–749. <https://doi.org/10.1126/science.166.3906.747>

Humphries, R. K., Eaves, A. C., & Eaves, C. J. (1981). Self-renewal of hemopoietic stem cells during mixed colony formation in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 78(6), 3629–3633. <https://doi.org/10.1073/pnas.78.6.3629>

Humphries, R. K., Jacky, P. B., Dill, F. J., Eaves, A. C., & Eaves, C. J. (1979). CFU-S in individual erythroid colonies derived in vitro from adult mouse marrow. *Nature*, 279(5715), 718–720. <https://doi.org/10.1038/279718a0>

Ichikawa, Y., Pluznik, D. H., & Sachs, L. (1966). In vitro control of the development of macrophage and granulocyte colonies. *Proceedings of the National Academy of Sciences of the United States of America*, 56(2), 488–495. <https://doi.org/10.1073/pnas.56.2.488>

Ichimaru, M., Tomonaga, M., Amenomori, T., & Matsuo, T. (1991). Atomic bomb and leukemia. *Journal of Radiation Research*, 32 Suppl 2, 14–19. https://doi.org/10.1269/jrr.32.supplement2_14

Ieyasu, A., Ishida, R., Kimura, T., Morita, M., Wilkinson, A. C., Sudo, K., Nishimura, T., Ohehara, J., Tajima, Y., Lai, C.-Y., Otsu, M., Nakamura, Y., Ema, H., Nakauchi, H., & Yamazaki, S. (2017). An all-recombinant protein-based culture system specifically identifies hematopoietic stem cell maintenance factors. *Stem Cell Reports*, 8(3), 500–508. <https://doi.org/10.1016/j.stemcr.2017.01.015>

Iscoe, N. (1984). Culture of lymphocytes and hemopoietic cells in serum-free medium. In B. D. S. D. Gh (Ed.), *Methods for Serum-Free Culture of Neuronal and Lymphoid Cells* (pp. 169–185). Alan R. Liss, Inc. <https://www.labs.uhnresearch.ca/labs/iscoe/Serum-Free84.pdf>

Iscoe, N. N., Guilbert, L. J., & Weyman, C. (1980). Complete replacement of serum in primary cultures of erythropoietin-dependent red cell precursors (CFU-E) by albumin, transferrin, iron, unsaturated fatty acid, lecithin and cholesterol. *Experimental Cell Research*, 126(1), 121–126. [https://doi.org/10.1016/0014-4827\(80\)90476-0](https://doi.org/10.1016/0014-4827(80)90476-0)

Iscoe, N. N., & Melchers, F. (1978). Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes. *The Journal of Experimental Medicine*, 147(3), 923–933. <https://doi.org/10.1084/jem.147.3.923>

Ishibashi, F., Shimizu, H., Nakata, T., Fujii, S., Suzuki, K., Kawamoto, A., Anzai, S., Kuno, R., Nagata, S., Ito, G., Murano, T., Mizutani, T., Oshima, S., Tsuchiya, K., Nakamura, T., Watanabe, M., & Okamoto, R. (2018). Contribution of ATOH1+ cells to the homeostasis, repair, and tumorigenesis of the colonic epithelium. *Stem Cell Reports*, 10(1), 27–42. <https://doi.org/10.1016/j.stemcr.2017.11.006>

Ishibashi, T., Yokota, T., Tanaka, H., Ichii, M., Sudo, T., Satoh, Y., Doi, Y., Ueda, T., Tanimura, A., Hamanaka, Y., Ezoe, S., Shibayama, H., Oritani, K., & Kanakura, Y. (2016). ESAM is a novel human hematopoietic stem cell marker associated with a subset of human leukemias. *Experimental Hematology*, 44(4), 269–81.e1. <https://doi.org/10.1016/j.exphem.2015.12.010>

Iwasaki, H., & Akashi, K. (2007). Myeloid lineage commitment from the hematopoietic stem cell. *Immunity*, 26(6), 726–740. <https://doi.org/10.1016/j.immuni.2007.06.004>

Jacobson, L. O., & Marks, E. K. (1949). The role of the spleen in radiation injury. *Proceedings of the Society for Experimental Biology and Medicine*. Society for Experimental Biology and Medicine (New York, N.Y.), 70(4), 740–742. <https://doi.org/10.3181/00379727-70-17053>

Jacobson, L. O., Simmons, E. L., Marks, E. K., & Eldredge, J. H. (1951). Recovery from radiation injury. *Science (New York, N.Y.)*, 113(2940), 510–511. <https://doi.org/10.1126/science.113.2940.510>

Jacquelin, S., Kramer, F., Mullally, A., & Lane, S. W. (2020). Murine models of myelofibrosis. *Cancers*, 12(9), 2381. <https://doi.org/10.3390/cancers12092381>

Jaganathan, B. G., Anjos-Afonso, F., Kumar, A., & Bonnet, D. (2013). Active RHOA favors retention of human hematopoietic stem/progenitor cells in their niche. *Journal of Biomedical Science*, 20, 66. <https://doi.org/10.1186/1423-0127-20-66>

Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P. V., Mar, B. G., Lindsley, R. C., Mermel, C. H., Burt, N., Chavez, A., Higgins, J. M., Moltchanov, V., Kuo, F. C., Kluk, M. J., Henderson, B., Kinnunen, L., Koistinen, H. A., Ladenvall, C., Getz, G., ... Ebert, B. L. (2014). Age-related clonal hematopoiesis associated with adverse outcomes. *The New England Journal of Medicine*, 371(26), 2488–2498. <https://doi.org/10.1056/NEJMoa1408617>

Jakobsen, N. A., Turkalj, S., Zeng, A. G. X., Stoilova, B., Metzner, M., Rahmig, S., Nagree, M. S., Shah, S., Moore, R., Usukhbayar, B., Angulo Salazar, M., Gafencu, G.-A., Kennedy, A., Newman, S., Kendrick, B. J. L., Taylor, A. H., Afinowi-Luitz, R., Gundle, R., Watkins, B., ... Vyas, P. (2024). Selective advantage of mutant stem cells in human clonal hematopoiesis is associated with attenuated response to inflammation and aging. *Cell Stem Cell*, 31(8), 1127–1144.e17. <https://doi.org/10.1016/j.stem.2024.05.010>

Jam, F. A., Morimune, T., Tsukamura, A., Tano, A., Tanaka, Y., Mori, Y., Yamamoto, T., Nishimura, M., Tooyama, I., & Mori, M. (2020). Neuroepithelial cell competition triggers loss of cellular juvenescence. *Scientific Reports*, 10(1), 18044. <https://doi.org/10.1038/s41598-020-74874-4>

James, C., Mazurier, F., Dupont, S., Chaligne, R., Lamrissi-Garcia, I., Tulliez, M., Lippert, E., Mahon, F.-X., Pasquet, J.-M., Etienne, G., Delhommeau, F., Giraudier, S., Vainchenker, W., & de Verneuil, H. (2008). The hematopoietic stem cell compartment of JAK2V617F-positive myeloproliferative disorders is a reflection of disease heterogeneity. *Blood*, 112(6), 2429–2438. <https://doi.org/10.1182/blood-2008-02-137877>

Jamieson, C. H. M., Gotlib, J., Durocher, J. A., Chao, M. P., Mariappan, M. R., Lay, M., Jones, C., Zehnder, J. L., Lilleberg, S. L., & Weissman, I. L. (2006). The JAK2 V617F mutation occurs in hematopoietic stem cells in polycythemia vera and predisposes toward erythroid differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, 103(16), 6224–6229. <https://doi.org/10.1073/pnas.0601462103>

Jankowska, A. M., Szpurka, H., Tiu, R. V., Makishima, H., Afable, M., Huh, J., O’Keefe, C. L., Ganetzky, R., McDevitt, M. A., & Maciejewski, J. P. (2009). Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. *Blood*, 113(25), 6403–6410. <https://doi.org/10.1182/blood-2009-02-205690>

Jassinskaja, M., Bode, D., Gonka, M., Roumeliotis, T. I., Hogg, A. J., Rubio Lara, J. A., Bennett, E., Milek, J., Theeuwes, B., Vijayabaskar, M. S., Cosme, L. C., Che, J. L. C., MacDonald, S., Ahmed, S., Hall, B. A., Vasey, G., Kooi, H., Belmonte, M., Shepherd, M. S., ... Kent, D. G. (2025). Selection of pre-leukemic hematopoietic stem cells driven by distinct extracellular matrix molecules. In *bioRxiv* (p. 2025.01.20.633881). <https://doi.org/10.1101/2025.01.20.633881>

Jenq, R. R., & van den Brink, M. R. M. (2010). Allogeneic haematopoietic stem cell transplantation: individualized stem cell and immune therapy of cancer. *Nature Reviews. Cancer*, 10(3), 213–221. <https://doi.org/10.1038/nrc2804>

Jian, D., Wang, W., Zhou, X., Jia, Z., Wang, J., Yang, M., Zhao, W., Jiang, Z., Hu, X., & Zhu, J. (2018). Interferon-induced protein 35 inhibits endothelial cell proliferation, migration and re-endothelialization of injured arteries by inhibiting the nuclear factor-kappa B pathway. *Acta Physiologica (Oxford, England)*, 223(3), e13037. <https://doi.org/10.1111/apha.13037>

Jiang, S. (2020). Tet2 at the interface between cancer and immunity. *Communications Biology*, 3(1), 667. <https://doi.org/10.1038/s42003-020-01391-5>

Johansson, A., Khalilnezhad, A., Takizawa, H., Mizuno, H., Suda, T., & Umemoto, T. (2024). Mobilization dynamics of bone marrow hematopoietic stem cells during hematopoietic regeneration. *Experimental Hematology*, 138(104281), 104281. <https://doi.org/10.1016/j.exphem.2024.104281>

Johnsen, J. M., Nickerson, D. A., & Reiner, A. P. (2013). Massively parallel sequencing: the new frontier of hematologic genomics. *Blood*, 122(19), 3268–3275. <https://doi.org/10.1182/blood-2013-07-460287>

Johnston, L. A., Prober, D. A., Edgar, B. A., Eisenman, R. N., & Gallant, P. (1999). *Drosophila myc* regulates cellular growth during development. *Cell*, 98(6), 779–790. [https://doi.org/10.1016/s0092-8674\(00\)81512-3](https://doi.org/10.1016/s0092-8674(00)81512-3)

Johnston, Laura A. (2014). Socializing with MYC: cell competition in development and as a model for premalignant cancer. *Cold Spring Harbor Perspectives in Medicine*, 4(4), a014274. <https://doi.org/10.1101/cshperspect.a014274>

Jonason, A. S., Kunala, S., Price, G. J., Restifo, R. J., Spinelli, H. M., Persing, J. A., Leffell, D. J., Tarone, R. E., & Brash, D. E. (1996). Frequent clones of p53-mutated keratinocytes in normal human skin. *Proceedings of the National Academy of Sciences of the United States of America*, 93(24), 14025–14029. <https://doi.org/10.1073/pnas.93.24.14025>

Jordan, C. T., & Lemischka, I. R. (1990). Clonal and systemic analysis of long-term hematopoiesis in the mouse. *Genes & Development*, 4(2), 220–232. <https://doi.org/10.1101/gad.4.2.220>

Jung, W.-C., Levesque, J.-P., & Ruitenberg, M. J. (2017). It takes nerve to fight back: The significance of neural innervation of the bone marrow and spleen for immune function. *Seminars in Cell & Developmental Biology*, 61, 60–70. <https://doi.org/10.1016/j.semcdb.2016.08.010>

Jurecic, R. (2019). Hematopoietic stem cell heterogeneity. *Advances in Experimental Medicine and Biology*, 1169, 195–211. https://doi.org/10.1007/978-3-030-24108-7_10

Justin, M., Jež, M., Košir, A., Miceska, S., Rožman, P., & Jazbec, K. (2021). Application of the 3R principles: Vertebrae as an additional source of murine bone-marrow cells. *Laboratory Animals*, 55(1), 43–52. <https://doi.org/10.1177/0023677220922573>

Jutzi, J. S., & Mullally, A. (2020). Remodeling the bone marrow microenvironment - A proposal for targeting pro-inflammatory contributors in MPN. *Frontiers in Immunology*, 11, 2093. <https://doi.org/10.3389/fimmu.2020.02093>

Kaasinen, E., Kuusmin, O., Rajamäki, K., Ristolainen, H., Aavikko, M., Kondelin, J., Saarinen, S., Berta, D. G., Katainen, R., Hirvonen, E. A. M., Karhu, A., Taira, A., Tanskanen, T., Alkods, A., Taipale, M., Morgunova, E., Franssila, K., Lehtonen, R., Mäkinen, M., ... Aaltonen, L. A. (2019). Impact of constitutional TET2 haploinsufficiency on molecular and clinical phenotype in humans. *Nature Communications*, 10(1), 1252. <https://doi.org/10.1038/s41467-019-09198-7>

Kameda, T., Shide, K., Yamaji, T., Kamiunten, A., Sekine, M., Taniguchi, Y., Hidaka, T., Kubuki, Y., Shimoda, H., Marutsuka, K., Sashida, G., Aoyama, K., Yoshimitsu, M., Harada, T., Abe, H., Miike, T., Iwakiri, H., Tahara, Y., Sueta, M., ... Shimoda, K. (2015). Loss of TET2 has

dual roles in murine myeloproliferative neoplasms: disease sustainer and disease accelerator. *Blood*, 125(2), 304–315. <https://doi.org/10.1182/blood-2014-04-555508>

Kamminga, L. M., van Os, R., Ausema, A., Noach, E. J. K., Weersing, E., Dontje, B., Vellenga, E., & de Haan, G. (2005). Impaired hematopoietic stem cell functioning after serial transplantation and during normal aging. *Stem Cells* (Dayton, Ohio), 23(1), 82–92. <https://doi.org/10.1634/stemcells.2004-0066>

Kapadia, C. D., Williams, N., Dawson, K. J., Watson, C., Yousefzadeh, M. J., Le, D., Nyamondo, K., Kodavali, S., Cagan, A., Waldvogel, S., Zhang, X., De La Fuente, J., Leongamornlert, D., Mitchell, E., Florez, M. A., Sosnowski, K., Aguilar, R., Martell, A., Guzman, A., ... Nangalia, J. (2025). Clonal dynamics and somatic evolution of haematopoiesis in mouse. *Nature*, 641(8063), 681–689. <https://doi.org/10.1038/s41586-025-08625-8>

Karamitros, D., Stoilova, B., Aboukhalil, Z., Hamey, F., Reinisch, A., Samitsch, M., Quek, L., Otto, G., Repapi, E., Doondeea, J., Usukhbayar, B., Calvo, J., Taylor, S., Goardon, N., Six, E., Pflumio, F., Porcher, C., Majeti, R., Göttgens, B., & Vyas, P. (2018). Single-cell analysis reveals the continuum of human lympho-myeloid progenitor cells. *Nature Immunology*, 19(1), 85–97. <https://doi.org/10.1038/s41590-017-0001-2>

Keller, G., Paige, C., Gilboa, E., & Wagner, E. F. (1985). Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. *Nature*, 318(6042), 149–154. <https://doi.org/10.1038/318149a0>

Kelly, L. S., Darden, D. B., Fenner, B. P., Efron, P. A., & Mohr, A. M. (2021). The hematopoietic stem/progenitor cell response to hemorrhage, injury, and sepsis: A review of pathophysiology. *Shock* (Augusta, Ga.), 56(1), 30–41. <https://doi.org/10.1097/SHK.0000000000001699>

Kent, D. G., Copley, M. R., Benz, C., Wöhrer, S., Dykstra, B. J., Ma, E., Cheyne, J., Zhao, Y., Bowie, M. B., Zhao, Y., Gasparetto, M., Delaney, A., Smith, C., Marra, M., & Eaves, C. J. (2009). Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential. *Blood*, 113(25), 6342–6350. <https://doi.org/10.1182/blood-2008-12-192054>

Kent, D. G., Dykstra, B. J., Cheyne, J., Ma, E., & Eaves, C. J. (2008). Steel factor coordinately regulates the molecular signature and biologic function of hematopoietic stem cells. *Blood*, 112(3), 560–567. <https://doi.org/10.1182/blood-2007-10-117820>

Kent, D. G., Dykstra, B. J., & Eaves, C. J. (2016). Isolation and assessment of single long-term reconstituting hematopoietic stem cells from adult mouse bone marrow. *Current Protocols in Stem Cell Biology*, 38(1), 2A.4.1-2A.4.24. <https://doi.org/10.1002/cpsc.10>

Kent, D. G., & Eaves, C. J. (2016). Adult hematopoiesis. In *Encyclopedia of Immunobiology* (pp. 15–25). Elsevier. <https://doi.org/10.1016/b978-0-12-374279-7.01003-1>

Kent, D. G., & Green, A. R. (2017). Order matters: The order of somatic mutations influences cancer evolution. *Cold Spring Harbor Perspectives in Medicine*, 7(4). <https://doi.org/10.1101/cshperspect.a027060>

Kent, D. G., Li, J., Tanna, H., Fink, J., Kirschner, K., Pask, D. C., Silber, Y., Hamilton, T. L., Sneade, R., Simons, B. D., & Green, A. R. (2013). Self-renewal of single mouse hematopoietic stem cells is reduced by JAK2V617F without compromising progenitor cell expansion. *PLoS Biology*, 11(6), e1001576. <https://doi.org/10.1371/journal.pbio.1001576>

Kfoury, Y. S., Ji, F., Jain, E., Mazzola, M., Schirolli, G., Papazian, A., Mercier, F., Sykes, D. B., Kiem, A., Randolph, M., Calvi, L. M., Abdel-Wahab, O., Sadreyev, R. I., & Scadden, D. T. (2023). The bone marrow stroma in human myelodysplastic syndrome reveals alterations that regulate disease progression. *Blood Advances*, 7(21), 6608–6623. <https://doi.org/10.1182/bloodadvances.2022008268>

Khan, J. A., Mendelson, A., Kunisaki, Y., Birbrair, A., Kou, Y., Arnal-Estapé, A., Pinho, S., Ciero, P., Nakahara, F., Ma'ayan, A., Bergman, A., Merad, M., & Frenette, P. S. (2016). Fetal liver hematopoietic stem cell niches associate with portal vessels. *Science (New York, N.Y.)*, 351(6269), 176–180. <https://doi.org/10.1126/science.aad0084>

Kiel, M. J., Radice, G. L., & Morrison, S. J. (2007). Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. *Cell Stem Cell*, 1(2), 204–217. <https://doi.org/10.1016/j.stem.2007.06.001>

Kiel, M. J., Yilmaz, O. H., Iwashita, T., Yilmaz, O. H., Terhorst, C., & Morrison, S. J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*, 121(7), 1109–1121. <https://doi.org/10.1016/j.cell.2005.05.026>

Kikuchi, K., Sakamoto, Y., Uezu, A., Yamamoto, H., Ishiguro, K.-I., Shimamura, K., Saito, T., Hisanaga, S.-I., & Nakanishi, H. (2022). Map7D2 and Map7D1 facilitate microtubule stabilization through distinct mechanisms in neuronal cells. *Life Science Alliance*, 5(8). <https://doi.org/10.26508/lsa.202201390>

Kim, B., Park, Y.-Y., & Lee, J.-H. (2024). CXCL10 promotes melanoma angiogenesis and tumor growth. *Animal Cells and Systems*, 28(1), 453–465. <https://doi.org/10.1080/19768354.2024.2402024>

Kim, M., Choi, H. Y., Woo, J. W., Chung, Y. R., & Park, S. Y. (2021). Role of CXCL10 in the progression of in situ to invasive carcinoma of the breast. *Scientific Reports*, 11(1), 18007. <https://doi.org/10.1038/s41598-021-97390-5>

Kimmel, C. B., Warga, R. M., & Schilling, T. F. (1990). Origin and organization of the zebrafish fate map. *Development (Cambridge, England)*, 108(4), 581–594. <https://doi.org/10.1242/dev.108.4.581>

Kimura, M. (1968). Evolutionary rate at the molecular level. *Nature*, 217(5129), 624–626. <https://doi.org/10.1038/217624a0>

King, K. Y., & Goodell, M. A. (2011). Inflammatory modulation of HSCs: viewing the HSC as a foundation for the immune response. *Nature Reviews. Immunology*, 11(10), 685–692. <https://doi.org/10.1038/nri3062>

Klampfl, T., Gisslinger, H., Harutyunyan, A. S., Nivarthi, H., Rumi, E., Milosevic, J. D., Them, N. C. C., Berg, T., Gisslinger, B., Pietra, D., Chen, D., Vladimer, G. I., Bagienski, K., Milanesi, C., Casetti, I. C., Sant'Antonio, E., Ferretti, V., Elena, C., Schischlik, F., ... Kralovics, R. (2013). Somatic mutations of calreticulin in myeloproliferative neoplasms. *The New England Journal of Medicine*, 369(25), 2379–2390. <https://doi.org/10.1056/NEJMoa1311347>

Klepin, H. D., Rao, A. V., & Pardee, T. S. (2014). Acute myeloid leukemia and myelodysplastic syndromes in older adults. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, 32(24), 2541–2552. <https://doi.org/10.1200/JCO.2014.55.1564>

Ko, M., Bandukwala, H. S., An, J., Lamperti, E. D., Thompson, E. C., Hastie, R., Tsangaratou, A., Rajewsky, K., Koralov, S. B., & Rao, A. (2011). Ten-Eleven-Translocation 2 (TET2) negatively regulates homeostasis and differentiation of hematopoietic stem cells in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 108(35), 14566–14571. <https://doi.org/10.1073/pnas.1112317108>

Ko, M., Huang, Y., Jankowska, A. M., Pape, U. J., Tahiliani, M., Bandukwala, H. S., An, J., Lamperti, E. D., Koh, K. P., Ganetzky, R., Liu, X. S., Aravind, L., Agarwal, S., Maciejewski, J. P., & Rao, A. (2010). Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature*, 468(7325), 839–843. <https://doi.org/10.1038/nature09586>

Kobayashi, H., Butler, J. M., O'Donnell, R., Kobayashi, M., Ding, B.-S., Bonner, B., Chiu, V. K., Nolan, D. J., Shido, K., Benjamin, L., & Rafii, S. (2010). Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nature Cell Biology*, 12(11), 1046–1056. <https://doi.org/10.1038/ncb2108>

Kodra, A. L., Singh, A. S., de la Cova, C., Ziosi, M., & Johnston, L. A. (2024). The *Drosophila* tumor necrosis factor Eiger promotes Myc supercompetition independent of canonical Jun N-terminal kinase signaling. *Genetics*, 228(1). <https://doi.org/10.1093/genetics/iyae107>

Koeffler, H. P., & Leong, G. (2017). Preleukemia: one name, many meanings. *Leukemia*, 31(3), 534–542. <https://doi.org/10.1038/leu.2016.364>

Kogan, S. C., Ward, J. M., Anver, M. R., Berman, J. J., Brayton, C., Cardiff, R. D., Carter, J. S., de Coronado, S., Downing, J. R., Fredrickson, T. N., Haines, D. C., Harris, A. W., Harris, N. L., Hiai, H., Jaffe, E. S., MacLennan, I. C. M., Pandolfi, P. P., Pattengale, P. K., Perkins, A. S., ... Hematopathology subcommittee of the Mouse Models of Human Cancers Consortium. (2002). Bethesda proposals for classification of nonlymphoid hematopoietic neoplasms in mice. *Blood*, 100(1), 238–245. <https://doi.org/10.1182/blood.v100.1.238>

Koh, B. I., Mohanakrishnan, V., Jeong, H.-W., Park, H., Kruse, K., Choi, Y. J., Nieminen-Kelhä, M., Kumar, R., Pereira, R. S., Adams, S., Lee, H. J., Bixel, M. G., Vajkoczy, P., Krause, D. S., & Adams, R. H. (2024). Adult skull bone marrow is an expanding and resilient haematopoietic reservoir. *Nature*, 636(8041), 172–181. <https://doi.org/10.1038/s41586-024-08163-9>

Köhler, G., & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, 256(5517), 495–497. <https://doi.org/10.1038/256495a0>

Kolabas, Z. I., Kuemmerle, L. B., Perneczky, R., Förstera, B., Ulukaya, S., Ali, M., Kapoor, S., Bartos, L. M., Büttner, M., Caliskan, O. S., Rong, Z., Mai, H., Höher, L., Jeridi, D., Molbay, M., Khalin, I., Deligiannis, I. K., Negwer, M., Roberts, K., ... Erturk, A. (2023). Distinct molecular profiles of skull bone marrow in health and neurological disorders. *Cell*, 186(17), 3706–3725.e29. <https://doi.org/10.1016/j.cell.2023.07.009>

Kong, T., Laranjeira, A. B. A., Letson, C. T., Yu, L., Lin, S., Fowles, J. S., Fisher, D. A. C., Ng, S., Yang, W., He, F., Youn, M., Mark, K., Jose, A. S., Liu, J., Kim, A. B., Cox, M. J., Fulbright, M. C., Jayanthan, A., Los, G., ... Oh, S. T. (2025). RSK1 is an exploitable dependency in myeloproliferative neoplasms and secondary acute myeloid leukemia. *Nature Communications*, 16(1), 492. <https://doi.org/10.1038/s41467-024-55643-7>

Konstantinov, I. E. (2000). In search of Alexander A. maximow: The man behind the Unitarian theory of hematopoiesis. *Perspectives in Biology and Medicine*, 43(2), 269–276. <https://doi.org/10.1353/pbm.2000.0006>

Korbecki, J., Bosiacki, M., Szatkowska, I., Kupnicka, P., Chlubek, D., & Baranowska-Bosiacka, I. (2024). The clinical significance and involvement in molecular cancer processes of chemokine CXCL1 in selected tumors. *International Journal of Molecular Sciences*, 25(8). <https://doi.org/10.3390/ijms25084365>

Korn, C., & Méndez-Ferrer, S. (2017). Myeloid malignancies and the microenvironment. *Blood*, 129(7), 811–822. <https://doi.org/10.1182/blood-2016-09-670224>

Kosmider, O., Gelsi-Boyer, V., Ciudad, M., Racoeur, C., Jooste, V., Vey, N., Quesnel, B., Fenaux, P., Bastie, J.-N., Beyne-Rauzy, O., Stamatoulas, A., Dreyfus, F., Ifrah, N., de Botton, S., Vainchenker, W., Bernard, O. A., Birnbaum, D., Fontenay, M., Solary, E., & Groupe Francophone des Myélodysplasies. (2009). TET2 gene mutation is a frequent and adverse event in chronic myelomonocytic leukemia. *Haematologica*, 94(12), 1676–1681. <https://doi.org/10.3324/haematol.2009.011205>

Kotani, S., Yoda, A., Kon, A., Kataoka, K., Ochi, Y., Shiozawa, Y., Hirsch, C., Takeda, J., Ueno, H., Yoshizato, T., Yoshida, K., Nakagawa, M. M., Nannya, Y., Kakiuchi, N., Yamauchi, T., Aoki, K., Shiraishi, Y., Miyano, S., Maeda, T., ... Makishima, H. (2019). Molecular pathogenesis of disease progression in MLL-rearranged AML. *Leukemia*, 33(3), 612–624. <https://doi.org/10.1038/s41375-018-0253-3>

Kralovics, R., Passamonti, F., Buser, A. S., Teo, S.-S., Tiedt, R., Passweg, J. R., Tichelli, A., Cazzola, M., & Skoda, R. C. (2005). A gain-of-function mutation of JAK2 in myeloproliferative disorders. *The New England Journal of Medicine*, 352(17), 1779–1790. <https://doi.org/10.1056/NEJMoa051113>

Krotenberg Garcia, A., Ledesma-Terrón, M., Lamprou, M., Vriend, J., van Luyk, M. E., & Suijkerbuijk, S. J. E. (2024). Cell competition promotes metastatic intestinal cancer through a multistage process. *iScience*, 27(5), 109718. <https://doi.org/10.1016/j.isci.2024.109718>

Kucinski, I., Dinan, M., Kolahgar, G., & Piddini, E. (2017). Chronic activation of JNK JAK/STAT and oxidative stress signalling causes the loser cell status. *Nature Communications*, 8(1), 136. <https://doi.org/10.1038/s41467-017-00145-y>

Kunisaki, Y., Bruns, I., Scheiermann, C., Ahmed, J., Pinho, S., Zhang, D., Mizoguchi, T., Wei, Q., Lucas, D., Ito, K., Mar, J. C., Bergman, A., & Frenette, P. S. (2013). Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature*, 502(7473), 637–643. <https://doi.org/10.1038/nature12612>

Kuntz, A., & Richins, C. A. (1945). Innervation of the bone marrow. *The Journal of Comparative Neurology*, 83(3), 213–222. <https://doi.org/10.1002/cne.900830302>

Lacout, C., Pisani, D. F., Tulliez, M., Gachelin, F. M., Vainchenker, W., & Villeval, J.-L. (2006). JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis. *Blood*, 108(5), 1652–1660. <https://doi.org/10.1182/blood-2006-02-002030>

Lakshmipathy, U., & Verfaillie, C. (2005). Stem cell plasticity. *Blood Reviews*, 19(1), 29–38. <https://doi.org/10.1016/j.blre.2004.03.001>

Lakso, M., Pichel, J. G., Gorman, J. R., Sauer, B., Okamoto, Y., Lee, E., Alt, F. W., & Westphal, H. (1996). Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proceedings of the National Academy of Sciences of the United States of America*, 93(12), 5860–5865. <https://doi.org/10.1073/pnas.93.12.5860>

Lala, T., & Hall, R. A. (2022). Adhesion G protein-coupled receptors: structure, signaling, physiology, and pathophysiology. *Physiological Reviews*, 102(4), 1587–1624. <https://doi.org/10.1152/physrev.00027.2021>

Lampreia, F. P., Carmelo, J. G., & Anjos-Afonso, F. (2017). Notch signaling in the regulation of hematopoietic stem cell. *Current Stem Cell Reports*, 3(3), 202–209. <https://doi.org/10.1007/s40778-017-0090-8>

Laneuville, P., Chang, W., Kamel-Reid, S., Fauser, A. A., & Dick, J. E. (1988). High-efficiency gene transfer and expression in normal human hematopoietic cells with retrovirus vectors. *Blood*, 71(3), 811–814. <https://doi.org/10.1182/blood.v71.3.811.811>

Langemeijer, S. M. C., Kuiper, R. P., Berends, M., Knops, R., Aslanyan, M. G., Massop, M., Stevens-Linders, E., van Hoogen, P., van Kessel, A. G., Raymakers, R. A. P., Kamping, E. J., Verhoef, G. E., Verburch, E., Hagemeijer, A., Vandenberghe, P., de Witte, T., van der Reijden, B. A., & Jansen, J. H. (2009). Acquired mutations in TET2 are common in myelodysplastic syndromes. *Nature Genetics*, 41(7), 838–842. <https://doi.org/10.1038/ng.391>

Lanikova, L., Babosova, O., & Prchal, J. T. (2019). Experimental modeling of myeloproliferative neoplasms. *Genes*, 10(10), 813. <https://doi.org/10.3390/genes10100813>

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M. A., & Dick, J. E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature*, 367(6464), 645–648. <https://doi.org/10.1038/367645a0>

Larsen, T. S., Christensen, J. H., Hasselbalch, H. C., & Pallisgaard, N. (2007). The JAK2 V617F mutation involves B- and T-lymphocyte lineages in a subgroup of patients with Philadelphia-chromosome negative chronic myeloproliferative disorders. *British Journal of Haematology*, 136(5), 745–751. <https://doi.org/10.1111/j.1365-2141.2007.06497.x>

Lassailly, F., Foster, K., Lopez-Onieva, L., Currie, E., & Bonnet, D. (2013). Multimodal imaging reveals structural and functional heterogeneity in different bone marrow compartments: functional implications on hematopoietic stem cells. *Blood*, 122(10), 1730–1740. <https://doi.org/10.1182/blood-2012-11-467498>

Laurenti, E., & Göttgens, B. (2018). From haematopoietic stem cells to complex differentiation landscapes. *Nature*, 553(7689), 418–426. <https://doi.org/10.1038/nature25022>

Lawrence, P. A., & Johnston, P. (1986). Observations on cell lineage of internal organs of *Drosophila*. *Journal of Embryology and Experimental Morphology*, 91, 251–266. <https://www.ncbi.nlm.nih.gov/pubmed/3711788>

Le, X. (2024). Partners in crime: Co-occurring genetic alterations in EGFR-mutant NSCLC. *Journal of Thoracic Oncology: Official Publication of the International Association for the Study of Lung Cancer*, 19(2), 190–192. <https://doi.org/10.1016/j.jtho.2023.11.009>

Leavy, O. (2016). The birth of monoclonal antibodies. *Nature Immunology*, 17(S1), S13–S13. <https://doi.org/10.1038/ni.3608>

Lee, J., Godfrey, A. L., & Nangalia, J. (2020). Genomic heterogeneity in myeloproliferative neoplasms and applications to clinical practice. *Blood Reviews*, 42(100708), 100708. <https://doi.org/10.1016/j.blre.2020.100708>

Lee, J. Y., & Hong, S.-H. (2020). Hematopoietic stem cells and their roles in tissue regeneration. *International Journal of Stem Cells*, 13(1), 1–12. <https://doi.org/10.15283/ijsc19127>

Lee-Six, H., & Kent, D. G. (2020). Tracking hematopoietic stem cells and their progeny using whole-genome sequencing. *Experimental Hematology*, 83, 12–24. <https://doi.org/10.1016/j.exphem.2020.01.004>

Lee-Six, H., Øbro, N. F., Shepherd, M. S., Grossmann, S., Dawson, K., Belmonte, M., Osborne, R. J., Huntly, B. J. P., Martincorena, I., Anderson, E., O'Neill, L., Stratton, M. R., Laurenti, E., Green, A. R., Kent, D. G., & Campbell, P. J. (2018). Population dynamics of normal human blood inferred from somatic mutations. *Nature*, 561(7724), 473–478. <https://doi.org/10.1038/s41586-018-0497-0>

Lemischka, I. R., Raulet, D. H., & Mulligan, R. C. (1986). Developmental potential and dynamic behavior of hematopoietic stem cells. *Cell*, 45(6), 917–927. [https://doi.org/10.1016/0092-8674\(86\)90566-0](https://doi.org/10.1016/0092-8674(86)90566-0)

Lendahl, U. (2022). 100 plus years of stem cell research-20 years of ISSCR. *Stem Cell Reports*, 17(6), 1248–1267. <https://doi.org/10.1016/j.stemcr.2022.04.004>

Lesire, L., Leroux, F., Deprez-Poulain, R., & Deprez, B. (2022). Insulin-degrading enzyme, an under-estimated potential target to treat cancer? *Cells (Basel, Switzerland)*, 11(7), 1228. <https://doi.org/10.3390/cells11071228>

Levine, R. L., Pardanani, A., Tefferi, A., & Gilliland, D. G. (2007). Role of JAK2 in the pathogenesis and therapy of myeloproliferative disorders. *Nature Reviews. Cancer*, 7(9), 673–683. <https://doi.org/10.1038/nrc2210>

Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., Wernig, G., Huntly, B. J. P., Boggon, T. J., Wlodarska, I., Clark, J. J., Moore, S., Adelsperger, J., Koo, S., Lee, J. C., Gabriel, S., Mercher, T., D'Andrea, A., Fröhling, S., Döhner, K., Marynen, P., ... Gilliland, D. G. (2005). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell*, 7(4), 387–397. <https://doi.org/10.1016/j.ccr.2005.03.023>

Ley, T. J., Mardis, E. R., Ding, L., Fulton, B., McLellan, M. D., Chen, K., Dooling, D., Dunford-Shore, B. H., McGrath, S., Hickenbotham, M., Cook, L., Abbott, R., Larson, D. E., Koboldt, D. C., Pohl, C., Smith, S., Hawkins, A., Abbott, S., Locke, D., ... Wilson, R. K. (2008). DNA sequencing of a cytogenetically normal acute myeloid leukaemia genome. *Nature*, 456(7218), 66–72. <https://doi.org/10.1038/nature07485>

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., & 1000 Genome Project Data Processing Subgroup. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics (Oxford, England)*, 25(16), 2078–2079. <https://doi.org/10.1093/bioinformatics/btp352>

Li, J., Kent, D. G., Chen, E., & Green, A. R. (2011). Mouse models of myeloproliferative neoplasms: JAK of all grades. *Disease Models & Mechanisms*, 4(3), 311–317. <https://doi.org/10.1242/dmm.006817>

Li, J., Kent, D. G., Godfrey, A. L., Manning, H., Nangalia, J., Aziz, A., Chen, E., Saeb-Parsy, K., Fink, J., Sneade, R., Hamilton, T. L., Pask, D. C., Silber, Y., Zhao, X., Ghevaert, C., Liu, P., & Green, A. R. (2014). JAK2V617F homozygosity drives a phenotypic switch in

myeloproliferative neoplasms, but is insufficient to sustain disease. *Blood*, 123(20), 3139–3151. <https://doi.org/10.1182/blood-2013-06-510222>

Li, J., Spensberger, D., Ahn, J. S., Anand, S., Beer, P. A., Ghevaert, C., Chen, E., Forrai, A., Scott, L. M., Ferreira, R., Campbell, P. J., Watson, S. P., Liu, P., Erber, W. N., Huntly, B. J. P., Ottersbach, K., & Green, A. R. (2010). JAK2 V617F impairs hematopoietic stem cell function in a conditional knock-in mouse model of JAK2 V617F-positive essential thrombocythemia. *Blood*, 116(9), 1528–1538. <https://doi.org/10.1182/blood-2009-12-259747>

Li, L., Bowling, S., McGeary, S. E., Yu, Q., Lemke, B., Alcedo, K., Jia, Y., Liu, X., Ferreira, M., Klein, A. M., Wang, S.-W., & Camargo, F. D. (2023). A mouse model with high clonal barcode diversity for joint lineage, transcriptomic, and epigenomic profiling in single cells. *Cell*, 186(23), 5183–5199.e22. <https://doi.org/10.1016/j.cell.2023.09.019>

Li, R., Colombo, M., Wang, G., Rodriguez-Romera, A., Benlabiod, C., Jooss, N. J., O’Sullivan, J., Brierley, C. K., Clark, S.-A., Pérez Sáez, J. M., Fernández, P. A., Schoof, E. M., Porse, B., Meng, Y., Khan, A. O., Wen, S., Dong, P., Zhou, W., Sousos, N., ... Psaila, B. (2024). A proinflammatory stem cell niche drives myelofibrosis through a targetable galectin-1 axis. *Science Translational Medicine*, 16(768), eadj7552. <https://doi.org/10.1126/scitranslmed.adj7552>

Li, X., Li, C., Zhang, W., Wang, Y., Qian, P., & Huang, H. (2023). Inflammation and aging: signaling pathways and intervention therapies. *Signal Transduction and Targeted Therapy*, 8(1), 239. <https://doi.org/10.1038/s41392-023-01502-8>

Li, Z., Cai, X., Cai, C.-L., Wang, J., Zhang, W., Petersen, B. E., Yang, F.-C., & Xu, M. (2011). Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. *Blood*, 118(17), 4509–4518. <https://doi.org/10.1182/blood-2010-12-325241>

Lima, A., Lubatti, G., Burgstaller, J., Hu, D., Green, A. P., Di Gregorio, A., Zawadzki, T., Pernaute, B., Mahammadov, E., Perez-Montero, S., Dore, M., Sanchez, J. M., Bowling, S., Sancho, M., Kolbe, T., Karimi, M. M., Carling, D., Jones, N., Srinivas, S., ... Rodriguez, T. A. (2021). Cell competition acts as a purifying selection to eliminate cells with mitochondrial defects during early mouse development. *Nature Metabolism*, 3(8), 1091–1108. <https://doi.org/10.1038/s42255-021-00422-7>

Lin, D. S., Kan, A., Gao, J., Crampin, E. J., Hodgkin, P. D., & Naik, S. H. (2018). DiSNE movie visualization and assessment of clonal kinetics reveal multiple trajectories of dendritic cell development. *Cell Reports*, 22(10), 2557–2566. <https://doi.org/10.1016/j.celrep.2018.02.046>

Lin, D. S., Zhang, S., Schreuder, J., Tran, J., Sargeant, T., Metcalf, D., Ng, A. P., Weber, T. S., & Naik, S. H. (2024). A multi-track landscape of haematopoiesis informed by cellular barcoding and agent-based modelling. In *bioRxiv*. <https://doi.org/10.1101/2024.03.28.587126>

Lio, C.-W. J., Yuita, H., & Rao, A. (2019). Dysregulation of the TET family of epigenetic regulators in lymphoid and myeloid malignancies. *Blood*, 134(18), 1487–1497. <https://doi.org/10.1182/blood.2019791475>

Little, A. C., Pathanjeli, P., Wu, Z., Bao, L., Goo, L. E., Yates, J. A., Oliver, C. R., Soellner, M. B., & Merajver, S. D. (2019). IL-4/IL-13 stimulated macrophages enhance breast cancer invasion via Rho-GTPase regulation of synergistic VEGF/CCL-18 signaling. *Frontiers in Oncology*, 9, 456. <https://doi.org/10.3389/fonc.2019.00456>

Liu, M., Guo, S., & Stiles, J. K. (2011). The emerging role of CXCL10 in cancer (Review). *Oncology Letters*, 2(4), 583–589. <https://doi.org/10.3892/ol.2011.300>

Liu, W., Du, W., Shang, X., Wang, L., Evelyn, C., Florian, M. C., A Ryan, M., Rayes, A., Zhao, X., Setchell, K., Meller, J., Guo, F., Nassar, N., Geiger, H., Pang, Q., & Zheng, Y. (2019). Rational identification of a Cdc42 inhibitor presents a new regimen for long-term hematopoietic stem cell mobilization. *Leukemia*, 33(3), 749–761. <https://doi.org/10.1038/s41375-018-0251-5>

Livet, J., Weissman, T. A., Kang, H., Draft, R. W., Lu, J., Bennis, R. A., Sanes, J. R., & Lichtman, J. W. (2007). Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature*, 450(7166), 56–62. <https://doi.org/10.1038/nature06293>

Lo Celso, C., & Scadden, D. (2007). Isolation and transplantation of hematopoietic stem cells (HSCs). *Journal of Visualized Experiments: JoVE*, 2, 157. <https://doi.org/10.3791/157>

Lord, B. I., Testa, N. G., & Hendry, J. H. (1975). The relative spatial distributions of CFUs and CFUc in the normal mouse femur. *Blood*, 46(1), 65–72. <https://doi.org/10.1182/blood.v46.1.65.65>

Lu, R., Neff, N. F., Quake, S. R., & Weissman, I. L. (2011). Tracking single hematopoietic stem cells in vivo using high-throughput sequencing in conjunction with viral genetic barcoding. *Nature Biotechnology*, 29(10), 928–933. <https://doi.org/10.1038/nbt.1977>

Lu, Y., Li, M., Gao, Z., Ma, H., Chong, Y., Hong, J., Wu, J., Wu, D., Xi, D., & Deng, W. (2025). Advances in whole genome sequencing: Methods, tools, and applications in population genomics. *International Journal of Molecular Sciences*, 26(1). <https://doi.org/10.3390/ijms26010372>

Ludwig, L. S., Lareau, C. A., Ulirsch, J. C., Christian, E., Muus, C., Li, L. H., Pelka, K., Ge, W., Oren, Y., Brack, A., Law, T., Rodman, C., Chen, J. H., Boland, G. M., Hacohen, N., Rozenblatt-Rosen, O., Aryee, M. J., Buenrostro, J. D., Regev, A., & Sankaran, V. G. (2019). Lineage tracing in humans enabled by mitochondrial mutations and single-cell genomics. *Cell*, 176(6), 1325–1339.e22. <https://doi.org/10.1016/j.cell.2019.01.022>

Lundberg, P., Karow, A., Nienhold, R., Looser, R., Hao-Shen, H., Nissen, I., Girsberger, S., Lehmann, T., Passweg, J., Stern, M., Beisel, C., Kralovics, R., & Skoda, R. C. (2014). Clonal evolution and clinical correlates of somatic mutations in myeloproliferative neoplasms. *Blood*, 123(14), 2220–2228. <https://doi.org/10.1182/blood-2013-11-537167>

Luque Paz, D., Kralovics, R., & Skoda, R. C. (2023). Genetic basis and molecular profiling in myeloproliferative neoplasms. *Blood*, 141(16), 1909–1921. <https://doi.org/10.1182/blood.2022017578>

Lynch, M. (2016). Mutation and human exceptionalism: Our future genetic load. *Genetics*, 202(3), 869–875. <https://doi.org/10.1534/genetics.115.180471>

Ma, X., Chow, J. M., Gri, G., Carra, G., Gerosa, F., Wolf, S. F., Dzialo, R., & Trinchieri, G. (1996). The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *The Journal of Experimental Medicine*, 183(1), 147–157. <https://doi.org/10.1084/jem.183.1.147>

Macaulay, I. C., Svensson, V., Labalette, C., Ferreira, L., Hamey, F., Voet, T., Teichmann, S. A., & Cvejic, A. (2016). Single-cell RNA-sequencing reveals a continuous spectrum of

differentiation in hematopoietic cells. *Cell Reports*, 14(4), 966–977. <https://doi.org/10.1016/j.celrep.2015.12.082>

Machado, H. E., Mitchell, E., Øbro, N. F., Kübler, K., Davies, M., Leongamornlert, D., Cull, A., Maura, F., Sanders, M. A., Cagan, A. T. J., McDonald, C., Belmonte, M., Shepherd, M. S., Vieira Braga, F. A., Osborne, R. J., Mahbubani, K., Martincorena, I., Laurenti, E., Green, A. R., ... Campbell, P. J. (2022). Diverse mutational landscapes in human lymphocytes. *Nature*, 608(7924), 724–732. <https://doi.org/10.1038/s41586-022-05072-7>

Maheden, K., Zhang, V. W., & Shakiba, N. (2022). The field of cell competition comes of age: Semantics and technological synergy. *Frontiers in Cell and Developmental Biology*, 10, 891569. <https://doi.org/10.3389/fcell.2022.891569>

Mamada, H., Sato, T., Ota, M., & Sasaki, H. (2015). Cell competition in mouse NIH3T3 embryonic fibroblasts is controlled by the activity of Tead family proteins and Myc. *Journal of Cell Science*, 128(4), 790–803. <https://doi.org/10.1242/jcs.163675>

Manders, F., van Boxtel, R., & Middelkamp, S. (2021). The dynamics of somatic Mutagenesis during life in humans. *Frontiers in Aging*, 2, 802407. <https://doi.org/10.3389/fragi.2021.802407>

Manshouri, T., Estrov, Z., Quintás-Cardama, A., Burger, J., Zhang, Y., Livun, A., Knez, L., Harris, D., Creighton, C. J., Kantarjian, H. M., & Verstovsek, S. (2011). Bone marrow stroma-secreted cytokines protect JAK2(V617F)-mutated cells from the effects of a JAK2 inhibitor. *Cancer Research*, 71(11), 3831–3840. <https://doi.org/10.1158/0008-5472.CAN-10-4002>

Martin, A. (2015). Ray Owen and the history of naturally acquired chimerism. *Chimerism*, 6(1–2), 2–7. <https://doi.org/10.1080/19381956.2016.1168561>

Martín, F. A., Herrera, S. C., & Morata, G. (2009). Cell competition, growth and size control in the *Drosophila* wing imaginal disc. *Development (Cambridge, England)*, 136(22), 3747–3756. <https://doi.org/10.1242/dev.038406>

Martin, H., Mali, R. S., Ma, P., Chatterjee, A., Ramdas, B., Sims, E., Munugalavadla, V., Ghosh, J., Mattingly, R. R., Visconte, V., Tiu, R. V., Vlaar, C. P., Dharmawardhane, S., & Kapur, R. (2013). Pak and Rac GTPases promote oncogenic KIT-induced neoplasms. *The Journal of Clinical Investigation*, 123(10), 4449–4463. <https://doi.org/10.1172/JCI67509>

Martin, J., Blackburn, D., & Wiley, E. O. (2010). Are node-based and stem-based clades equivalent? Insights from graph theory. *PLoS Currents*, 2, RRN1196. <https://doi.org/10.1371/currents.RRN1196>

Martincorena, I., & Campbell, P. J. (2015). Somatic mutation in cancer and normal cells. *Science (New York, N.Y.)*, 349(6255), 1483–1489. <https://doi.org/10.1126/science.aab4082>

Martincorena, I., Fowler, J. C., Wabik, A., Lawson, A. R. J., Abascal, F., Hall, M. W. J., Cagan, A., Murai, K., Mahbubani, K., Stratton, M. R., Fitzgerald, R. C., Handford, P. A., Campbell, P. J., Saeb-Parsy, K., & Jones, P. H. (2018). Somatic mutant clones colonize the human esophagus with age. *Science (New York, N.Y.)*, 362(6417), 911–917. <https://doi.org/10.1126/science.aau3879>

Martin-Rufino, J. D., & Sankaran, V. G. (2021). Deciphering transcriptional and functional heterogeneity in hematopoiesis with single-cell genomics. *Current Opinion in Hematology*, 28(4), 269–276. <https://doi.org/10.1097/MOH.0000000000000657>

Marty, C., Lacout, C., Martin, A., Hasan, S., Jacquot, S., Birling, M.-C., Vainchenker, W., & Villeval, J.-L. (2010). Myeloproliferative neoplasm induced by constitutive expression of JAK2V617F in knock-in mice. *Blood*, 116(5), 783–787. <https://doi.org/10.1182/blood-2009-12-257063>

Marusyk, A., Porter, C. C., Zaberezhnyy, V., & DeGregori, J. (2010). Irradiation selects for p53-deficient hematopoietic progenitors. *PLoS Biology*, 8(3), e1000324. <https://doi.org/10.1371/journal.pbio.1000324>

Mascarenhas, J., Gleitz, H. F. E., Chifotides, H. T., Harrison, C. N., Verstovsek, S., Vannucchi, A. M., Rampal, R. K., Kiladjian, J.-J., Vainchenker, W., Hoffman, R., Schneider, R. K., & List, A. F. (2023). Biological drivers of clinical phenotype in myelofibrosis. *Leukemia*, 37(2), 255–264. <https://doi.org/10.1038/s41375-022-01767-y>

Maslah, N., Benajiba, L., Giraudier, S., Kiladjian, J.-J., & Cassinat, B. (2023). Clonal architecture evolution in Myeloproliferative Neoplasms: from a driver mutation to a complex heterogeneous mutational and phenotypic landscape. *Leukemia*, 37(5), 957–963. <https://doi.org/10.1038/s41375-023-01886-0>

Mason, E. F., Kuo, F. C., Hasserjian, R. P., Seegmiller, A. C., & Pozdnyakova, O. (2018). A distinct immunophenotype identifies a subset of NPM1-mutated AML with TET2 or IDH1/2 mutations and improved outcome. *American Journal of Hematology*, 93(4), 504–510. <https://doi.org/10.1002/ajh.25018>

Matsuoka, S., Facchini, R., Luis, T. C., Carrelha, J., Woll, P. S., Mizukami, T., Wu, B., Boukarabila, H., Buono, M., Norfo, R., Arai, F., Suda, T., Mead, A. J., Nerlov, C., & Jacobsen, S. E. W. (2023). Loss of endothelial membrane KIT ligand affects systemic KIT ligand levels but not bone marrow hematopoietic stem cells. *Blood*, 142(19), 1622–1632. <https://doi.org/10.1182/blood.2022019018>

Maximow, A. A. (1924). Relation of blood cells to connective tissues and endothelium. *Physiological Reviews*, 4(4), 533–563. <https://doi.org/10.1152/physrev.1924.4.4.533>

Mazzitelli, J. A., Pulous, F. E., Smyth, L. C. D., Kaya, Z., Rustenhoven, J., Moskowitz, M. A., Kipnis, J., & Nahrendorf, M. (2023). Skull bone marrow channels as immune gateways to the central nervous system. *Nature Neuroscience*, 26(12), 2052–2062. <https://doi.org/10.1038/s41593-023-01487-1>

McClatchy, J., Strogantsev, R., Wolfe, E., Lin, H. Y., Mohammadhosseini, M., Davis, B. A., Eden, C., Goldman, D., Fleming, W. H., Conley, P., Wu, G., Cimmimo, L., Mohammed, H., & Agarwal, A. (2023). Clonal hematopoiesis related TET2 loss-of-function impedes IL1 β -mediated epigenetic reprogramming in hematopoietic stem and progenitor cells. *Nature Communications*, 14(1), 8102. <https://doi.org/10.1038/s41467-023-43697-y>

McKenna, A., Findlay, G. M., Gagnon, J. A., Horwitz, M. S., Schier, A. F., & Shendure, J. (2016). Whole-organism lineage tracing by combinatorial and cumulative genome editing. *Science (New York, N.Y.)*, 353(6298), aaf7907. <https://doi.org/10.1126/science.aaf7907>

McKinney-Freeman, S., & Goodell, M. A. (2004). Circulating hematopoietic stem cells do not efficiently home to bone marrow during homeostasis. *Experimental Hematology*, 32(9), 868–876. <https://doi.org/10.1016/j.exphem.2004.06.010>

McLennan, D. A. (2010). How to read a phylogenetic tree. *Evolution*, 3(4), 506–519. <https://doi.org/10.1007/s12052-010-0273-6>

McMullin, M. F., & Anderson, L. A. (2020). Aetiology of myeloproliferative neoplasms. *Cancers*, 12(7), 1810. <https://doi.org/10.3390/cancers12071810>

Meaker, G. A., & Wilkinson, A. C. (2024). Ex vivo hematopoietic stem cell expansion technologies: recent progress, applications, and open questions. *Experimental Hematology*, 130(104136), 104136. <https://doi.org/10.1016/j.exphem.2023.12.001>

Medinger, M., & Passweg, J. (2014). Angiogenesis in myeloproliferative neoplasms, new markers and future directions. *Memo*, 7(4), 206–210. <https://doi.org/10.1007/s12254-014-0142-z>

Medvinsky, A. L., & Dzierzak, E. A. (1998). Development of the definitive hematopoietic hierarchy in the mouse. *Developmental and Comparative Immunology*, 22(3), 289–301. [https://doi.org/10.1016/s0145-305x\(98\)00007-x](https://doi.org/10.1016/s0145-305x(98)00007-x)

Melo-Cardenas, J., Bezavada, L., Crawford, J. C., Gurbuxani, S., Cotton, A., Kang, G., Gossett, J., Marinaccio, C., Weinberg, R., Hoffman, R., Migliaccio, A. R., Zheng, Y., Derecka, M., Rinaldi, C. R., & Crispino, J. D. (2022). IL-13/IL-4 signaling contributes to fibrotic progression of the myeloproliferative neoplasms. *Blood*, 140(26), 2805–2817. <https://doi.org/10.1182/blood.2022017326>

Mendez Luque, L. F., Blackmon, A. L., Ramanathan, G., & Fleischman, A. G. (2019). Key role of inflammation in myeloproliferative neoplasms: Instigator of disease initiation, progression. And symptoms. *Current Hematologic Malignancy Reports*, 14(3), 145–153. <https://doi.org/10.1007/s11899-019-00508-w>

Méndez-Ferrer, S., Bonnet, D., Steensma, D. P., Hasserjian, R. P., Ghobrial, I. M., Gribben, J. G., Andreeff, M., & Krause, D. S. (2020). Bone marrow niches in haematological malignancies. *Nature Reviews. Cancer*, 20(5), 285–298. <https://doi.org/10.1038/s41568-020-0245-2>

Méndez-Ferrer, S., Lucas, D., Battista, M., & Frenette, P. S. (2008). Haematopoietic stem cell release is regulated by circadian oscillations. *Nature*, 452(7186), 442–447. <https://doi.org/10.1038/nature06685>

Mercher, T., Quivoron, C., Couronné, L., Bastard, C., Vainchenker, W., & Bernard, O. A. (2012). TET2, a tumor suppressor in hematological disorders. *Biochimica et Biophysica Acta*, 1825(2), 173–177. <https://doi.org/10.1016/j.bbcan.2011.12.002>

Merlo, L. M. F., Pepper, J. W., Reid, B. J., & Maley, C. C. (2006). Cancer as an evolutionary and ecological process. *Nature Reviews. Cancer*, 6(12), 924–935. <https://doi.org/10.1038/nrc2013>

Mesa, R., Miller, C. B., Thyne, M., Mangan, J., Goldberger, S., Fazal, S., Ma, X., Wilson, W., Paranagama, D. C., Dubinski, D. G., Boyle, J., & Mascarenhas, J. O. (2016). Myeloproliferative neoplasms (MPNs) have a significant impact on patients' overall health and productivity: the MPN Landmark survey. *BMC Cancer*, 16(1), 167. <https://doi.org/10.1186/s12885-016-2208-2>

Mesfin, F. M., Manohar, K., Hunter, C. E., Shelley, W. C., Brokaw, J. P., Liu, J., Ma, M., & Markel, T. A. (2023). Stem cell derived therapies to preserve and repair the developing intestine. *Seminars in Perinatology*, 47(3), 151727. <https://doi.org/10.1016/j.semperi.2023.151727>

Metcalf, D. (2010). The colony-stimulating factors and cancer. *Nature Reviews. Cancer*, 10(6), 425–434. <https://doi.org/10.1038/nrc2843>

Miao, R., Chun, H., Feng, X., Gomes, A. C., Choi, J., & Pereira, J. P. (2022). Competition between hematopoietic stem and progenitor cells controls hematopoietic stem cell compartment size. *Nature Communications*, 13(1), 4611. <https://doi.org/10.1038/s41467-022-32228-w>

Mikkola, H. K. A., Gekas, C., Orkin, S. H., & Dieterlen-Lievre, F. (2005). Placenta as a site for hematopoietic stem cell development. *Experimental Hematology*, 33(9), 1048–1054. <https://doi.org/10.1016/j.exphem.2005.06.011>

Mikkola, H. K. A., & Orkin, S. H. (2006). The journey of developing hematopoietic stem cells. *Development (Cambridge, England)*, 133(19), 3733–3744. <https://doi.org/10.1242/dev.02568>

Miller, C. L., & Eaves, C. J. (1997). Expansion in vitro of adult murine hematopoietic stem cells with transplantable lympho-myeloid reconstituting ability. *Proceedings of the National Academy of Sciences of the United States of America*, 94(25), 13648–13653. <https://doi.org/10.1073/pnas.94.25.13648>

Mills, J. C., Stanger, B. Z., & Sander, M. (2019). Nomenclature for cellular plasticity: are the terms as plastic as the cells themselves? *The EMBO Journal*, 38(19), e103148. <https://doi.org/10.15252/emboj.2019103148>

Milosevic Feenstra, J. D., Nivarthi, H., Gisslinger, H., Leroy, E., Rumi, E., Chachoua, I., Bagienski, K., Kubesova, B., Pietra, D., Gisslinger, B., Milanesi, C., Jäger, R., Chen, D., Berg, T., Schalling, M., Schuster, M., Bock, C., Constantinescu, S. N., Cazzola, M., & Kralovics, R. (2016). Whole-exome sequencing identifies novel MPL and JAK2 mutations in triple-negative myeloproliferative neoplasms. *Blood*, 127(3), 325–332. <https://doi.org/10.1182/blood-2015-07-661835>

Moignard, V., Woodhouse, S., Haghverdi, L., Lilly, A. J., Tanaka, Y., Wilkinson, A. C., Buettner, F., Macaulay, I. C., Jawaid, W., Diamanti, E., Nishikawa, S.-I., Piterman, N., Kouskoff, V., Theis, F. J., Fisher, J., & Göttgens, B. (2015). Decoding the regulatory network of early blood development from single-cell gene expression measurements. *Nature Biotechnology*, 33(3), 269–276. <https://doi.org/10.1038/nbt.3154>

Mölder, F., Jablonski, K. P., Letcher, B., Hall, M. B., Tomkins-Tinch, C. H., Sochat, V., Forster, J., Lee, S., Twardziok, S. O., Kanitz, A., Wilm, A., Holtgrewe, M., Rahmann, S., Nahnsen, S., & Köster, J. (2021). Sustainable data analysis with Snakemake. *F1000Research*, 10(33), 33. <https://doi.org/10.12688/f1000research.29032.2>

Mondet, J., Hussein, K., & Mossuz, P. (2015). Circulating cytokine levels as markers of inflammation in Philadelphia negative myeloproliferative neoplasms: Diagnostic and prognostic interest. *Mediators of Inflammation*, 2015(1), 670580. <https://doi.org/10.1155/2015/670580>

Monga, I., Kaur, K., & Dhanda, S. K. (2022). Revisiting hematopoiesis: applications of the bulk and single-cell transcriptomics dissecting transcriptional heterogeneity in hematopoietic stem cells. *Briefings in Functional Genomics*, 21(3), 159–176. <https://doi.org/10.1093/bfpg/elac002>

Moon, B., Yang, S.-J., Park, S. M., Lee, S.-H., Song, K. S., Jeong, E.-J., Park, M., Kim, J.-S., Yeom, Y. I., & Kim, J.-A. (2020). LAD1 expression is associated with the metastatic potential of colorectal cancer cells. *BMC Cancer*, 20(1), 1180. <https://doi.org/10.1186/s12885-020-07660-0>

Moore, L., Cagan, A., Coorens, T. H. H., Neville, M. D. C., Sanghvi, R., Sanders, M. A., Oliver, T. R. W., Leongamornlert, D., Ellis, P., Noorani, A., Mitchell, T. J., Butler, T. M., Hooks, Y., Warren, A. Y., Jorgensen, M., Dawson, K. J., Menzies, A., O'Neill, L., Latimer, C., ... Rahbari, R. (2021). The mutational landscape of human somatic and germline cells. *Nature*, 597(7876), 381–386. <https://doi.org/10.1038/s41586-021-03822-7>

Moran-Crusio, K., Reavie, L., Shih, A., Abdel-Wahab, O., Ndiaye-Lobry, D., Lobry, C., Figueroa, M. E., Vasanthakumar, A., Patel, J., Zhao, X., Perna, F., Pandey, S., Madzo, J., Song, C., Dai, Q., He, C., Ibrahim, S., Beran, M., Zavadil, J., ... Levine, R. L. (2011). Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. *Cancer Cell*, 20(1), 11–24. <https://doi.org/10.1016/j.ccr.2011.06.001>

Morata, G., & Ripoll, P. (1975). Minutes: mutants of drosophila autonomously affecting cell division rate. *Developmental Biology*, 42(2), 211–221. [https://doi.org/10.1016/0012-1606\(75\)90330-9](https://doi.org/10.1016/0012-1606(75)90330-9)

Morata, Ginés. (2021). Cell competition: A historical perspective. *Developmental Biology*, 476, 33–40. <https://doi.org/10.1016/j.ydbio.2021.02.012>

Morcos, M. N. F., Schoedel, K. B., Hoppe, A., Behrendt, R., Basak, O., Clevers, H. C., Roers, A., & Gerbaulet, A. (2017). SCA-1 expression level identifies quiescent hematopoietic stem and progenitor cells. *Stem Cell Reports*, 8(6), 1472–1478. <https://doi.org/10.1016/j.stemcr.2017.04.012>

Moreno, E., & Basler, K. (2004). dMyc transforms cells into super-competitors. *Cell*, 117(1), 117–129. [https://doi.org/10.1016/s0092-8674\(04\)00262-4](https://doi.org/10.1016/s0092-8674(04)00262-4)

Moreno, E., Basler, K., & Morata, G. (2002). Cells compete for decapentaplegic survival factor to prevent apoptosis in *Drosophila* wing development. *Nature*, 416(6882), 755–759. <https://doi.org/10.1038/416755a>

Morita, Y., Ema, H., & Nakauchi, H. (2010). Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *The Journal of Experimental Medicine*, 207(6), 1173–1182. <https://doi.org/10.1084/jem.20091318>

Morrison, S. J., & Weissman, I. L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity*, 1(8), 661–673. [https://doi.org/10.1016/1074-7613\(94\)90037-x](https://doi.org/10.1016/1074-7613(94)90037-x)

Mullally, A., Lane, S. W., Ball, B., Megerdichian, C., Okabe, R., Al-Shahrour, F., Paktinat, M., Haydu, J. E., Housman, E., Lord, A. M., Wernig, G., Kharas, M. G., Mercher, T., Kutok, J. L., Gilliland, D. G., & Ebert, B. L. (2010). Physiological Jak2V617F expression causes a lethal myeloproliferative neoplasm with differential effects on hematopoietic stem and progenitor cells. *Cancer Cell*, 17(6), 584–596. <https://doi.org/10.1016/j.ccr.2010.05.015>

Mullally, A., Lane, S. W., Brumme, K., & Ebert, B. L. (2012). Myeloproliferative neoplasm animal models. *Hematology/Oncology Clinics of North America*, 26(5), 1065–1081. <https://doi.org/10.1016/j.hoc.2012.07.007>

Müller-Sieburg, C. E., Cho, R. H., Thoman, M., Adkins, B., & Sieburg, H. B. (2002). Deterministic regulation of hematopoietic stem cell self-renewal and differentiation. *Blood*, 100(4), 1302–1309. https://doi.org/10.1182/blood.v100.4.1302.h81602001302_1302_1309

Muller-Sieburg, C. E., Whitlock, C. A., & Weissman, I. L. (1986). Isolation of two early B lymphocyte progenitors from mouse marrow: a committed pre-pre-B cell and a clonogenic Thy-1-lo hematopoietic stem cell. *Cell*, 44(4), 653–662. [https://doi.org/10.1016/0092-8674\(86\)90274-6](https://doi.org/10.1016/0092-8674(86)90274-6)

Murata, T., Noguchi, P. D., & Puri, R. K. (1996). IL-13 induces phosphorylation and activation of JAK2 Janus kinase in human colon carcinoma cell lines: similarities between IL-4 and IL-13 signaling. *The Journal of Immunology*, 156(8), 2972–2978. <https://doi.org/10.4049/jimmunol.156.8.2972>

Naik, S. H., Perié, L., Swart, E., Gerlach, C., van Rooij, N., de Boer, R. J., & Schumacher, T. N. (2013). Diverse and heritable lineage imprinting of early haematopoietic progenitors. *Nature*, 496(7444), 229–232. <https://doi.org/10.1038/nature12013>

Naji, N. S., Sathish, M., & Karantanos, T. (2024). Inflammation and related signaling pathways in acute myeloid leukemia. *Cancers*, 16(23). <https://doi.org/10.3390/cancers16233974>

Nakamuta, A., Yoshido, K., & Naoki, H. (2022). Stem cell homeostasis regulated by hierarchy and neutral competition. *Communications Biology*, 5(1), 1268. <https://doi.org/10.1038/s42003-022-04218-7>

Nakao, Y., Nakagawa, S., Yamashita, Y.-I., Umezaki, N., Okamoto, Y., Ogata, Y., Yasuda-Yoshihara, N., Itoyama, R., Yusa, T., Yamashita, K., Miyata, T., Okabe, H., Hayashi, H., Imai, K., & Baba, H. (2021). High ARHGEF2 (GEF-H1) expression is associated with poor prognosis via cell cycle regulation in patients with pancreatic cancer. *Annals of Surgical Oncology*, 28(8), 4733–4743. <https://doi.org/10.1245/s10434-020-09383-9>

Nakazawa, H., English, D., Randell, P. L., Nakazawa, K., Martel, N., Armstrong, B. K., & Yamasaki, H. (1994). UV and skin cancer: specific p53 gene mutation in normal skin as a biologically relevant exposure measurement. *Proceedings of the National Academy of Sciences of the United States of America*, 91(1), 360–364. <https://doi.org/10.1073/pnas.91.1.360>

Nangalia, J., Massie, C. E., Baxter, E. J., Nice, F. L., Gundem, G., Wedge, D. C., Avezov, E., Li, J., Kollmann, K., Kent, D. G., Aziz, A., Godfrey, A. L., Hinton, J., Martincorena, I., Van Loo, P., Jones, A. V., Guglielmelli, P., Tarpey, P., Harding, H. P., ... Green, A. R. (2013). Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. *The New England Journal of Medicine*, 369(25), 2391–2405. <https://doi.org/10.1056/NEJMoa1312542>

Nathan, C. F. (1987). Secretory products of macrophages. *The Journal of Clinical Investigation*, 79(2), 319–326. <https://doi.org/10.1172/JCI112815>

Nayak, R. C., Chang, K.-H., Vaitinadin, N.-S., & Cancelas, J. A. (2013). Rho GTPases control specific cytoskeleton-dependent functions of hematopoietic stem cells. *Immunological Reviews*, 256(1), 255–268. <https://doi.org/10.1111/imr.12119>

Nestorowa, S., Hamey, F. K., Pijuan Sala, B., Diamanti, E., Shepherd, M., Laurenti, E., Wilson, N. K., Kent, D. G., & Göttgens, B. (2016). A single-cell resolution map of mouse hematopoietic stem and progenitor cell differentiation. *Blood*, 128(8), e20-31. <https://doi.org/10.1182/blood-2016-05-716480>

Neumann, E. (1868). Über die Bedeutung des Knochenmarks für die Blutbildung. *Zentralblatt Für Die Medizinischen Wissenschaften*, 689(6).

Ng, E. S., Sarila, G., Li, J. Y., Edirisinghe, H. S., Saxena, R., Sun, S., Bruveris, F. F., Labonne, T., Sleebs, N., Maytum, A., Yow, R. Y., Inguanti, C., Motazedian, A., Calvanese, V., Capellera-Garcia, S., Ma, F., Nim, H. T., Ramialison, M., Bonifer, C., ... Elefanty, A. G. (2024). Long-term engrafting multilineage hematopoietic cells differentiated from human induced pluripotent stem cells. *Nature Biotechnology*, 1–14. <https://doi.org/10.1038/s41587-024-02360-7>

Ng, P. C., & Kirkness, E. F. (2010). Whole genome sequencing. *Methods in Molecular Biology* (Clifton, N.J.), 628, 215–226. https://doi.org/10.1007/978-1-60327-367-1_12

Ngo, S., Papazoglou, D., Huerga Encabo, H., & Bonnet, D. (2024). Exploring the intricate cross-talk between clonal expansion and the bone marrow niche. *Frontiers in Hematology*, 3. <https://doi.org/10.3389/frhem.2024.1334807>

Nguyen, D. H., Ball, E. D., & Varki, A. (2006). Myeloid precursors and acute myeloid leukemia cells express multiple CD33-related Siglecs. *Experimental Hematology*, 34(6), 728–735. <https://doi.org/10.1016/j.exphem.2006.03.003>

Nichols, J., Lima, A., & Rodríguez, T. A. (2022). Cell competition and the regulative nature of early mammalian development. *Cell Stem Cell*, 29(7), 1018–1030. <https://doi.org/10.1016/j.stem.2022.06.003>

Niizuma, K., Morikawa, S., Gars, E., Xiang, J., Matsubara-Takahashi, T., Saito, R., Takematsu, E., Wang, Y., Xu, H., Wakimoto, A., Tan, T. K., Kubota, Y., Chan, C. K. F., Weissman, I. L., Nakagawa, T., Wilkinson, A. C., Nakauchi, H., & Yamamoto, R. (2025). Elevated hematopoietic stem cell frequency in mouse alveolar bone marrow. *Stem Cell Reports*, 20(1), 102374. <https://doi.org/10.1016/j.stemcr.2024.11.004>

Nik-Zainal, S., Alexandrov, L. B., Wedge, D. C., Van Loo, P., Greenman, C. D., Raine, K., Jones, D., Hinton, J., Marshall, J., Stebbings, L. A., Menzies, A., Martin, S., Leung, K., Chen, L., Leroy, C., Ramakrishna, M., Rance, R., Lau, K. W., Mudie, L. J., ... Breast Cancer Working Group of the International Cancer Genome Consortium. (2012). Mutational processes molding the genomes of 21 breast cancers. *Cell*, 149(5), 979–993. <https://doi.org/10.1016/j.cell.2012.04.024>

Nimer, S. D., & Uchida, H. (1995). Regulation of granulocyte-macrophage colony-stimulating factor and interleukin 3 expression. *Stem Cells* (Dayton, Ohio), 13(4), 324–335. <https://doi.org/10.1002/stem.5530130402>

Nombela-Arrieta, C., & Manz, M. G. (2017). Quantification and three-dimensional microanatomical organization of the bone marrow. *Blood Advances*, 1(6), 407–416. <https://doi.org/10.1182/bloodadvances.2016003194>

North, T. E., Goessling, W., Peeters, M., Li, P., Ceol, C., Lord, A. M., Weber, G. J., Harris, J., Cutting, C. C., Huang, P., Dzierzak, E., & Zon, L. I. (2009). Hematopoietic stem cell development is dependent on blood flow. *Cell*, 137(4), 736–748. <https://doi.org/10.1016/j.cell.2009.04.023>

Notta, F., Zandi, S., Takayama, N., Dobson, S., Gan, O. I., Wilson, G., Kaufmann, K. B., McLeod, J., Laurenti, E., Dunant, C. F., McPherson, J. D., Stein, L. D., Dror, Y., & Dick, J. E. (2016). Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science* (New York, N.Y.), 351(6269), aab2116. <https://doi.org/10.1126/science.aab2116>

Novick, L. R., Stull, A. T., & Catley, K. M. (2012). Reading phylogenetic trees: The effects of tree orientation and text processing on comprehension. *Bioscience*, 62(8), 757–764. <https://doi.org/10.1525/bio.2012.62.8.8>

Nowell, P. C. (1976). The clonal evolution of tumor cell populations. *Science (New York, N.Y.)*, 194(4260), 23–28. <https://doi.org/10.1126/science.959840>

Nowell, P. C., & Hungerford, D. A. (1961). Chromosome studies in human leukemia. II. Chronic granulocytic leukemia. *Journal of the National Cancer Institute*, 27, 1013–1035. <https://doi.org/10.1093/jnci/27.5.1013>

Nowell, Peter C. (2007). Discovery of the Philadelphia chromosome: a personal perspective. *The Journal of Clinical Investigation*, 117(8), 2033–2035. <https://doi.org/10.1172/JCI31771>

Nussinov, R., Tsai, C.-J., & Jang, H. (2021). Anticancer drug resistance: An update and perspective. *Drug Resistance Updates: Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy*, 59(100796), 100796. <https://doi.org/10.1016/j.drug.2021.100796>

Øbro, N. F., Grinfeld, J., Belmonte, M., Irvine, M., Shepherd, M. S., Rao, T. N., Karow, A., Riedel, L. M., Harris, O. B., Baxter, E. J., Nangalia, J., Godfrey, A., Harrison, C. N., Li, J., Skoda, R. C., Campbell, P. J., Green, A. R., & Kent, D. G. (2020). Longitudinal cytokine profiling identifies GRO- α and EGF as potential biomarkers of disease progression in essential thrombocythemia. *HemaSphere*, 4(3), e371. <https://doi.org/10.1097/HS9.0000000000000371>

Ochi, K., Morita, M., Wilkinson, A. C., Iwama, A., & Yamazaki, S. (2021). Non-conditioned bone marrow chimeric mouse generation using culture-based enrichment of hematopoietic stem and progenitor cells. *Nature Communications*, 12(1), 3568. <https://doi.org/10.1038/s41467-021-23763-z>

O'Connell, K. E., Mikkola, A. M., Stepanek, A. M., Vernet, A., Hall, C. D., Sun, C. C., Yildirim, E., Staropoli, J. F., Lee, J. T., & Brown, D. E. (2015). Practical murine hematopathology: a comparative review and implications for research. *Comparative Medicine*, 65(2), 96–113. <https://www.ncbi.nlm.nih.gov/pubmed/25926395>

O'Donoghue, K., & Fisk, N. M. (2004). Fetal stem cells. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 18(6), 853–875. <https://doi.org/10.1016/j.bpobgyn.2004.06.010>

Oedekoven, C. A., Belmonte, M., Bode, D., Hamey, F. K., Shepherd, M. S., Che, J. L. C., Boyd, G., McDonald, C., Belluschi, S., Diamanti, E., Bastos, H. P., Bridge, K. S., Göttgens, B., Laurenti, E., & Kent, D. G. (2021). Hematopoietic stem cells retain functional potential and molecular identity in hibernation cultures. *Stem Cell Reports*, 16(6), 1614–1628. <https://doi.org/10.1016/j.stemcr.2021.04.002>

Oguro, H., Ding, L., & Morrison, S. J. (2013). SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. *Cell Stem Cell*, 13(1), 102–116. <https://doi.org/10.1016/j.stem.2013.05.014>

Ortmann, C. A., Kent, D. G., Nangalia, J., Silber, Y., Wedge, D. C., Grinfeld, J., Baxter, E. J., Massie, C. E., Papaemmanuil, E., Menon, S., Godfrey, A. L., Dimitropoulou, D., Guglielmelli, P., Bellosillo, B., Besses, C., Döhner, K., Harrison, C. N., Vassiliou, G. S., Vannucchi, A., ... Green, A. R. (2015). Effect of mutation order on myeloproliferative neoplasms. *The New England Journal of Medicine*, 372(7), 601–612. <https://doi.org/10.1056/NEJMoa1412098>

- Osawa, M., Hanada, K., Hamada, H., & Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science (New York, N.Y.)*, 273(5272), 242–245. <https://doi.org/10.1126/science.273.5272.242>
- Osborne, G. W. (2011). Recent advances in flow cytometric cell sorting. *Methods in Cell Biology*, 102, 533–556. <https://doi.org/10.1016/B978-0-12-374912-3.00021-3>
- Osorio, F. G., Rosendahl Huber, A., Oka, R., Verheul, M., Patel, S. H., Hasaart, K., de la Fonteijne, L., Varela, I., Camargo, F. D., & van Boxtel, R. (2018). Somatic mutations reveal lineage relationships and age-related Mutagenesis in human hematopoiesis. *Cell Reports*, 25(9), 2308–2316.e4. <https://doi.org/10.1016/j.celrep.2018.11.014>
- Ostojic, A., Vrhovac, R., & Verstovsek, S. (2011). Ruxolitinib: a new JAK1/2 inhibitor that offers promising options for treatment of myelofibrosis. *Future Oncology (London, England)*, 7(9), 1035–1043. <https://doi.org/10.2217/fon.11.81>
- Ostrander, E. L., Kramer, A. C., Mallaney, C., Celik, H., Koh, W. K., Fairchild, J., Haussler, E., Zhang, C. R. C., & Challen, G. A. (2020). Divergent effects of Dnmt3a and Tet2 mutations on hematopoietic progenitor cell fitness. *Stem Cell Reports*, 14(4), 551–560. <https://doi.org/10.1016/j.stemcr.2020.02.011>
- Ostroverkhova, D., Przytycka, T. M., & Panchenko, A. R. (2023). Cancer driver mutations: predictions and reality. *Trends in Molecular Medicine*, 29(7), 554–566. <https://doi.org/10.1016/j.molmed.2023.03.007>
- Ott, S. M. (2018). Cortical or trabecular bone: What's the difference? *American Journal of Nephrology*, 47(6), 373–375. <https://doi.org/10.1159/000489672>
- Ou, L., Andrade, A., Alberto, R. A., Bakker, A., & Bechger, T. (2020). Identifying qualitative between-subject and within-subject variability: A method for clustering regime-switching dynamics. *Frontiers in Psychology*, 11, 1136. <https://doi.org/10.3389/fpsyg.2020.01136>
- P. C. Nowell & D. A. Hungerford. (1960). A minute chromosome in human chronic granulocytic leukemia. *Science*, 142(1497).
- Padda, J., Khalid, K., Yadav, J., Almanie, A. H., Mehta, K. A., Al Hennawi, H., Boddeti, N. L., Campos, V. Y. M., & Jean-Charles, G. (2021). JAK2 and TET2 mutation in polycythemia Vera. *Cureus*, 13(9), e17854. <https://doi.org/10.7759/cureus.17854>
- Page, R. D. M., & Holmes, E. C. (1991). *Molecular evolution: A phylogenetic approach*. Wiley-Blackwell.
- Palmer-Crocker, R. L., Hughes, C. C., & Pober, J. S. (8 1996). IL-4 and IL-13 activate the JAK2 tyrosine kinase and Stat6 in cultured human vascular endothelial cells through a common pathway that does not involve the gamma c chain. *The Journal of Clinical Investigation*, 98(3), 604–609. <https://doi.org/10.1172/JCI118829>
- Papaemmanuil, E., Gerstung, M., Malcovati, L., Tauro, S., Gundem, G., Van Loo, P., Yoon, C. J., Ellis, P., Wedge, D. C., Pellagatti, A., Shlien, A., Groves, M. J., Forbes, S. A., Raine, K., Hinton, J., Mudie, L. J., McLaren, S., Hardy, C., Latimer, C., ... Chronic Myeloid Disorders Working Group of the International Cancer Genome Consortium. (2013). Clinical and biological implications of driver mutations in myelodysplastic syndromes. *Blood*, 122(22), 3616–3627; quiz 3699. <https://doi.org/10.1182/blood-2013-08-518886>

Pardanani, A. D., Levine, R. L., Lasho, T., Pikman, Y., Mesa, R. A., Wadleigh, M., Steensma, D. P., Elliott, M. A., Wolanskyj, A. P., Hogan, W. J., McClure, R. F., Litzow, M. R., Gilliland, D. G., & Tefferi, A. (2006). MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood*, 108(10), 3472–3476. <https://doi.org/10.1182/blood-2006-04-018879>

Patterson, S. D., & Copland, M. (2023). The bone marrow immune microenvironment in CML: Treatment responses, treatment-free remission, and therapeutic vulnerabilities. *Current Hematologic Malignancy Reports*, 18(2), 19–32. <https://doi.org/10.1007/s11899-023-00688-6>

Paul, F., Arkin, Y., Giladi, A., Jaitin, D. A., Kenigsberg, E., Keren-Shaul, H., Winter, D., Lara-Astiaso, D., Gury, M., Weiner, A., David, E., Cohen, N., Lauridsen, F. K. B., Haas, S., Schlitzer, A., Mildner, A., Ginhoux, F., Jung, S., Trumpp, A., ... Amit, I. (2015). Transcriptional heterogeneity and lineage commitment in myeloid progenitors. *Cell*, 163(7), 1663–1677. <https://doi.org/10.1016/j.cell.2015.11.013>

Pei, W., Feyerabend, T. B., Rössler, J., Wang, X., Postrach, D., Busch, K., Rode, I., Klapproth, K., Dietlein, N., Quedenau, C., Chen, W., Sauer, S., Wolf, S., Höfer, T., & Rodewald, H.-R. (2017). Polylox barcoding reveals haematopoietic stem cell fates realized in vivo. *Nature*, 548(7668), 456–460. <https://doi.org/10.1038/nature23653>

Pei, W., Shang, F., Wang, X., Fanti, A.-K., Greco, A., Busch, K., Klapproth, K., Zhang, Q., Quedenau, C., Sauer, S., Feyerabend, T. B., Höfer, T., & Rodewald, H.-R. (2020). Resolving fates and single-cell transcriptomes of hematopoietic stem cell clones by PolyloxExpress barcoding. *Cell Stem Cell*, 27(3), 383-395.e8. <https://doi.org/10.1016/j.stem.2020.07.018>

Peña, O. A., & Martin, P. (2024). Cellular and molecular mechanisms of skin wound healing. *Nature Reviews. Molecular Cell Biology*, 25(8), 599–616. <https://doi.org/10.1038/s41580-024-00715-1>

Pereira, A. L., Galli, S., & Nombela-Arrieta, C. (2024). Bone marrow niches for hematopoietic stem cells. *HemaSphere*, 8(8), e133. <https://doi.org/10.1002/hem3.133>

Perlman, R. L. (2016). Mouse models of human disease: An evolutionary perspective. *Evolution, Medicine, and Public Health*, 2016(1), 170–176. <https://doi.org/10.1093/emph/eow014>

Perry, J. M., & Li, L. (2010). Functional assays for hematopoietic stem cell self-renewal. *Methods in Molecular Biology (Clifton, N.J.)*, 636, 45–54. https://doi.org/10.1007/978-1-60761-691-7_3

Petersen, B. E., Bowen, W. C., Patrene, K. D., Mars, W. M., Sullivan, A. K., Murase, N., Boggs, S. S., Greenberger, J. S., & Goff, J. P. (1999). Bone marrow as a potential source of hepatic oval cells. *Science (New York, N.Y.)*, 284(5417), 1168–1170. <https://doi.org/10.1126/science.284.5417.1168>

Peterson, J. R., Lebensohn, A. M., Pelish, H. E., & Kirschner, M. W. (2006). Biochemical suppression of small-molecule inhibitors: a strategy to identify inhibitor targets and signaling pathway components. *Chemistry & Biology*, 13(4), 443–452. <https://doi.org/10.1016/j.chembiol.2006.02.009>

Petzer, A. L., Eaves, C. J., Barnett, M. J., & Eaves, A. C. (1997). Selective expansion of primitive normal hematopoietic cells in cytokine-supplemented cultures of purified cells from patients with chronic myeloid leukemia. *Blood*, 90(1), 64–69. <https://doi.org/10.1182/blood.v90.1.64>

- Petzer, A. L., Eaves, C. J., Lansdorp, P. M., Ponchio, L., Barnett, M. J., & Eaves, A. C. (1996). Characterization of primitive subpopulations of normal and leukemic cells present in the blood of patients with newly diagnosed as well as established chronic myeloid leukemia. *Blood*, 88(6), 2162–2171. <https://doi.org/10.1182/blood.v88.6.2162.bloodjournal8862162>
- Petzer, A. L., Hogge, D. E., Landsdorp, P. M., Reid, D. S., & Eaves, C. J. (1996). Self-renewal of primitive human hematopoietic cells (long-term-culture-initiating cells) in vitro and their expansion in defined medium. *Proceedings of the National Academy of Sciences of the United States of America*, 93(4), 1470–1474. <https://doi.org/10.1073/pnas.93.4.1470>
- Pharoah, D. S., Varsani, H., Tatham, R. W., Newton, K. R., de Jager, W., Prakken, B. J., Klein, N., & Wedderburn, L. R. (2006). Expression of the inflammatory chemokines CCL5, CCL3 and CXCL10 in juvenile idiopathic arthritis, and demonstration of CCL5 production by an atypical subset of CD8+ T cells. *Arthritis Research & Therapy*, 8(2), R50. <https://doi.org/10.1186/ar1913>
- Piccaluga, P. P., Agostinelli, C., Righi, S., Zinzani, P. L., & Pileri, S. A. (2007). Expression of CD52 in peripheral T-cell lymphoma. *Haematologica*, 92(4), 566–567. <https://doi.org/10.3324/haematol.10767>
- Picelli, S., Björklund, Å. K., Faridani, O. R., Sagasser, S., Winberg, G., & Sandberg, R. (2013). Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nature Methods*, 10(11), 1096–1098. <https://doi.org/10.1038/nmeth.2639>
- Pietrzyk, M. E., Priestley, G. V., & Wolf, N. S. (1985). Normal cycling patterns of hematopoietic stem cell subpopulations: an assay using long-term in vivo BrdU infusion. *Blood*, 66(6), 1460–1462. <https://doi.org/10.1182/blood.v66.6.1460.1460>
- Pikman, Y., Lee, B. H., Mercher, T., McDowell, E., Ebert, B. L., Gozo, M., Cuker, A., Wernig, G., Moore, S., Galinsky, I., DeAngelo, D. J., Clark, J. J., Lee, S. J., Golub, T. R., Wadleigh, M., Gilliland, D. G., & Levine, R. L. (2006). MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia. *PLoS Medicine*, 3(7), e270. <https://doi.org/10.1371/journal.pmed.0030270>
- Pillai, A. A., Fazal, S., Mukkamalla, S. K. R., & Babiker, H. M. (2025). Polycythemia. In *StatPearls*. StatPearls Publishing. <https://www.ncbi.nlm.nih.gov/books/NBK526081/>
- Pivovarova, O., Höhn, A., Grune, T., Pfeiffer, A. F. H., & Rudovich, N. (2016). Insulin-degrading enzyme: new therapeutic target for diabetes and Alzheimer's disease? *Annals of Medicine*, 48(8), 614–624. <https://doi.org/10.1080/07853890.2016.1197416>
- Pluznik, D. H., & Sachs, L. (1965). The cloning of normal 'mast' cells in tissue culture. *Journal of Cellular Physiology*, 66(3), 319–324. <https://doi.org/10.1002/jcp.1030660309>
- Pluznik, D. H., & Sachs, L. (1966). The induction of clones of normal mast cells by a substance from conditioned medium. *Experimental Cell Research*, 43(3), 553–563. [https://doi.org/10.1016/0014-4827\(66\)90026-7](https://doi.org/10.1016/0014-4827(66)90026-7)
- Podani, J. (2013). Tree thinking, time and topology: comments on the interpretation of tree diagrams in evolutionary/phylogenetic systematics. *Cladistics: The International Journal of the Willi Hennig Society*, 29(3), 315–327. <https://doi.org/10.1111/j.1096-0031.2012.00423.x>
- Pontikoglou, C. G., Matheakakis, A., & Papadaki, H. A. (2023). The mesenchymal compartment in myelodysplastic syndrome: Its role in the pathogenesis of the disorder and its

therapeutic targeting. *Frontiers in Oncology*, 13, 1102495. <https://doi.org/10.3389/fonc.2023.1102495>

Posfai, E., Schell, J. P., Janiszewski, A., Rovic, I., Murray, A., Bradshaw, B., Yamakawa, T., Pardon, T., El Bakkali, M., Talon, I., De Geest, N., Kumar, P., To, S. K., Petropoulos, S., Jurisicova, A., Pasque, V., Lanner, F., & Rossant, J. (2021). Evaluating totipotency using criteria of increasing stringency. *Nature Cell Biology*, 23(1), 49–60. <https://doi.org/10.1038/s41556-020-00609-2>

Prakash, S., Hoffman, R., Barouk, S., Wang, Y. L., Knowles, D. M., & Orazi, A. (2012). Splenic extramedullary hematopoietic proliferation in Philadelphia chromosome-negative myeloproliferative neoplasms: heterogeneous morphology and cytological composition. *Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc*, 25(6), 815–827. <https://doi.org/10.1038/modpathol.2012.33>

Prančlová, V., Hönig, V., Zemanová, M., Růžek, D., & Palus, M. (2024). Robust CXCL10/IP-10 and CCL5/RANTES production induced by tick-borne encephalitis virus in human brain pericytes despite weak infection. *International Journal of Molecular Sciences*, 25(14), 7892. <https://doi.org/10.3390/ijms25147892>

Pronk, E., & Raaijmakers, M. H. G. P. (2019). The mesenchymal niche in MDS. *Blood*, 133(10), 1031–1038. <https://doi.org/10.1182/blood-2018-10-844639>

Proserpio, V., & Lönnberg, T. (2016). Single-cell technologies are revolutionizing the approach to rare cells. *Immunology and Cell Biology*, 94(3), 225–229. <https://doi.org/10.1038/icb.2015.106>

Purton, L. E., & Scadden, D. T. (2007). Limiting factors in murine hematopoietic stem cell assays. *Cell Stem Cell*, 1(3), 263–270. <https://doi.org/10.1016/j.stem.2007.08.016>

Putcha, G. V., & Johnson, E. M., Jr. (2004). Men are but worms: neuronal cell death in *C. elegans* and vertebrates. *Cell Death and Differentiation*, 11(1), 38–48. <https://doi.org/10.1038/sj.cdd.4401352>

Qiao, W., & Zandstra, P. W. (2015). Bringing blood stem cell phenotype, genotype, and function closer together. *Cell Stem Cell*, 16(6), 574–575. <https://doi.org/10.1016/j.stem.2015.05.001>

Qin, X., & Tape, C. J. (2024). Functional analysis of cell plasticity using single-cell technologies. *Trends in Cell Biology*, 34(10), 854–864. <https://doi.org/10.1016/j.tcb.2024.01.006>

Qiu, C., Ge, Z., Cui, W., Yu, L., & Li, J. (2020). Human amniotic epithelial stem cells: A promising seed cell for clinical applications. *International Journal of Molecular Sciences*, 21(20), 7730. <https://doi.org/10.3390/ijms21207730>

Quijada-Álamo, M., Pérez-Carretero, C., Hernández-Sánchez, M., Rodríguez-Vicente, A.-E., Herrero, A.-B., Hernández-Sánchez, J.-M., Martín-Izquierdo, M., Santos-Mínguez, S., Del Rey, M., González, T., Rubio-Martínez, A., García de Coca, A., Dávila-Valls, J., Hernández-Rivas, J.-Á., Parker, H., Strefford, J. C., Benito, R., Ordóñez, J.-L., & Hernández-Rivas, J.-M. (2021). Dissecting the role of TP53 alterations in del(11q) chronic lymphocytic leukemia. *Clinical and Translational Medicine*, 11(2), e304. <https://doi.org/10.1002/ctm2.304>

Quivoron, C., Couronné, L., Della Valle, V., Lopez, C. K., Plo, I., Wagner-Ballon, O., Do Cruzeiro, M., Delhommeau, F., Arnulf, B., Stern, M.-H., Godley, L., Opolon, P., Tilly, H., Solary,

E., Duffourd, Y., Dessen, P., Merle-Beral, H., Nguyen-Khac, F., Fontenay, M., ... Bernard, O. A. (2011). TET2 inactivation results in pleiotropic hematopoietic abnormalities in mouse and is a recurrent event during human lymphomagenesis. *Cancer Cell*, 20(1), 25–38. <https://doi.org/10.1016/j.ccr.2011.06.003>

Rademakers, T., Goedhart, M., Hoogenboezem, M., García Ponce, A., van Rijssel, J., Samus, M., Schnoor, M., Butz, S., Huveneers, S., Vestweber, D., Nolte, M. A., Voermans, C., & van Buul, J. D. (2020). Hematopoietic stem and progenitor cells use podosomes to transcellularly cross the bone marrow endothelium. *Haematologica*, 105(12), 2746–2756. <https://doi.org/10.3324/haematol.2018.196329>

Radvoyevitch, T., Gale, R. P., & Kalaycio, M. E. (2024). What do atomic bomb survivors teach us about therapy-free remission in people with chronic myeloid leukaemia? *Leukemia*, 38(1), 207–209. <https://doi.org/10.1038/s41375-023-02081-x>

Ramalingam, P., Poulos, M. G., & Butler, J. M. (2017). Regulation of the hematopoietic stem cell lifecycle by the endothelial niche. *Current Opinion in Hematology*, 24(4), 289–299. <https://doi.org/10.1097/MOH.0000000000000350>

Ramdas, B., Lakshmi Reddy, P., Mali, R. S., Pasupuleti, S. K., Zhang, J., Kelley, M. R., Paczesny, S., Zhang, C., & Kapur, R. (9 2022). Combined heterozygosity of FLT3 ITD, TET2, and DNMT3A results in aggressive leukemia. *JCI Insight*, 7(17). <https://doi.org/10.1172/jci.insight.162016>

Ramos, C. V., Ballesteros-Arias, L., Silva, J. G., Paiva, R. A., Nogueira, M. F., Carneiro, J., Gjini, E., & Martins, V. C. (2020). Cell competition, the kinetics of thymopoiesis, and thymus cellularity are regulated by double-negative 2 to 3 early thymocytes. *Cell Reports*, 32(3), 107910. <https://doi.org/10.1016/j.celrep.2020.107910>

Ramos, C. V., & Martins, V. C. (2021). Cell competition in hematopoietic cells: Quality control in homeostasis and its role in leukemia. *Developmental Biology*, 475, 1–9. <https://doi.org/10.1016/j.ydbio.2021.02.013>

Rashidi, N. M., Scott, M. K., Scherf, N., Krinner, A., Kalchschmidt, J. S., Gounaris, K., Selkirk, M. E., Roeder, I., & Lo Celso, C. (2014). In vivo time-lapse imaging shows diverse niche engagement by quiescent and naturally activated hematopoietic stem cells. *Blood*, 124(1), 79–83. <https://doi.org/10.1182/blood-2013-10-534859>

Reagan, M. R., & Rosen, C. J. (2016). Navigating the bone marrow niche: translational insights and cancer-driven dysfunction. *Nature Reviews. Rheumatology*, 12(3), 154–168. <https://doi.org/10.1038/nrrheum.2015.160>

Ren, P., Dong, X., & Vijg, J. (2022). Age-related somatic mutation burden in human tissues. *Frontiers in Aging*, 3, 1018119. <https://doi.org/10.3389/fragi.2022.1018119>

Ren, P., Zhang, J., & Vijg, J. (2024). Somatic mutations in aging and disease. *GeroScience*, 46(5), 5171–5189. <https://doi.org/10.1007/s11357-024-01113-3>

Renders, S., Svendsen, A. F., Panten, J., Rama, N., Maryanovich, M., Sommerkamp, P., Ladel, L., Redavid, A. R., Gibert, B., Lazare, S., Ducarouge, B., Schönberger, K., Narr, A., Tourbez, M., Dethmers-Ausema, B., Zwart, E., Hotz-Wagenblatt, A., Zhang, D., Korn, C., ... Trumpp, A. (2021). Niche derived netrin-1 regulates hematopoietic stem cell dormancy via its receptor neogenin-1. *Nature Communications*, 12(1), 608. <https://doi.org/10.1038/s41467-020-20801-0>

Reschke, R., Yu, J., Flood, B., Higgs, E. F., Hatogai, K., & Gajewski, T. F. (2021). Immune cell and tumor cell-derived CXCL10 is indicative of immunotherapy response in metastatic melanoma. *Journal for Immunotherapy of Cancer*, 9(9), e003521. <https://doi.org/10.1136/jitc-2021-003521>

Ribatti, D. (2018). An historical note on the cell theory. *Experimental Cell Research*, 364(1), 1–4. <https://doi.org/10.1016/j.yexcr.2018.01.038>

Rindy, L. J., & Chambers, A. R. (2025). Bone marrow aspiration and biopsy. In StatPearls. StatPearls Publishing. <https://www.ncbi.nlm.nih.gov/books/NBK559232/>

Robbe, P., & Schuh, A. (2023). Genomic stratification of hematological malignancies. *HemaSphere*, 7(6), e902. <https://doi.org/10.1097/HS9.0000000000000902>

Robin, C., Ottersbach, K., de Bruijn, M., Ma, X., van der Horn, K., & Dzierzak, E. (2003). Developmental origins of hematopoietic stem cells. *Oncology Research*, 13(6–10), 315–321. <https://doi.org/10.3727/096504003108748519>

Robinson, W., Metcalf, D., & Bradley, T. R. (1967). Stimulation by normal and leukemic mouse sera of colony formation in vitro by mouse bone marrow cells. *Journal of Cellular Physiology*, 69(1), 83–91. <https://doi.org/10.1002/jcp.1040690111>

Rodriguez-Fraticelli, A. E., Wolock, S. L., Weinreb, C. S., Panero, R., Patel, S. H., Jankovic, M., Sun, J., Calogero, R. A., Klein, A. M., & Camargo, F. D. (2018). Clonal analysis of lineage fate in native haematopoiesis. *Nature*, 553(7687), 212–216. <https://doi.org/10.1038/nature25168>

Rontauroli, S., Carretta, C., Parenti, S., Bertesi, M., & Manfredini, R. (2022). Novel molecular insights into leukemic evolution of myeloproliferative neoplasms: A single cell perspective. *International Journal of Molecular Sciences*, 23(23), 15256. <https://doi.org/10.3390/ijms232315256>

Rosendaal, M., Hodgson, G. S., & Bradley, T. R. (1979). Organization of haemopoietic stem cells: the generation-age hypothesis. *Cell and Tissue Kinetics*, 12(1), 17–29. <https://doi.org/10.1111/j.1365-2184.1979.tb00110.x>

Rosner, M., Horer, S., Feichtinger, M., & Hengstschläger, M. (2023). Multipotent fetal stem cells in reproductive biology research. *Stem Cell Research & Therapy*, 14(1), 157. <https://doi.org/10.1186/s13287-023-03379-4>

Rotondi, M., Rosati, A., Buonamano, A., Lasagni, L., Lazzeri, E., Pradella, F., Fossombroni, V., Cirami, C., Liotta, F., La Villa, G., Serio, M., Berton, E., Salvadori, M., & Romagnani, P. (2004). High pretransplant serum levels of CXCL10/IP-10 are related to increased risk of renal allograft failure. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons*, 4(9), 1466–1474. <https://doi.org/10.1111/j.1600-6143.2004.00525.x>

Rougerie, P., & Delon, J. (2012). Rho GTPases: masters of T lymphocyte migration and activation. *Immunology Letters*, 142(1–2), 1–13. <https://doi.org/10.1016/j.imlet.2011.12.003>

Roux, J.-P., Duboeuf, F., Sornay-Rendu, E., Rinaudo, L., Ulivieri, F. M., Wegrzyn, J., & Chapurlat, R. (2024). The relationship between bone strain index, bone mass, microarchitecture and mechanical behavior in human vertebrae: an ex vivo study. *Osteoporosis International: A Journal Established as Result of Cooperation between the*

European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA, 35(6), 1069–1075. <https://doi.org/10.1007/s00198-024-07066-9>

Rowley, J. D. (1973). Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature*, 243(5405), 290–293. <https://doi.org/10.1038/243290a0>

Rubio-Lara, J. A., Igarashi, K. J., Sood, S., Johansson, A., Sommerkamp, P., Yamashita, M., & Lin, D. S. (2023). Expanding hematopoietic stem cell ex vivo: recent advances and technical considerations. *Experimental Hematology*, 125–126, 6–15. <https://doi.org/10.1016/j.exphem.2023.07.006>

Rupec, R. A., Jundt, F., Rebholz, B., Eckelt, B., Weindl, G., Herzinger, T., Flaig, M. J., Moosmann, S., Plewig, G., Dörken, B., Förster, I., Huss, R., & Pfeffer, K. (2005). Stroma-mediated dysregulation of myelopoiesis in mice lacking I kappa B alpha. *Immunity*, 22(4), 479–491. <https://doi.org/10.1016/j.immuni.2005.02.009>

Rydell-Törmänen, K., & Johnson, J. R. (2019). The applicability of mouse models to the study of human disease. *Methods in Molecular Biology* (Clifton, N.J.), 1940, 3–22. https://doi.org/10.1007/978-1-4939-9086-3_1

Sabin, F. R., Cunningham, R. S., Doan, C. A., and Kindwall, J. A. (1925). The normal rhythm of the white blood cells. *Bull. Johns Hopk. Hosp.*, 37, 231–280.

Saenz, D. T., Fiskus, W., Manshouri, T., Rajapakshe, K., Krieger, S., Sun, B., Mill, C. P., DiNardo, C., Pemmaraju, N., Kadia, T., Parmar, S., Sharma, S., Coarfa, C., Qiu, P., Verstovsek, S., & Bhalla, K. N. (2017). BET protein bromodomain inhibitor-based combinations are highly active against post-myeloproliferative neoplasm secondary AML cells. *Leukemia*, 31(3), 678–687. <https://doi.org/10.1038/leu.2016.260>

Safina, K., & van Galen, P. (2024). New frameworks for hematopoiesis derived from single-cell genomics. *Blood*, 144(10), 1039–1047. <https://doi.org/10.1182/blood.2024024006>

Saint-Martin, C., Leroy, G., Delhommeau, F., Panelatti, G., Dupont, S., James, C., Plo, I., Bordessoule, D., Chomienne, C., Delannoy, A., Devidas, A., Gardembas-Pain, M., Isnard, F., Plumelle, Y., Bernard, O., Vainchenker, W., Najman, A., Bellanné-Chantelot, C., & French Group of Familial Myeloproliferative Disorders. (2009). Analysis of the ten-eleven translocation 2 (TET2) gene in familial myeloproliferative neoplasms. *Blood*, 114(8), 1628–1632. <https://doi.org/10.1182/blood-2009-01-197525>

Sakurai, M., Ishitsuka, K., Becker, H. J., & Yamazaki, S. (2024). Ex vivo expansion of human hematopoietic stem cells and clinical applications. *Cancer Science*, 115(3), 698–705. <https://doi.org/10.1111/cas.16066>

Sakurai, M., Ishitsuka, K., Ito, R., Wilkinson, A. C., Kimura, T., Mizutani, E., Nishikii, H., Sudo, K., Becker, H. J., Takemoto, H., Sano, T., Kataoka, K., Takahashi, S., Nakamura, Y., Kent, D. G., Iwama, A., Chiba, S., Okamoto, S., Nakauchi, H., & Yamazaki, S. (2023). Chemically defined cytokine-free expansion of human haematopoietic stem cells. *Nature*, 615(7950), 127–133. <https://doi.org/10.1038/s41586-023-05739-9>

Sandberg, R. (2014). Entering the era of single-cell transcriptomics in biology and medicine. *Nature Methods*, 11(1), 22–24. <https://doi.org/10.1038/nmeth.2764>

Sandén, C., Lilljebjörn, H., Orsmark Pietras, C., Henningsson, R., Saba, K. H., Landberg, N., Thorsson, H., von Palffy, S., Peña-Martinez, P., Högberg, C., Rissler, M., Gisselsson, D.,

Lazarevic, V., Juliusson, G., Ågerstam, H., & Fioretos, T. (2020). Clonal competition within complex evolutionary hierarchies shapes AML over time. *Nature Communications*, 11(1), 579. <https://doi.org/10.1038/s41467-019-14106-0>

Sanjuan-Pla, A., Macaulay, I. C., Jensen, C. T., Woll, P. S., Luis, T. C., Mead, A., Moore, S., Carella, C., Matsuoka, S., Bouriez Jones, T., Chowdhury, O., Stenson, L., Lutteropp, M., Green, J. C. A., Facchini, R., Boukarabila, H., Grover, A., Gambardella, A., Thongjuea, S., ... Jacobsen, S. E. W. (2013). Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature*, 502(7470), 232–236. <https://doi.org/10.1038/nature12495>

Santos, F. P. S., Kantarjian, H. M., Jain, N., Manshouri, T., Thomas, D. A., Garcia-Manero, G., Kennedy, D., Estrov, Z., Cortes, J., & Verstovsek, S. (2010). Phase 2 study of CEP-701, an orally available JAK2 inhibitor, in patients with primary or post-polycythemia vera/essential thrombocythemia myelofibrosis. *Blood*, 115(6), 1131–1136. <https://doi.org/10.1182/blood-2009-10-246363>

Sarkaria, S. M., Decker, M., & Ding, L. (2018). Bone marrow micro-environment in normal and deranged hematopoiesis: Opportunities for regenerative medicine and therapies. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*, 40(3). <https://doi.org/10.1002/bies.201700190>

Saruhan-Direskeneli, G., Gürses, C., Demirbilek, V., Yentür, S. P., Yilmaz, G., Onal, E., Yapici, Z., Yalçinkaya, C., Cokar, O., Akman-Demir, G., & Gökyiğit, A. (2005). Elevated interleukin-12 and CXCL10 in subacute sclerosing panencephalitis. *Cytokine*, 32(2), 104–110. <https://doi.org/10.1016/j.cyto.2005.08.004>

Saunders, A. E., & Johnson, P. (2010). Modulation of immune cell signalling by the leukocyte common tyrosine phosphatase, CD45. *Cellular Signalling*, 22(3), 339–348. <https://doi.org/10.1016/j.cellsig.2009.10.003>

Sauvageau, G., Thorsteinsdottir, U., Eaves, C. J., Lawrence, H. J., Largman, C., Lansdorp, P. M., & Humphries, R. K. (1995). Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. *Genes & Development*, 9(14), 1753–1765. <https://doi.org/10.1101/gad.9.14.1753>

Sauvageau, Guy, Iscove, N. N., & Humphries, R. K. (2004). In vitro and in vivo expansion of hematopoietic stem cells. *Oncogene*, 23(43), 7223–7232. <https://doi.org/10.1038/sj.onc.1207942>

Sawamiphak, S., Kontarakis, Z., & Stainier, D. Y. R. (2014). Interferon gamma signaling positively regulates hematopoietic stem cell emergence. *Developmental Cell*, 31(5), 640–653. <https://doi.org/10.1016/j.devcel.2014.11.007>

Scadden, D. T. (2014). Nice neighborhood: emerging concepts of the stem cell niche. *Cell*, 157(1), 41–50. <https://doi.org/10.1016/j.cell.2014.02.013>

Schaub, F. X., Looser, R., Li, S., Hao-Shen, H., Lehmann, T., Tichelli, A., & Skoda, R. C. (2010). Clonal analysis of TET2 and JAK2 mutations suggests that TET2 can be a late event in the progression of myeloproliferative neoplasms. *Blood*, 115(10), 2003–2007. <https://doi.org/10.1182/blood-2009-09-245381>

Schepers, K., Campbell, T. B., & Passegué, E. (2015). Normal and leukemic stem cell niches: insights and therapeutic opportunities. *Cell Stem Cell*, 16(3), 254–267. <https://doi.org/10.1016/j.stem.2015.02.014>

Schepers, K., Pietras, E. M., Reynaud, D., Flach, J., Binnewies, M., Garg, T., Wagers, A. J., Hsiao, E. C., & Passegué, E. (2013). Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. *Cell Stem Cell*, 13(3), 285–299. <https://doi.org/10.1016/j.stem.2013.06.009>

Schmitt, C. E., Lizama, C. O., & Zovein, A. C. (2014). From transplantation to transgenics: mouse models of developmental hematopoiesis. *Experimental Hematology*, 42(8), 707–716. <https://doi.org/10.1016/j.exphem.2014.06.008>

Schnöder, T. M., Eberhardt, J., Koehler, M., Bierhoff, H. B., Weinert, S., Pandey, A. D., Nimmagadda, S. C., Wolleschak, D., Jöhrens, K., Fischer, T., & Heidel, F. H. (2017). Cell autonomous expression of CXCL-10 in JAK2V617F-mutated MPN. *Journal of Cancer Research and Clinical Oncology*, 143(5), 807–820. <https://doi.org/10.1007/s00432-017-2354-1>

Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells*, 4(1–2), 7–25. <https://www.ncbi.nlm.nih.gov/pubmed/747780>

Schulte, R., Wilson, N. K., Prick, J. C. M., Cossetti, C., Maj, M. K., Gottgens, B., & Kent, D. G. (2015). Index sorting resolves heterogeneous murine hematopoietic stem cell populations. *Experimental Hematology*, 43(9), 803–811. <https://doi.org/10.1016/j.exphem.2015.05.006>

Seita, J., & Weissman, I. L. (2010). Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdisciplinary Reviews. Systems Biology and Medicine*, 2(6), 640–653. <https://doi.org/10.1002/wsbm.86>

Sender, R., & Milo, R. (2021). The distribution of cellular turnover in the human body. *Nature Medicine*, 27(1), 45–48. <https://doi.org/10.1038/s41591-020-01182-9>

Shakiba, N., Fahmy, A., Jayakumaran, G., McGibbon, S., David, L., Trcka, D., Elbaz, J., Puri, M. C., Nagy, A., van der Kooy, D., Goyal, S., Wrana, J. L., & Zandstra, P. W. (2019). Cell competition during reprogramming gives rise to dominant clones. *Science (New York, N.Y.)*, 364(6438), eaan0925. <https://doi.org/10.1126/science.aan0925>

Shang, X., Marchioni, F., Sipes, N., Evelyn, C. R., Jerabek-Willemsen, M., Duhr, S., Seibel, W., Wortman, M., & Zheng, Y. (2012). Rational design of small molecule inhibitors targeting RhoA subfamily Rho GTPases. *Chemistry & Biology*, 19(6), 699–710. <https://doi.org/10.1016/j.chembiol.2012.05.009>

Shaposhnikov, V. L. (1979). Distribution of bone marrow cells in the mouse skeleton. *Bulletin of Experimental Biology and Medicine*, 87(5), 510–512. <https://doi.org/10.1007/bf00806699>

Shastri, A., Will, B., Steidl, U., & Verma, A. (2017). Stem and progenitor cell alterations in myelodysplastic syndromes. *Blood*, 129(12), 1586–1594. <https://doi.org/10.1182/blood-2016-10-696062>

Shepherd, M. S., Li, J., Wilson, N. K., Oedekoven, C. A., Li, J., Belmonte, M., Fink, J., Prick, J. C. M., Pask, D. C., Hamilton, T. L., Loeffler, D., Rao, A., Schröder, T., Göttgens, B., Green, A. R., & Kent, D. G. (2018). Single-cell approaches identify the molecular network driving malignant hematopoietic stem cell self-renewal. *Blood*, 132(8), 791–803. <https://doi.org/10.1182/blood-2017-12-821066>

Shide, K., Shimoda, H. K., Kumano, T., Karube, K., Kameda, T., Takenaka, K., Oku, S., Abe, H., Katayose, K. S., Kubuki, Y., Kusumoto, K., Hasuike, S., Tahara, Y., Nagata, K., Matsuda,

T., Ohshima, K., Harada, M., & Shimoda, K. (2008). Development of ET, primary myelofibrosis and PV in mice expressing JAK2 V617F. *Leukemia*, 22(1), 87–95. <https://doi.org/10.1038/sj.leu.2405043>

Shii, K., Yokono, K., Baba, S., & Roth, R. A. (1986). Purification and characterization of insulin-degrading enzyme from human erythrocytes. *Diabetes*, 35(6), 675–683. <https://doi.org/10.2337/diab.35.6.675>

Shimomura, O., Johnson, F. H., & Saiga, Y. (1962). Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *Journal of Cellular and Comparative Physiology*, 59(3), 223–239. <https://doi.org/10.1002/jcp.1030590302>

Shin, J. Y., Hu, W., Naramura, M., & Park, C. Y. (2014). High c-Kit expression identifies hematopoietic stem cells with impaired self-renewal and megakaryocytic bias. *The Journal of Experimental Medicine*, 211(2), 217–231. <https://doi.org/10.1084/jem.20131128>

Shino, M. Y., Todd, J. L., Neely, M. L., Kirchner, J., Frankel, C. W., Snyder, L. D., Pavlisko, E. N., Fishbein, G. A., Schaenman, J. M., Mason, K., Kesler, K., Martinu, T., Singer, L. G., Tsuang, W., Budev, M., Shah, P. D., Reynolds, J. M., Williams, N., Robien, M. A., ... Belperio, J. A. (2022). Plasma CXCL9 and CXCL10 at allograft injury predict chronic lung allograft dysfunction. *American Journal of Transplantation: Official Journal of the American Society of Transplantation and the American Society of Transplant Surgeons*, 22(9), 2169–2179. <https://doi.org/10.1111/ajt.17108>

Shurin, G. V., Yurkovetsky, Z. R., Chatta, G. S., Tourkova, I. L., Shurin, M. R., & Lokshin, A. E. (2007). Dynamic alteration of soluble serum biomarkers in healthy aging. *Cytokine*, 39(2), 123–129. <https://doi.org/10.1016/j.cyto.2007.06.006>

Sidorenko, S. P., & Clark, E. A. (2003). The dual-function CD150 receptor subfamily: the viral attraction. *Nature Immunology*, 4(1), 19–24. <https://doi.org/10.1038/ni0103-19>

Siegel, F. P., Tauscher, J., & Petrides, P. E. (2013). Aquagenic pruritus in polycythemia vera: characteristics and influence on quality of life in 441 patients. *American Journal of Hematology*, 88(8), 665–669. <https://doi.org/10.1002/ajh.23474>

Siminovitch, L., McCulloch, E. A., & Till, J. E. (1963). The distribution of colony-forming cells among spleen colonies. *Journal of Cellular and Comparative Physiology*, 62(3), 327–336. <https://doi.org/10.1002/jcp.1030620313>

Singh, I., Fernandez-Perez, D., Sanchez, P. S., & Rodriguez-Fraticelli, A. E. (2025). Pre-existing stem cell heterogeneity dictates clonal responses to the acquisition of leukemic driver mutations. *Cell Stem Cell*, 32(4), 564-580.e6. <https://doi.org/10.1016/j.stem.2025.01.012>

Singh, K. P., Casado, F. L., Opanashuk, L. A., & Gasiewicz, T. A. (2009). The aryl hydrocarbon receptor has a normal function in the regulation of hematopoietic and other stem/progenitor cell populations. *Biochemical Pharmacology*, 77(4), 577–587. <https://doi.org/10.1016/j.bcp.2008.10.001>

Singh, V. K., Saini, A., Kalsan, M., Kumar, N., & Chandra, R. (2016). Describing the stem cell potency: The various methods of functional assessment and in silico diagnostics. *Frontiers in Cell and Developmental Biology*, 4, 134. <https://doi.org/10.3389/fcell.2016.00134>

Sinkala, M. (2023). Mutational landscape of cancer-driver genes across human cancers. *Scientific Reports*, 13(1), 12742. <https://doi.org/10.1038/s41598-023-39608-2>

Smit, T. H., Odgaard, A., & Schneider, E. (1997). Structure and function of vertebral trabecular bone. *Spine*, 22(24), 2823–2833. <https://doi.org/10.1097/00007632-199712150-00005>

Smith, L. G., Weissman, I. L., & Heimfeld, S. (1991). Clonal analysis of hematopoietic stem-cell differentiation in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 88(7), 2788–2792. <https://doi.org/10.1073/pnas.88.7.2788>

Song, M.-K., Park, B.-B., & Uhm, J.-E. (2018). Understanding splenomegaly in myelofibrosis: Association with molecular pathogenesis. *International Journal of Molecular Sciences*, 19(3). <https://doi.org/10.3390/ijms19030898>

Sonnenschein, C., & Soto, A. M. (2000). Somatic mutation theory of carcinogenesis: why it should be dropped and replaced. *Molecular Carcinogenesis*, 29(4), 205–211. [https://doi.org/10.1002/1098-2744\(200012\)29:4<205::aid-mc1002>3.0.co;2-w](https://doi.org/10.1002/1098-2744(200012)29:4<205::aid-mc1002>3.0.co;2-w)

Soto, A. M., & Sonnenschein, C. (2014). One hundred years of somatic mutation theory of carcinogenesis: is it time to switch? *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*, 36(1), 118–120. <https://doi.org/10.1002/bies.201300160>

Sovani, V. (2021). Normal bone marrow, its structure and function. *Diagnostic Histopathology (Oxford, England)*, 27(9), 349–356. <https://doi.org/10.1016/j.mpdhp.2021.06.001>

Spalding, K. L., Bhardwaj, R. D., Buchholz, B. A., Druid, H., & Frisén, J. (2005). Retrospective birth dating of cells in humans. *Cell*, 122(1), 133–143. <https://doi.org/10.1016/j.cell.2005.04.028>

Spangrude, G. J., & Brooks, D. M. (1992). Phenotypic analysis of mouse hematopoietic stem cells shows a Thy-1-negative subset. *Blood*, 80(8), 1957–1964. <https://doi.org/10.1182/blood.v80.8.1957.1957>

Spangrude, G. J., Heimfeld, S., & Weissman, I. L. (1988). Purification and characterization of mouse hematopoietic stem cells. *Science (New York, N.Y.)*, 241(4861), 58–62. <https://doi.org/10.1126/science.2898810>

Spanjaard, B., Hu, B., Mitic, N., Olivares-Chauvet, P., Janjuha, S., Ninov, N., & Junker, J. P. (2018). Simultaneous lineage tracing and cell-type identification using CRISPR-Cas9-induced genetic scars. *Nature Biotechnology*, 36(5), 469–473. <https://doi.org/10.1038/nbt.4124>

Spemann, H., & Mangold, H. (1924). über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. *Development genes and evolution*, 100(3–4), 599–638. <https://doi.org/10.1007/bf02108133>

Steensma, D. P., & Kyle, R. A. (2021). James till and Ernest McCulloch: Hematopoietic stem cell discoverers. *Mayo Clinic Proceedings*. Mayo Clinic, 96(3), 830–831. <https://doi.org/10.1016/j.mayocp.2021.01.016>

Sternberg, N., Sauer, B., Hoess, R., & Abremski, K. (1986). Bacteriophage P1 cre gene and its regulatory region. Evidence for multiple promoters and for regulation by DNA methylation. *Journal of Molecular Biology*, 187(2), 197–212. [https://doi.org/10.1016/0022-2836\(86\)90228-7](https://doi.org/10.1016/0022-2836(86)90228-7)

Stetka, J., & Skoda, R. C. (2021). Mouse models of myeloproliferative neoplasms for pre-clinical testing of novel therapeutic agents. *Biomedical Papers of the Medical Faculty of the*

University Palacky, Olomouc, Czechoslovakia, 165(1), 26–33.
<https://doi.org/10.5507/bp.2021.004>

Stowers, R. S., & Schwarz, T. L. (1999). A genetic method for generating *Drosophila* eyes composed exclusively of mitotic clones of a single genotype. *Genetics*, 152(4), 1631–1639. <https://doi.org/10.1093/genetics/152.4.1631>

Stratton, M. R., Campbell, P. J., & Futreal, P. A. (2009). The cancer genome. *Nature*, 458(7239), 719–724. <https://doi.org/10.1038/nature07943>

Su, R., Lin, H.-S., Zhang, X.-H., Yin, X.-L., Ning, H.-M., Liu, B., Zhai, P.-F., Gong, J.-N., Shen, C., Song, L., Chen, J., Wang, F., Zhao, H.-L., Ma, Y.-N., Yu, J., & Zhang, J.-W. (2015). MiR-181 family: regulators of myeloid differentiation and acute myeloid leukemia as well as potential therapeutic targets. *Oncogene*, 34(25), 3226–3239. <https://doi.org/10.1038/onc.2014.274>

Subramaniam, A., Žemaitis, K., Talkhoncheh, M. S., Yudovich, D., Bäckström, A., Debnath, S., Chen, J., Jain, M. V., Galeev, R., Gaetani, M., Zubarev, R. A., & Larsson, J. (2020). Lysine-specific demethylase 1A restricts ex vivo propagation of human HSCs and is a target of UM171. *Blood*, 136(19), 2151–2161. <https://doi.org/10.1182/blood.2020005827>

Suda, T., Suda, J., & Ogawa, M. (1984). Disparate differentiation in mouse hemopoietic colonies derived from paired progenitors. *Proceedings of the National Academy of Sciences of the United States of America*, 81(8), 2520–2524. <https://doi.org/10.1073/pnas.81.8.2520>

Sudo, K., Ema, H., Morita, Y., & Nakauchi, H. (2000). Age-associated characteristics of murine hematopoietic stem cells. *The Journal of Experimental Medicine*, 192(9), 1273–1280. <https://doi.org/10.1084/jem.192.9.1273>

Sugiyama, T., Kohara, H., Noda, M., & Nagasawa, T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity*, 25(6), 977–988. <https://doi.org/10.1016/j.immuni.2006.10.016>

Sui, L., Ye, L., Sanders, A. J., Yang, Y., Hao, C., Hargest, R., & Jiang, W. G. (2021). Expression of death associated proteins DAP1 and DAP3 in human pancreatic cancer. *Anticancer Research*, 41(5), 2357–2362. <https://doi.org/10.21873/anticancer.15010>

Sun, D., Luo, M., Jeong, M., Rodriguez, B., Xia, Z., Hannah, R., Wang, H., Le, T., Faull, K. F., Chen, R., Gu, H., Bock, C., Meissner, A., Göttgens, B., Darlington, G. J., Li, W., & Goodell, M. A. (2014). Epigenomic profiling of young and aged HSCs reveals concerted changes during aging that reinforce self-renewal. *Cell Stem Cell*, 14(5), 673–688. <https://doi.org/10.1016/j.stem.2014.03.002>

Swierczek, S. I., Yoon, D., Bellanné-Chantelot, C., Kim, S. J., Saint-Martin, C., Delhommeau, F., Najman, A., & Prchal, J. T. (2011). Extent of hematopoietic involvement by TET2 mutations in JAK2V617F polycythemia vera. *Haematologica*, 96(5), 775–778. <https://doi.org/10.3324/haematol.2010.029678>

Sykes, S. M., & Scadden, D. T. (2013). Modeling human hematopoietic stem cell biology in the mouse. *Seminars in Hematology*, 50(2), 92–100. <https://doi.org/10.1053/j.seminhematol.2013.03.029>

Szilvassy, S. J., Lansdorp, P. M., Humphries, R. K., Eaves, A. C., & Eaves, C. J. (1989). Isolation in a single step of a highly enriched murine hematopoietic stem cell population with

competitive long-term repopulating ability. *Blood*, 74(3), 930–939. <https://doi.org/10.1182/blood.v74.3.930.930>

Tabansky, I., & Stern, J. N. H. (2016). Basics of stem cell biology as applied to the brain. In *Stem Cells in Neuroendocrinology* (pp. 11–24). Springer International Publishing. https://doi.org/10.1007/978-3-319-41603-8_2

Tak, T., Eisele, A. S., & Perié, L. (2021). In vivo tracking of hematopoietic stem and progenitor cell ontogeny by cellular barcoding. *Methods in Molecular Biology* (Clifton, N.J.), 2308, 281–300. https://doi.org/10.1007/978-1-0716-1425-9_21

Talarmain, L., Clarke, M. A., Shorthouse, D., Cabrera-Cosme, L., Kent, D. G., Fisher, J., & Hall, B. A. (2022). HOXA9 has the hallmarks of a biological switch with implications in blood cancers. *Nature Communications*, 13(1), 5829. <https://doi.org/10.1038/s41467-022-33189-w>

Tang, W., Li, Z., Li, X., & Huo, Z. (2020). High CXCR2 expression predicts poor prognosis in adult patients with acute myeloid leukemia. *Therapeutic Advances in Hematology*, 11, 2040620720958586. <https://doi.org/10.1177/2040620720958586>

Tang, X., Wang, J., Chen, J., Liu, W., Qiao, P., Quan, H., Li, Z., Dang, E., Wang, G., & Shao, S. (2024). Epidermal stem cells: skin surveillance and clinical perspective. *Journal of Translational Medicine*, 22(1), 779. <https://doi.org/10.1186/s12967-024-05600-1>

Taswell, C. (1984). Limiting dilution assays for the determination of immunocompetent cell frequencies. III. Validity tests for the single-hit Poisson model. *Journal of Immunological Methods*, 72(1), 29–40. [https://doi.org/10.1016/0022-1759\(84\)90430-7](https://doi.org/10.1016/0022-1759(84)90430-7)

Tátrai, P., & Gergely, F. (2022). Centrosome function is critical during terminal erythroid differentiation. *The EMBO Journal*, 41(14), e108739. <https://doi.org/10.15252/embj.2021108739>

Taylor, E., Taoudi, S., & Medvinsky, A. (2010). Hematopoietic stem cell activity in the aorta-gonad-mesonephros region enhances after mid-day 11 of mouse development. *The International Journal of Developmental Biology*, 54(6–7), 1055–1060. <https://doi.org/10.1387/ijdb.103152et>

Tefferi, A. (2010). Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1. *Leukemia*, 24(6), 1128–1138. <https://doi.org/10.1038/leu.2010.69>

Tefferi, A., Pardanani, A., Lim, K.-H., Abdel-Wahab, O., Lasho, T. L., Patel, J., Gangat, N., Finke, C. M., Schwager, S., Mullally, A., Li, C.-Y., Hanson, C. A., Mesa, R., Bernard, O., Delhommeau, F., Vainchenker, W., Gilliland, D. G., & Levine, R. L. (2009). TET2 mutations and their clinical correlates in polycythemia vera, essential thrombocythemia and myelofibrosis. *Leukemia*, 23(5), 905–911. <https://doi.org/10.1038/leu.2009.47>

Tefferi, Ayalew, & Barbui, T. (2023). Polycythemia vera: 2024 update on diagnosis, risk-stratification, and management. *American Journal of Hematology*, 98(9), 1465–1487. <https://doi.org/10.1002/ajh.27002>

Tefferi, Ayalew, Vaidya, R., Caramazza, D., Finke, C., Lasho, T., & Pardanani, A. (2011). Circulating interleukin (IL)-8, IL-2R, IL-12, and IL-15 levels are independently prognostic in primary myelofibrosis: a comprehensive cytokine profiling study. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, 29(10), 1356–1363. <https://doi.org/10.1200/JCO.2010.32.9490>

Tian, L., Tomei, S., Schreuder, J., Weber, T. S., Amann-Zalcenstein, D., Lin, D. S., Tran, J., Audiger, C., Chu, M., Jarratt, A., Willson, T., Hilton, A., Pang, E. S., Patton, T., Kelly, M., Su, S., Gouil, Q., Diakumis, P., Bahlo, M., ... Naik, S. H. (2021). Clonal multi-omics reveals Bcor as a negative regulator of emergency dendritic cell development. *Immunity*, 54(6), 1338–1351.e9. <https://doi.org/10.1016/j.immuni.2021.03.012>

Tiedt, R., Hao-Shen, H., Sobas, M. A., Looser, R., Dirnhofer, S., Schwaller, J., & Skoda, R. C. (2008). Ratio of mutant JAK2-V617F to wild-type Jak2 determines the MPD phenotypes in transgenic mice. *Blood*, 111(8), 3931–3940. <https://doi.org/10.1182/blood-2007-08-107748>

Till, J. E., & McCULLOCH, E. A. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiation Research*, 14(2), 213–222. <https://doi.org/10.2307/3570892>

Torres, D. G., Paes, J., da Costa, A. G., Malheiro, A., Silva, G. V., Mourão, L. P. de S., & Tarragô, A. M. (2022). JAK2 variant signaling: Genetic, hematologic and immune implication in chronic myeloproliferative neoplasms. *Biomolecules*, 12(2), 291. <https://doi.org/10.3390/biom12020291>

Trevisan, M., & Iscove, N. N. (1995). Phenotypic analysis of murine long-term hemopoietic reconstituting cells quantitated competitively in vivo and comparison with more advanced colony-forming progeny. *The Journal of Experimental Medicine*, 181(1), 93–103. <https://doi.org/10.1084/jem.181.1.93>

Trumpp, A., & Haas, S. (2022). Cancer stem cells: The adventurous journey from hematopoietic to leukemic stem cells. *Cell*, 185(8), 1266–1270. <https://doi.org/10.1016/j.cell.2022.03.025>

Tsai, F. D., & Lindsley, R. C. (2020). Clonal hematopoiesis in the inherited bone marrow failure syndromes. *Blood*, 136(14), 1615–1622. <https://doi.org/10.1182/blood.2019000990>

Tsiouplis, N. J., Bailey, D. W., Chiou, L. F., Wissink, F. J., & Tsagaratou, A. (2020). TET-mediated epigenetic regulation in immune cell development and disease. *Frontiers in Cell and Developmental Biology*, 8, 623948. <https://doi.org/10.3389/fcell.2020.623948>

Tsukuba, T., Yanagawa, M., Okamoto, K., Okamoto, Y., Yasuda, Y., Nakayama, K. I., Kadowaki, T., & Yamamoto, K. (2009). Impaired chemotaxis and cell adhesion due to decrease in several cell-surface receptors in cathepsin E-deficient macrophages. *The Journal of Biochemistry*, 145(5), 565–573. <https://doi.org/10.1093/jb/mvp016>

Tu, T. C., Brown, N. K., Kim, T.-J., Wroblewska, J., Yang, X., Guo, X., Lee, S. H., Kumar, V., Lee, K.-M., & Fu, Y.-X. (2015). CD160 is essential for NK-mediated IFN- γ production. *The Journal of Experimental Medicine*, 212(3), 415–429. <https://doi.org/10.1084/jem.20131601>

Tucker, T., Marra, M., & Friedman, J. M. (2009). Massively parallel sequencing: the next big thing in genetic medicine. *The American Journal of Human Genetics*, 85(2), 142–154. <https://doi.org/10.1016/j.ajhg.2009.06.022>

Tümpel, S., & Rudolph, K. L. (2019). Quiescence: Good and bad of stem cell aging. *Trends in Cell Biology*, 29(8), 672–685. <https://doi.org/10.1016/j.tcb.2019.05.002>

Turner, C. H. (1998). Three rules for bone adaptation to mechanical stimuli. *Bone*, 23(5), 399–407. [https://doi.org/10.1016/s8756-3282\(98\)00118-5](https://doi.org/10.1016/s8756-3282(98)00118-5)

Tweedell, K. S. (2017). The adaptability of somatic stem cells: A review. *Journal of Stem Cells & Regenerative Medicine*, 13(1), 3–13. <https://www.ncbi.nlm.nih.gov/pubmed/28684892>

Tyler, D. M., Li, W., Zhuo, N., Pellock, B., & Baker, N. E. (2007). Genes affecting cell competition in *Drosophila*. *Genetics*, 175(2), 643–657. <https://doi.org/10.1534/genetics.106.061929>

Tyner, J. W., Tognon, C. E., Bottomly, D., Wilmot, B., Kurtz, S. E., Savage, S. L., Long, N., Schultz, A. R., Traer, E., Abel, M., Agarwal, A., Blucher, A., Borate, U., Bryant, J., Burke, R., Carlos, A., Carpenter, R., Carroll, J., Chang, B. H., ... Druker, B. J. (2018). Functional genomic landscape of acute myeloid leukaemia. *Nature*, 562(7728), 526–531. <https://doi.org/10.1038/s41586-018-0623-z>

Uchida, F., Uzawa, K., Kasamatsu, A., Takatori, H., Sakamoto, Y., Ogawara, K., Shiiba, M., Tanzawa, H., & Bukawa, H. (2012). Overexpression of cell cycle regulator CDCA3 promotes oral cancer progression by enhancing cell proliferation with prevention of G1 phase arrest. *BMC Cancer*, 12(1), 321. <https://doi.org/10.1186/1471-2407-12-321>

Uchida, N., & Weissman, I. L. (1992). Searching for hematopoietic stem cells: evidence that Thy-1.1^{lo} Lin⁻ Sca-1⁺ cells are the only stem cells in C57BL/Ka-Thy-1.1 bone marrow. *The Journal of Experimental Medicine*, 175(1), 175–184. <https://doi.org/10.1084/jem.175.1.175>

Ueda, T., Tsuji, K., Yoshino, H., Ebihara, Y., Yagasaki, H., Hisakawa, H., Mitsui, T., Manabe, A., Tanaka, R., Kobayashi, K., Ito, M., Yasukawa, K., & Nakahata, T. (4 2000). Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. *The Journal of Clinical Investigation*, 105(7), 1013–1021. <https://doi.org/10.1172/JCI8583>

Ueda, Tomoaki, Yokota, T., Okuzaki, D., Uno, Y., Mashimo, T., Kubota, Y., Sudo, T., Ishibashi, T., Shingai, Y., Doi, Y., Ozawa, T., Nakai, R., Tanimura, A., Ichii, M., Ezoe, S., Shibayama, H., Oritani, K., & Kanakura, Y. (2019). Endothelial cell-selective adhesion molecule contributes to the development of definitive hematopoiesis in the fetal liver. *Stem Cell Reports*, 13(6), 992–1005. <https://doi.org/10.1016/j.stemcr.2019.11.002>

Ulloa, B. A., Habbsa, S. S., Potts, K. S., Lewis, A., McKinstry, M., Payne, S. G., Flores, J. C., Nizhnik, A., Feliz Norberto, M., Mosimann, C., & Bowman, T. V. (2021). Definitive hematopoietic stem cells minimally contribute to embryonic hematopoiesis. *Cell Reports*, 36(11), 109703. <https://doi.org/10.1016/j.celrep.2021.109703>

Umemura, Y., Ishiko, T., Yamauchi, T., Kurono, M., & Mashiko, S. (1997). Five jumps per day increase bone mass and breaking force in rats. *Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research*, 12(9), 1480–1485. <https://doi.org/10.1359/jbmr.1997.12.9.1480>

Urbán, N., & Cheung, T. H. (2021). Stem cell quiescence: the challenging path to activation. *Development (Cambridge, England)*, 148(3), dev165084. <https://doi.org/10.1242/dev.165084>

Urs, A. P., Goda, C., & Kulkarni, R. (2024). Remodeling of the bone marrow microenvironment during acute myeloid leukemia progression. *Annals of Translational Medicine*, 12(4), 63. <https://doi.org/10.21037/atm-23-1824>

Urso, P., & Congdon, C. C. (1957). The effect of the amount of isologous bone marrow injected on the recovery of hematopoietic organs, survival and body weight after lethal irradiation injury in mice. *Blood*, 12(3), 251–260. <https://doi.org/10.1182/blood.v12.3.251.251>

Vachhani, P., Mascarenhas, J., Bose, P., Hobbs, G., Yacoub, A., Palmer, J. M., Gerds, A. T., Masarova, L., Kuykendall, A. T., Rampal, R. K., Mesa, R., & Verstovsek, S. (2024). Interferons in the treatment of myeloproliferative neoplasms. *Therapeutic Advances in Hematology*, 15, 20406207241229588. <https://doi.org/10.1177/20406207241229588>

Vaidya, R., Gangat, N., Jimma, T., Finke, C. M., Lasho, T. L., Pardananani, A., & Tefferi, A. (2012). Plasma cytokines in polycythemia vera: phenotypic correlates, prognostic relevance, and comparison with myelofibrosis. *American Journal of Hematology*, 87(11), 1003–1005. <https://doi.org/10.1002/ajh.23295>

van Neerven, S. M., & Vermeulen, L. (2023). Cell competition in development, homeostasis and cancer. *Nature Reviews. Molecular Cell Biology*, 24(3), 221–236. <https://doi.org/10.1038/s41580-022-00538-y>

VanHorn, S., & Morris, S. A. (2021). Next-generation lineage tracing and fate mapping to interrogate development. *Developmental Cell*, 56(1), 7–21. <https://doi.org/10.1016/j.devcel.2020.10.021>

Vasquez, R. E., Xin, L., & Soong, L. (2008). Effects of CXCL10 on dendritic cell and CD4+ T-cell functions during *Leishmania amazonensis* infection. *Infection and Immunity*, 76(1), 161–169. <https://doi.org/10.1128/IAI.00825-07>

Vazin, T., & Freed, W. J. (2010). Human embryonic stem cells: derivation, culture, and differentiation: a review. *Restorative Neurology and Neuroscience*, 28(4), 589–603. <https://doi.org/10.3233/RNN-2010-0543>

Velten, L., Haas, S. F., Raffel, S., Blaszkiewicz, S., Islam, S., Hennig, B. P., Hirche, C., Lutz, C., Buss, E. C., Nowak, D., Boch, T., Hofmann, W.-K., Ho, A. D., Huber, W., Trumpp, A., Essers, M. A. G., & Steinmetz, L. M. (2017). Human haematopoietic stem cell lineage commitment is a continuous process. *Nature Cell Biology*, 19(4), 271–281. <https://doi.org/10.1038/ncb3493>

Verovskaya, E., Broekhuis, M. J. C., Zwart, E., Ritsema, M., van Os, R., de Haan, G., & Bystrykh, L. V. (2013). Heterogeneity of young and aged murine hematopoietic stem cells revealed by quantitative clonal analysis using cellular barcoding. *Blood*, 122(4), 523–532. <https://doi.org/10.1182/blood-2013-01-481135>

Verovskaya, E., Broekhuis, M. J. C., Zwart, E., Weersing, E., Ritsema, M., Bosman, L. J., van Poele, T., de Haan, G., & Bystrykh, L. V. (2014). Asymmetry in skeletal distribution of mouse hematopoietic stem cell clones and their equilibration by mobilizing cytokines. *The Journal of Experimental Medicine*, 211(3), 487–497. <https://doi.org/10.1084/jem.20131804>

Vincent, J. P., Girdham, C. H., & O'Farrell, P. H. (1994). A cell-autonomous, ubiquitous marker for the analysis of *Drosophila* genetic mosaics. *Developmental Biology*, 164(1), 328–331. <https://doi.org/10.1006/dbio.1994.1203>

Vishwakarma, M., & Piddini, E. (2020). Outcompeting cancer. *Nature Reviews. Cancer*, 20(3), 187–198. <https://doi.org/10.1038/s41568-019-0231-8>

Visser, J. W., Bauman, J. G., Mulder, A. H., Eliason, J. F., & de Leeuw, A. M. (1984). Isolation of murine pluripotent hemopoietic stem cells. *The Journal of Experimental Medicine*, 159(6), 1576–1590. <https://doi.org/10.1084/jem.159.6.1576>

Visvader, J. E., & Clevers, H. (2016). Tissue-specific designs of stem cell hierarchies. *Nature Cell Biology*, 18(4), 349–355. <https://doi.org/10.1038/ncb3332>

Voit, R. A., Tao, L., Yu, F., Cato, L. D., Cohen, B., Fleming, T. J., Antoszewski, M., Liao, X., Fiorini, C., Nandakumar, S. K., Wahlster, L., Teichert, K., Regev, A., & Sankaran, V. G. (2023). A genetic disorder reveals a hematopoietic stem cell regulatory network co-opted in leukemia. *Nature Immunology*, 24(1), 69–83. <https://doi.org/10.1038/s41590-022-01370-4>

von Bubnoff, A. (2024). The Spemann-Mangold organizer discovery and society. *Cells & Development*, 178(203906), 203906. <https://doi.org/10.1016/j.cdev.2024.203906>

Vukotić, M., Kapor, S., Dragojević, T., Đikić, D., Mitrović Ajtić, O., Diklić, M., Subotički, T., Živković, E., Beleslin Čokić, B., Vojvodić, A., Santibáñez, J. F., Gotić, M., & Čokić, V. P. (2022). Inhibition of proinflammatory signaling impairs fibrosis of bone marrow mesenchymal stromal cells in myeloproliferative neoplasms. *Experimental & Molecular Medicine*, 54(3), 273–284. <https://doi.org/10.1038/s12276-022-00742-y>

Waddington, C. H. (2012). The epigenotype. 1942. *International Journal of Epidemiology*, 41(1), 10–13. <https://doi.org/10.1093/ije/dyr184>

Waddington, C. H. (2014). *The strategy of the genes*. Routledge. <https://doi.org/10.4324/9781315765471>

Wagers, A. J., & Weissman, I. L. (2004). Plasticity of adult stem cells. *Cell*, 116(5), 639–648. [https://doi.org/10.1016/s0092-8674\(04\)00208-9](https://doi.org/10.1016/s0092-8674(04)00208-9)

Walasek, M. A., van Os, R., & de Haan, G. (2012). Hematopoietic stem cell expansion: challenges and opportunities. *Annals of the New York Academy of Sciences*, 1266(1), 138–150. <https://doi.org/10.1111/j.1749-6632.2012.06549.x>

Wang, I. M., Contursi, C., Masumi, A., Ma, X., Trinchieri, G., & Ozato, K. (2000). An IFN- γ -inducible transcription factor, IFN consensus sequence binding protein (ICSBP), stimulates IL-12 p40 expression in macrophages. *The Journal of Immunology*, 165(1), 271–279. <https://doi.org/10.4049/jimmunol.165.1.271>

Wang, J., Rao, Q., Wang, M., Wei, H., Xing, H., Liu, H., Wang, Y., Tang, K., Peng, L., Tian, Z., & Wang, J. (2009). Overexpression of Rac1 in leukemia patients and its role in leukemia cell migration and growth. *Biochemical and Biophysical Research Communications*, 386(4), 769–774. <https://doi.org/10.1016/j.bbrc.2009.06.125>

Wang, J.-Y., Yu, P., Chen, S., Xing, H., Chen, Y., Wang, M., Tang, K., Tian, Z., Rao, Q., & Wang, J. (2013). Activation of Rac1 GTPase promotes leukemia cell chemotherapy resistance, quiescence and niche interaction. *Molecular Oncology*, 7(5), 907–916. <https://doi.org/10.1016/j.molonc.2013.05.001>

Wang, S., Hu, S., Luo, X., Bao, X., Li, J., Liu, M., Lv, Y., Zhao, C., Zeng, M., Chen, X., Unsworth, A., Jones, S., Johnson, T. W., White, S. J., Jia, H., & Yu, B. (2022). Prevalence and prognostic significance of DNMT3A- and TET2- clonal haematopoiesis-driver mutations in patients presenting with ST-segment elevation myocardial infarction. *EBioMedicine*, 78(103964), 103964. <https://doi.org/10.1016/j.ebiom.2022.103964>

Wang, W., Zhang, Y., Dettinger, P., Reimann, A., Kull, T., Loeffler, D., Manz, M. G., Lengerke, C., & Schroeder, T. (2021). Cytokine combinations for human blood stem cell expansion induce cell-type- and cytokine-specific signaling dynamics. *Blood*, 138(10), 847–857. <https://doi.org/10.1182/blood.2020008386>

- Wang, Y., & Zuo, X. (2019). Cytokines frequently implicated in myeloproliferative neoplasms. *Cytokine*, 116, 100005. <https://doi.org/10.1016/j.cytox.2019.100005>
- Wang, Z., Fang, Y., Wang, R., Kong, L., Liang, S., & Tao, S. (2024). Reconstructing tumor clonal heterogeneity and evolutionary relationships based on tumor DNA sequencing data. *Briefings in Bioinformatics*, 25(6). <https://doi.org/10.1093/bib/bbae516>
- Watson, C. J., Poon, G. Y. P., MacGregor, H. A. J., Fonseca, A. V. A., Apostolidou, S., Gentry-Maharaj, A., Menon, U., & Blundell, J. R. (2024). Evolutionary dynamics in the decades preceding acute myeloid leukaemia. In *bioRxiv*. <https://doi.org/10.1101/2024.07.05.602251>
- Weber, T. S., Dukes, M., Miles, D. C., Glaser, S. P., Naik, S. H., & Duffy, K. R. (2016). Site-specific recombinatorics: in situ cellular barcoding with the Cre Lox system. *BMC Systems Biology*, 10(1), 43. <https://doi.org/10.1186/s12918-016-0290-3>
- Wehling, A., Loeffler, D., Zhang, Y., Kull, T., Donato, C., Szczerba, B., Camargo Ortega, G., Lee, M., Moor, A., Göttgens, B., Aceto, N., & Schroeder, T. (2022). Combining single-cell tracking and omics improves blood stem cell fate regulator identification. *Blood*, 140(13), 1482–1495. <https://doi.org/10.1182/blood.2022016880>
- Weijts, B., Yvernogeau, L., & Robin, C. (2021). Recent advances in developmental hematopoiesis: Diving deeper with new technologies. *Frontiers in Immunology*, 12, 790379. <https://doi.org/10.3389/fimmu.2021.790379>
- Weinreb, C., Rodriguez-Fraticelli, A., Camargo, F. D., & Klein, A. M. (2020). Lineage tracing on transcriptional landscapes links state to fate during differentiation. *Science (New York, N.Y.)*, 367(6479), eaaw3381. <https://doi.org/10.1126/science.aaw3381>
- Weirich, G., Mengele, K., Yfanti, C., Gkazepis, A., Hellmann, D., Welk, A., Giersig, C., Kuo, W.-L., Rosner, M. R., Tang, W.-J., & Schmitt, M. (2008). Immunohistochemical evidence of ubiquitous distribution of the metalloendoprotease insulin-degrading enzyme (IDE; insulysin) in human non-malignant tissues and tumor cell lines. *Biological Chemistry*, 389(11), 1441–1445. <https://doi.org/10.1515/BC.2008.157>
- Weisblat, D. A., Sawyer, R. T., & Stent, G. S. (1978). Cell lineage analysis by intracellular injection of a tracer enzyme. *Science (New York, N.Y.)*, 202(4374), 1295–1298. <https://doi.org/10.1126/science.725606>
- Weiss, M. L., & Troyer, D. L. (2006). Stem cells in the umbilical cord. *Stem Cell Reviews*, 2(2), 155–162. <https://doi.org/10.1007/s12015-006-0022-y>
- Weissman, I. L. (2000). Stem cells: units of development, units of regeneration, and units in evolution. *Cell*, 100(1), 157–168. [https://doi.org/10.1016/s0092-8674\(00\)81692-x](https://doi.org/10.1016/s0092-8674(00)81692-x)
- Weissman, Irving L. (2002). The road ended up at stem cells. *Immunological Reviews*, 185(1), 159–174. <https://doi.org/10.1034/j.1600-065x.2002.18514.x>
- Weissman, Irving L., & Shizuru, J. A. (2008). The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. *Blood*, 112(9), 3543–3553. <https://doi.org/10.1182/blood-2008-08-078220>
- Weksberg, D. C., Chambers, S. M., Boles, N. C., & Goodell, M. A. (2008). CD150- side population cells represent a functionally distinct population of long-term hematopoietic stem cells. *Blood*, 111(4), 2444–2451. <https://doi.org/10.1182/blood-2007-09-115006>

Welch, J. S., Ley, T. J., Link, D. C., Miller, C. A., Larson, D. E., Koboldt, D. C., Wartman, L. D., Lamprecht, T. L., Liu, F., Xia, J., Kandoth, C., Fulton, R. S., McLellan, M. D., Dooling, D. J., Wallis, J. W., Chen, K., Harris, C. C., Schmidt, H. K., Kalicki-Veizer, J. M., ... Wilson, R. K. (2012). The origin and evolution of mutations in acute myeloid leukemia. *Cell*, 150(2), 264–278. <https://doi.org/10.1016/j.cell.2012.06.023>

Weng, C., Yu, F., Yang, D., Poeschla, M., Liggett, L. A., Jones, M. G., Qiu, X., Wahlster, L., Caulier, A., Hussmann, J. A., Schnell, A., Yost, K. E., Koblan, L. W., Martin-Rufino, J. D., Min, J., Hammond, A., Ssozi, D., Bueno, R., Mallidi, H., ... Sankaran, V. G. (2024). Deciphering cell states and genealogies of human haematopoiesis. *Nature*, 627(8003), 389–398. <https://doi.org/10.1038/s41586-024-07066-z>

Wernig, G., Mercher, T., Okabe, R., Levine, R. L., Lee, B. H., & Gilliland, D. G. (2006). Expression of Jak2V617F causes a polycythemia vera-like disease with associated myelofibrosis in a murine bone marrow transplant model. *Blood*, 107(11), 4274–4281. <https://doi.org/10.1182/blood-2005-12-4824>

Wijewardhane, N., Dressler, L., & Ciccarelli, F. D. (2021). Normal somatic mutations in cancer transformation. *Cancer Cell*, 39(2), 125–129. <https://doi.org/10.1016/j.ccell.2020.11.002>

Wilkinson, A. C., Dever, D. P., Baik, R., Camarena, J., Hsu, I., Charlesworth, C. T., Morita, C., Nakauchi, H., & Porteus, M. H. (2021). Cas9-AAV6 gene correction of beta-globin in autologous HSCs improves sickle cell disease erythropoiesis in mice. *Nature Communications*, 12(1), 686. <https://doi.org/10.1038/s41467-021-20909-x>

Wilkinson, A. C., Ishida, R., Kikuchi, M., Sudo, K., Morita, M., Crisostomo, R. V., Yamamoto, R., Loh, K. M., Nakamura, Y., Watanabe, M., Nakauchi, H., & Yamazaki, S. (2019). Long-term ex vivo haematopoietic-stem-cell expansion allows nonconditioned transplantation. *Nature*, 571(7763), 117–121. <https://doi.org/10.1038/s41586-019-1244-x>

Wilkinson, A. C., Ishida, R., Nakauchi, H., & Yamazaki, S. (2020). Long-term ex vivo expansion of mouse hematopoietic stem cells. *Nature Protocols*, 15(2), 628–648. <https://doi.org/10.1038/s41596-019-0263-2>

Williams, D. A., Lemischka, I. R., Nathan, D. G., & Mulligan, R. C. (1984). Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse. *Nature*, 310(5977), 476–480. <https://doi.org/10.1038/310476a0>

Williams, N., Lee, J., Mitchell, E., Moore, L., Baxter, E. J., Hewinson, J., Dawson, K. J., Menzies, A., Godfrey, A. L., Green, A. R., Campbell, P. J., & Nangalia, J. (2022). Life histories of myeloproliferative neoplasms inferred from phylogenies. *Nature*, 602(7895), 162–168. <https://doi.org/10.1038/s41586-021-04312-6>

Wilson, N. K., Kent, D. G., Buettner, F., Shehata, M., Macaulay, I. C., Calero-Nieto, F. J., Sánchez Castillo, M., Oedekoven, C. A., Diamanti, E., Schulte, R., Ponting, C. P., Voet, T., Caldas, C., Stingl, J., Green, A. R., Theis, F. J., & Göttgens, B. (2015). Combined single-cell functional and gene expression analysis resolves heterogeneity within stem cell populations. *Cell Stem Cell*, 16(6), 712–724. <https://doi.org/10.1016/j.stem.2015.04.004>

Woods, K., & Guezguez, B. (2021). Dynamic changes of the bone marrow niche: Mesenchymal stromal cells and their progeny during aging and leukemia. *Frontiers in Cell and Developmental Biology*, 9, 714716. <https://doi.org/10.3389/fcell.2021.714716>

Worton, R. G., McCulloch, E. A., & Till, J. E. (1969). Physical separation of hemopoietic stem cells differing in their capacity for self-renewal. *The Journal of Experimental Medicine*, 130(1), 91–103. <https://doi.org/10.1084/jem.130.1.91>

Wu, J. Z., Sinsel, E. W., Shroyer, J. F., Warren, C. M., Welcome, D. E., Zhao, K. D., An, K.-N., & Buczek, F. L. (2014). Analysis of the musculoskeletal loading of the thumb during pipetting--a pilot study. *Journal of Biomechanics*, 47(2), 392–399. <https://doi.org/10.1016/j.jbiomech.2013.11.015>

Wu, M., Li, L., Hamaker, M., Small, D., & Duffield, A. S. (2019). FLT3-ITD cooperates with Rac1 to modulate the sensitivity of leukemic cells to chemotherapeutic agents via regulation of DNA repair pathways. *Haematologica*, 104(12), 2418–2428. <https://doi.org/10.3324/haematol.2018.208843>

Wu, Q., Zhang, J., Kumar, S., Shen, S., Kincaid, M., Johnson, C. B., Zhang, Y. S., Turcotte, R., Alt, C., Ito, K., Homan, S., Sherman, B. E., Shao, T.-Y., Slaughter, A., Weinhaus, B., Song, B., Filippi, M. D., Grimes, H. L., Lin, C. P., ... Lucas, D. (2024). Resilient anatomy and local plasticity of naive and stress haematopoiesis. *Nature*, 627(8005), 839–846. <https://doi.org/10.1038/s41586-024-07186-6>

Wu, Q., Zhang, J., & Lucas, D. (2021). Anatomy of hematopoiesis and local microenvironments in the bone marrow. Where to? *Frontiers in Immunology*, 12, 768439. <https://doi.org/10.3389/fimmu.2021.768439>

Xiao, Y., McGuinness, C. S., Doherty-Boyd, W. S., Salmeron-Sanchez, M., Donnelly, H., & Dalby, M. J. (2022). Current insights into the bone marrow niche: From biology in vivo to bioengineering ex vivo. *Biomaterials*, 286(121568), 121568. <https://doi.org/10.1016/j.biomaterials.2022.121568>

Xie, T., & Spradling, A. C. (1998). decapentaplegic is essential for the maintenance and division of germline stem cells in the *Drosophila* ovary. *Cell*, 94(2), 251–260. [https://doi.org/10.1016/s0092-8674\(00\)81424-5](https://doi.org/10.1016/s0092-8674(00)81424-5)

Xing, S., Wanting, T. H., Zhao, W., Ma, J., Wang, S., Xu, X., Li, Q., Fu, X., Xu, M., & Zhao, Z. J. (2008). Transgenic expression of JAK2V617F causes myeloproliferative disorders in mice. *Blood*, 111(10), 5109–5117. <https://doi.org/10.1182/blood-2007-05-091579>

Xiong, H., Zhang, H., Bai, J., Li, Y., Li, L., & Zhang, L. (2024). Associations of the circulating levels of cytokines with the risk of myeloproliferative neoplasms: a bidirectional mendelian-randomization study. *BMC Cancer*, 24(1), 531. <https://doi.org/10.1186/s12885-024-12301-x>

Yagi, M., Ritchie, K. A., Sitnicka, E., Storey, C., Roth, G. J., & Bartelmez, S. (1999). Sustained ex vivo expansion of hematopoietic stem cells mediated by thrombopoietin. *Proceedings of the National Academy of Sciences of the United States of America*, 96(14), 8126–8131. <https://doi.org/10.1073/pnas.96.14.8126>

Yamamoto, R., Morita, Y., Ooehara, J., Hamanaka, S., Onodera, M., Rudolph, K. L., Ema, H., & Nakauchi, H. (2013). Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell*, 154(5), 1112–1126. <https://doi.org/10.1016/j.cell.2013.08.007>

Yamamoto, R., Wilkinson, A. C., Ooehara, J., Lan, X., Lai, C.-Y., Nakauchi, Y., Pritchard, J. K., & Nakauchi, H. (2018). Large-scale clonal analysis resolves aging of the mouse hematopoietic stem cell compartment. *Cell Stem Cell*, 22(4), 600–607.e4. <https://doi.org/10.1016/j.stem.2018.03.013>

- Yamashita, M., & Passegué, E. (2019). TNF- α coordinates hematopoietic stem cell survival and myeloid regeneration. *Cell Stem Cell*, 25(3), 357-372.e7. <https://doi.org/10.1016/j.stem.2019.05.019>
- Yang, D., Sun, W., Gao, L., Zhao, K., Zhuang, Q., & Cai, Y. (2025). Cell competition as an emerging mechanism and therapeutic target in cancer. *Biochimica et Biophysica Acta. Molecular Basis of Disease*, 1871(5), 167769. <https://doi.org/10.1016/j.bbadis.2025.167769>
- Yang, F. C., Atkinson, S. J., Gu, Y., Borneo, J. B., Roberts, A. W., Zheng, Y., Pennington, J., & Williams, D. A. (2001). Rac and Cdc42 GTPases control hematopoietic stem cell shape, adhesion, migration, and mobilization. *Proceedings of the National Academy of Sciences of the United States of America*, 98(10), 5614–5618. <https://doi.org/10.1073/pnas.101546898>
- Yang, F., Nourse, C., Helgason, G. V., & Kirschner, K. (2023). Unraveling heterogeneity in the aging hematopoietic stem cell compartment: An insight from single-cell approaches. *HemaSphere*, 7(6), e895. <https://doi.org/10.1097/HS9.0000000000000895>
- Yang, L., Wang, L., Geiger, H., Cancelas, J. A., Mo, J., & Zheng, Y. (2007). Rho GTPase Cdc42 coordinates hematopoietic stem cell quiescence and niche interaction in the bone marrow. *Proceedings of the National Academy of Sciences of the United States of America*, 104(12), 5091–5096. <https://doi.org/10.1073/pnas.0610819104>
- Yang, S. X., Hollingshead, M., Rubinstein, L., Nguyen, D., Larenjeira, A. B. A., Kinders, R. J., Difilippantonio, M., & Doroshow, J. H. (2021). TET2 and DNMT3A mutations and exceptional response to 4'-thio-2'-deoxycytidine in human solid tumor models. *Journal of Hematology & Oncology*, 14(1), 83. <https://doi.org/10.1186/s13045-021-01091-5>
- Yang, Y., Cathelin, S., Liu, A. C. H., Subedi, A., Maher, A., Hosseini, M., Manikoth Ayyathan, D., Vanner, R., & Chan, S. M. (2025). TET2 deficiency increases the competitive advantage of hematopoietic stem and progenitor cells through upregulation of thrombopoietin receptor signaling. *Nature Communications*, 16(1), 2384. <https://doi.org/10.1038/s41467-025-57614-y>
- Yang, Z., Dong, R., Mao, X., He, X. C., & Li, L. (2024). Stress-protecting harbors for hematopoietic stem cells. *Current Opinion in Cell Biology*, 86(102284), 102284. <https://doi.org/10.1016/j.ceb.2023.102284>
- Yao, Y., & Wang, C. (2020). Dedifferentiation: inspiration for devising engineering strategies for regenerative medicine. *Npj Regenerative Medicine*, 5(1), 14. <https://doi.org/10.1038/s41536-020-00099-8>
- Yarmolinsky, M., & Hoess, R. (2015). The legacy of Nat Sternberg: The genesis of Cre-lox technology. *Annual Review of Virology*, 2(1), 25–40. <https://doi.org/10.1146/annurev-virology-100114-054930>
- Yfanti, C., Mengele, K., Gkazepis, A., Weirich, G., Giersig, C., Kuo, W.-L., Tang, W.-J., Rosner, M., & Schmitt, M. (2008). Expression of metalloprotease insulin-degrading enzyme insulysin in normal and malignant human tissues. *International Journal of Molecular Medicine*, 22(4), 421–431. https://doi.org/10.3892/ijmm_00000038
- Yokomizo, T., Ideue, T., Morino-Koga, S., Tham, C. Y., Sato, T., Takeda, N., Kubota, Y., Kurokawa, M., Komatsu, N., Ogawa, M., Araki, K., Osato, M., & Suda, T. (2022). Independent origins of fetal liver haematopoietic stem and progenitor cells. *Nature*, 609(7928), 779–784. <https://doi.org/10.1038/s41586-022-05203-0>

Yoneyama, H., Narumi, S., Zhang, Y., Murai, M., Baggiolini, M., Lanzavecchia, A., Ichida, T., Asakura, H., & Matsushima, K. (2002). Pivotal role of dendritic cell-derived CXCL10 in the retention of T helper cell 1 lymphocytes in secondary lymph nodes. *The Journal of Experimental Medicine*, 195(10), 1257–1266. <https://doi.org/10.1084/jem.20011983>

Young, K., Borikar, S., Bell, R., Kuffler, L., Philip, V., & Trowbridge, J. J. (2016). Progressive alterations in multipotent hematopoietic progenitors underlie lymphoid cell loss in aging. *The Journal of Experimental Medicine*, 213(11), 2259–2267. <https://doi.org/10.1084/jem.20160168>

Yu, X., Song, Z., Rao, L., Tu, Q., Zhou, J., Yin, Y., & Chen, D. (2020). Synergistic induction of CCL5, CXCL9 and CXCL10 by IFN- γ and NLRs ligands on human fibroblast-like synoviocytes-A potential immunopathological mechanism for joint inflammation in rheumatoid arthritis. *International Immunopharmacology*, 82(106356), 106356. <https://doi.org/10.1016/j.intimp.2020.106356>

Yusupova, M., & Fuchs, Y. (2023). To not love thy neighbor: mechanisms of cell competition in stem cells and beyond. *Cell Death and Differentiation*, 30(4), 979–991. <https://doi.org/10.1038/s41418-023-01114-3>

Zaleskas, V. M., Krause, D. S., Lazarides, K., Patel, N., Hu, Y., Li, S., & Van Etten, R. A. (2006). Molecular pathogenesis and therapy of polycythemia induced in mice by JAK2 V617F. *PloS One*, 1(1), e18. <https://doi.org/10.1371/journal.pone.0000018>

Zeller, C., Richter, D., Jurinovic, V., Valtierra-Gutiérrez, I. A., Jayavelu, A. K., Mann, M., Bagnoli, J. W., Hellmann, I., Herold, T., Enard, W., Vick, B., & Jeremias, I. (2022). Adverse stem cell clones within a single patient's tumor predict clinical outcome in AML patients. *Journal of Hematology & Oncology*, 15(1), 25. <https://doi.org/10.1186/s13045-022-01232-4>

Zhang, D., Kan, X., Huss, S. E., Jiang, L., Chen, L.-Q., & Hu, Y. (2018). Using phylogenetic analysis to investigate eukaryotic gene origin. *Journal of Visualized Experiments: JoVE*, 138. <https://doi.org/10.3791/56684>

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.-G., Ross, J., Haug, J., Johnson, T., Feng, J. Q., Harris, S., Wiedemann, L. M., Mishina, Y., & Li, L. (2003). Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*, 425(6960), 836–841. <https://doi.org/10.1038/nature02041>

Zhang, P., Li, X., Pan, C., Zheng, X., Hu, B., Xie, R., Hu, J., Shang, X., & Yang, H. (2022). Single-cell RNA sequencing to track novel perspectives in HSC heterogeneity. *Stem Cell Research & Therapy*, 13(1), 39. <https://doi.org/10.1186/s13287-022-02718-1>

Zhang, Qian, Zhang, Y., Zhang, Q., Li, L., & Zhao, S. (2023). Division promotes adult stem cells to perform active niche competition. *Genetics*, 224(1). <https://doi.org/10.1093/genetics/iyad035>

Zhang, Qinyu, Olofzon, R., Konturek-Ciesla, A., Yuan, O., & Bryder, D. (2024). Ex vivo expansion potential of murine hematopoietic stem cells is a rare property only partially predicted by phenotype. *eLife*, 12, RP91826. <https://doi.org/10.7554/eLife.91826>

Zhang, Qiuwan, & Lai, D. (2020). Application of human amniotic epithelial cells in regenerative medicine: a systematic review. *Stem Cell Research & Therapy*, 11(1), 439. <https://doi.org/10.1186/s13287-020-01951-w>

Zhang, Xiaotian, Su, J., Jeong, M., Ko, M., Huang, Y., Park, H. J., Guzman, A., Lei, Y., Huang, Y.-H., Rao, A., Li, W., & Goodell, M. A. (2016). DNMT3A and TET2 compete and cooperate to repress lineage-specific transcription factors in hematopoietic stem cells. *Nature Genetics*, 48(9), 1014–1023. <https://doi.org/10.1038/ng.3610>

Zhang, Xinchao, Zhang, Y., Wang, C., & Wang, X. (2023). TET (Ten-eleven translocation) family proteins: structure, biological functions and applications. *Signal Transduction and Targeted Therapy*, 8(1), 297. <https://doi.org/10.1038/s41392-023-01537-x>

Zhao, R., & Xi, R. (2010). Stem cell competition for niche occupancy: emerging themes and mechanisms. *Stem Cell Reviews*, 6(3), 345–350. <https://doi.org/10.1007/s12015-010-9128-3>

Zheng, G. X. Y., Terry, J. M., Belgrader, P., Ryvkin, P., Bent, Z. W., Wilson, R., Ziraldo, S. B., Wheeler, T. D., McDermott, G. P., Zhu, J., Gregory, M. T., Shuga, J., Montesclaros, L., Underwood, J. G., Masquelier, D. A., Nishimura, S. Y., Schnall-Levin, M., Wyatt, P. W., Hindson, C. M., ... Bielas, J. H. (2017). Massively parallel digital transcriptional profiling of single cells. *Nature Communications*, 8(1), 14049. <https://doi.org/10.1038/ncomms14049>

Zheng, Q., Li, M., Li, R., Shen, H., Zhang, Z., & Tian, X. (2022). IGSF6 is a prognostic biomarker associated with the anti-tumor activity of macrophages in lung adenocarcinoma. In *Research Square*. <https://doi.org/10.21203/rs.3.rs-1837141/v1>

Zhou, H.-S., Carter, B. Z., & Andreeff, M. (2016). Bone marrow niche-mediated survival of leukemia stem cells in acute myeloid leukemia: Yin and Yang. *Cancer Biology & Medicine*, 13(2), 248–259. <https://doi.org/10.20892/j.issn.2095-3941.2016.0023>

Zhou, X., Florian, M. C., Arumugam, P., Chen, X., Cancelas, J. A., Lang, R., Malik, P., Geiger, H., & Zheng, Y. (2013). RhoA GTPase controls cytokinesis and programmed necrosis of hematopoietic progenitors. *The Journal of Experimental Medicine*, 210(11), 2371–2385. <https://doi.org/10.1084/jem.20122348>

Zhu, Y., Wunderlich, Z., & Lander, A. D. (2025). Epithelial cell competition is promoted by signaling from immune cells. *Nature Communications*, 16(1), 3710. <https://doi.org/10.1038/s41467-025-59130-5>

Zijlmans, J. M., Visser, J. W., Kleiverda, K., Kluin, P. M., Willemze, R., & Fibbe, W. E. (1995). Modification of rhodamine staining allows identification of hematopoietic stem cells with preferential short-term or long-term bone marrow-repopulating ability. *Proceedings of the National Academy of Sciences of the United States of America*, 92(19), 8901–8905. <https://doi.org/10.1073/pnas.92.19.8901>

Ziosi, M., Baena-López, L. A., Grifoni, D., Frolidi, F., Pession, A., Garoia, F., Trotta, V., Bellosta, P., Cavicchi, S., & Pession, A. (2010). dMyc functions downstream of Yorkie to promote the supercompetitive behavior of hippo pathway mutant cells. *PLoS Genetics*, 6(9), e1001140. <https://doi.org/10.1371/journal.pgen.1001140>