

Investigating the Specific Roles of the Growth Factor Kit Ligand in the Regulation of Murine Haematopoiesis



Raffaella Facchini

St Cross College

Haematopoietic Stem Cell Laboratory

The Weatherall Institute of Molecular Medicine

University of Oxford

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Abstract

The bone marrow (BM) niche microenvironment provides haematopoietic stem (HSC) and progenitor cells with different exogenous cues, which maintain a tight regulation of haematopoiesis. These signals are in part orchestrated by cytokines, which can be bound to the cell surface of niche stromal cells or are freely diffusible soluble proteins. Herein, this work aimed to investigate the cytokine kit ligand (Kitl), specifically the roles of its membrane-associated (mKitl) and soluble (sKitl) isoforms within haematopoiesis.

Using the *Sl/Sl^d* mutant as a starting point, I sought to study using novel mouse models, the impact on haematopoiesis, following the specific loss of *mKitl* from all tissues, loss of *mKitl* or total *Kitl* (*mKitl* + *sKitl*) from specific stromal cell populations and loss of *mKitl* or total *Kitl* from BM in the presence of unaffected systemic sKitl in kidney subcapsular explant studies. Additionally, to gain further understanding of Kitl within niche biology, the spermatogonial niche was also investigated by a cell-specific KO approach.

Total germline deletion of *mKitl* disrupted the stem/progenitor-stromal cell interaction within the BM and resulted in reduced HSCs and erythroid progenitors. Specific deletion of *mKitl* from individual *Kitl*-expressing stromal cell populations in young mice had a limited effect upon haematopoiesis, however deletion of *mKitl* from multiple cell types simultaneously resulted in anaemia and reductions in certain myeloid progenitor cell populations. The phenotypes seen in corresponding aged mice were more defined and also impacted HSC numbers.

Analysis of mice with cell-specific loss of total *Kitl* revealed more marked haematopoietic defects, notably fewer phenotypic HSCs. The loss of either *mKitl* or total *Kitl* in a number of models also resulted in significant reductions in circulating sKitl levels, indicating a role for mKitl in the regulation of overall sKitl expression. The data generated also confirmed the importance of sKitl to HSC maintenance.

Within the BM, the combined action of mKitl, systemic and local sKitl all contribute to maintaining normal murine haematopoiesis in the BM niche. In the spermatogonial niche, deletion of *mKitl* in the Sertoli cells resulted in a significant reduction in the number of c-Kit⁺ spermatogonial progenitor cells. This effect was localised to the testes and occurred despite unaffected levels of systemic sKitl.

Overall, the work reported here has for the first time suggested different requirements for the two isoforms of the cytokine Kitl in spatially and functionally distinct niches *in vivo*.

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Commonly Used Abbreviations

3D – Three-dimensional

7-AAD – 7-Aminoactinomycin D

Ang-1 – Angiopoietin 1

ABC – ATP- binding cassette

ADAM – A disintegrin and metalloproteinase

APC – Allophycocyanin

AKT1 – v-akt murine thymoma viral oncogene homolog 1

AMH – Anti-Müllerian hormone

AML – Acute myeloid leukaemia

Ang - Angiopoietin

BCL-2 – B cell lymphoma 2

BFU-E – Burst forming unit-erythroid

BLC – Bone lining cell

BM – Bone marrow

BMI – Bmi1 polycomb ring finger

BMPR2 – Bone morphogenic protein receptor

bp – Base pair

BV – Brilliant Violet

CAR – CXCL12 abundant reticular

CC – Cell cycle

CBM – Central bone marrow

CD – Cluster of differentiation

cDNA – Complementary deoxyribonucleic acid

CFU-E – Colony-forming-unit erythroid

CML – Chronic myeloid leukaemia

CMP – Common myeloid progenitor

CNS – Central nervous system

CO₂ – Carbon dioxide

CSF-1 – Colony stimulating factor-1

CXCL12 – CXC-chemokine ligand 12

CXCR4 – CXC chemokine receptor 4

dKO – Double KO

DAPI – 4', 6-diamino-2-phenylindole

DMEM – Dulbecco's modified eagle medium

DN – Double-negative thymocyte progenitor

DNMT – DNA methyltransferase

dNTP – Deoxyribonucleotide triphosphate

dpc – Days post conception

E – Embryonic day

EC – Endothelial cell

EDTA – Ethylene-diamine-tetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

EPO – Erythropoietin

ES – Embryonic stem

ETP – Early thymic progenitor

Ery Pro – Erythroid progenitor

ExC – Extracellular

FABP4 – Fatty acid binding protein 4

FACS – Fluorescence-activated cell sorting

FBS – Foetal bovine serum

FCS – Foetal calf serum

Flt3 – Fms-related tyrosine kinase 3

Flt3l – Fms-related tyrosine kinase 3 ligand

FMO – Fluorescence minus one

FSC – Forward scatter

g – Gram

G – Gauge

G-CSF – Granulocyte colony-stimulating factor

GFAP – Glial fibrillary acidic protein

gDNA – Genomic deoxyribonucleic acid

GFR α 1 – GDNF family receptor alpha-1

GIST – Gastrointestinal stromal tumour

GM-CSF – Granulocyte macrophage colony-stimulating factor

GFP – Green fluorescent protein

GMP – Granulocyte-macrophage progenitor

HCT – Haematocrit

HEK – Human embryonic kidney

Hgb – Haemoglobin

HIF – Hypoxia-inducible transcription factor

HPC – Haematopoietic progenitor cell

Hr – Hours

HSC – Haematopoietic stem cell

HSPC – Haematopoietic stem and progenitor cells

HUVEC – Human umbilical endothelial cell

IC – Intracellular

ICC – Interstitial cells of Cajal

IFN- γ – Interferon gamma

IHC – Immunohistochemistry

IL – Interleukin

iRES – Internal ribosome entry site

kb – Kilobase

Kitl – Kit ligand (see also SCF)

KO – Knockout

KSE – Kidney subcapsular explantation

LB – Luria-Bertani

LepR – Leptin receptor

Lin⁻ – Lineage negative

LMPP – Lymphoid primed multipotent progenitor

LPA – Linear polyacrylamide

LSK – Lin⁻Sca1⁺KIT⁺

LT – Long term

MAb – Monoclonal antibody

MAPK – Mitogen-activated protein kinase

MCH – Mean cell haemoglobin

MCL-1 – Myeloid cell leukaemia 1

M-CSF – Macrophage-colony stimulating factor

MCV – Mean cell volume

min – Minutes

mKitl – Membrane-bound Kit ligand

MkP – Megakaryocyte progenitor

MMP – Matrix metalloprotease

MNC – Mononuclear cell

MPC – Myeloid progenitor cell

MPP – Multi-potent progenitor

mRNA – Messenger ribonucleic acid

mTOR – Mammalian target of rapamycin

MSC – Mesenchymal stem cell

MTS – [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]

Nes – Nestin

ng – Nanogram

OB – Osteoblast

OC – Osteoclast

O/N – Overnight

PB – Peripheral blood

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PDGF – Platelet-derived growth factor

PDGFR – Platelet-derived growth factor receptor

PE – Phycoerythrin

PFA – Paraformaldehyde

pg – Picogram

PI3K – Phosphoinositide 3-kinase

PKC – Protein kinase C

Plt – Platelet

pO_2 – Oxygen tension

Pre CFU-E– Pre-colony-forming-unit erythroid

Pre GM – Pre-granulocyte/monocyte precursor

Pre MegE – Pre-megakaryocyte/erythroid precursor

PTH – Parathyroid hormone

PTHrP – PTH-related protein

PS – Penicilin streptomycin

PVC – Perivascular cell

qPCR – Quantitative polmerase chain reaction

RANK – Receptor Activator of Nuclear Factor κ B

RBC – Red blood cell

rcf – Relative Centrifugal Force

Rh – Rhodamine 123

RNA – Ribonucleic acid

RT – Reverse transcriptase

RT– STA – Reverse transcriptase specific target amplification

Runx1 – Runt-related transcription factor 1

s – Seconds

SCF – Stem cell factor (Kit ligand)

SEM – Standard error of the mean

sKitl – Soluble Kit ligand

sKO – Single knock out

S/ – Steel

S^d – Steel dickie

SLAM – Signalling lymphocyte activation molecule

SNS – Sympathetic nervous system

SPC – Spermatogonial progenitor cell

SSC – Spermatogonial stem cell

ST – Short term

STAT – Signal transducers and activation of transcription

TAE – Tris-Acetate EDTA

TF – Transcription factor

TGF α – Transforming growth factor alpha

TGF β – Transforming growth factor beta

TKR – Tyrosine kinase receptor

TM – Transmembrane

THPO – Thrombopoietin

TNF α – Tumour necrosis factor alpha

TSC1 – Tuberous sclerosis 1

UV – Ultra violet

V – Volts

VCAM – Vascular cell adhesion molecule

VE-Cad – VE-Cadherin

VEGFR – Vascular endothelial growth factor receptor

VHL – von Hippel-Lindau tumour suppressor

VLA – Very late antigen

WBC – White blood cell

wpc – Weeks post conception

WT – Wild type

YFP – Yellow fluorescent protein

μ l – Microlitre

μ M – Micromolar

ΔC_t – Cycle threshold

1 Introduction

1.1 Haematopoiesis

Haematopoiesis, derived from the ancient Greek “haima” (αἷμα) meaning "blood" and “poietin” (ποιεῖν) meaning "to make", encompasses the diverse processes of blood cell formation. It is a dynamic process which needs to replenish greater than 7×10^9 blood cells per kilogram body weight per day¹. Blood is comprised of many cell types, which derive from haematopoietic stem cells (HSCs). HSCs undergo lineage commitment via stepwise processes of cell division and differentiation to give rise to all mature blood cells. As these cells commit towards a lineage, they successively lose the ability to self-renew and their proliferative capacity². Intrinsic and extrinsic regulators such as cytokines and transcription factors (TFs) tightly regulate the processes involved in self renewal and lineage commitment³. These are precisely regulated to maintain specific numbers of mature blood cells during steady-state haematopoiesis, although the system is highly flexible permitting rapid cellular proliferation, production and migration during periods of haematopoietic stress⁴.

Haematopoiesis being one of the best-studied stem cell renewal and differentiation processes has long acted as a paradigm for understanding tissue hierarchies and disease states both in blood and in other tissues. Continued elucidation of the regulation of haematopoiesis is crucial to understanding tissue renewal during both normal biological processes and after injury/disease, which is essential for improving medical procedures such as bone marrow (BM) transplantation. Haematopoietic processes are dependent on the interaction of HSCs with the components of the BM niche. This is a specialised microenvironment comprised of HSCs, non-haematopoietic stromal cells (endothelial cells (ECs), mesenchymal stem cells (MSCs), osteoblasts (OBs) and perivascular cells (PVCs)), specific temperatures,

oxygen tension, ion gradients and the presence of both membrane-bound and soluble cytokines which have been shown to regulate the maintenance, migration, proliferation, differentiation and quiescence of haematopoietic stem and progenitor cells (HSPCs)⁵⁻⁹.

1.2 Haematopoietic stem cells

HSCs are multipotent, primitive haematopoietic cells which are defined by their capacity for self-renewal, ability to differentiate into all lineages of haemato-lymphoid cells in addition to possessing long-term (>12 months) engraftment potential upon transplantation^{10,11}. The definition of HSCs was initially established in the 1960s by Till and McCulloch who demonstrated the presence of re-populating cells, which had been isolated from mouse BM. By injecting bulk BM cells into irradiated mice, proliferating colonies of spleen-colony forming cells (CFU-S; capable of reconstituting short-term haematopoiesis) formed in the spleen¹². CFU-S were extensively used in early studies, but these cells are now considered to be a more mature progenitor population than 'true HSCs'. Nevertheless, these initial studies opened up the field and led the way for others to increase our understanding of HSCs by investigating their self-renewal, multi-lineage differentiation and re-population capabilities¹³⁻¹⁵.

1.3 Embryonic emergence of haematopoietic stem cells

In mammals, the development of haematopoiesis occurs in two distinct phases¹⁰. Primitive haematopoiesis is first detected extra-embryonically in the yolk sac (YS) at embryonic day (E)7.5 in mice and as early as 30 days post conception (dpc) in humans^{16,17}. In mice, haematopoiesis begins with the production of macrophage progenitors and red blood cells (RBCs) containing embryonic haemoglobin (Hgb)¹⁸. These primitive haematopoietic cells are thought to derive from haemangioblasts,

which are bi-potent progenitors for the endothelial and haematopoietic cell lineages¹⁸⁻²⁰. Additional myeloid progenitors emerge around E8.5 in the YS, allantois and para-aortic-splanchnopleura region (PAS), which becomes the aorta-gonad-mesonephros (AGM)^{16,21}.

The first HSCs with re-populating ability (definitive HSCs) arise in the ventral wall of the dorsal aorta in the AGM region (around E10.5 in mice and 4 weeks post conception (wpc) in humans) to initiate definitive haematopoiesis and later appear in the YS and placenta²²⁻²⁴. HSCs migrate from the AGM to the foetal liver (FL) around E11.5 in mice and 5 wpc in humans¹⁰. The thymus is seeded at approximately E11¹⁶. The FL becomes the main site of haematopoiesis until approximately the second trimester (in humans), at which point the HSCs translocate via the peripheral circulation to seed additional tissues and organs. HSCs will enter the spleen at E14 in mice and then the BM at approximately E17.5 (and 12 wpc in humans)^{25,26}. By birth, the BM is the major site for haematopoiesis in humans and mice, although in the latter, splenic haematopoiesis continues for several weeks post-natally¹⁰. The emergence of murine HSCs is summarised in **Figure 1.1**.

The BM remains the main site of haematopoiesis throughout life for both mice and humans. The ability of HSCs to self-renew and differentiate before the formation of BM cavities during embryogenesis, suggests that HSCs arise and are maintained in niches which do not contain bone throughout most of gestation. This is supported by reports of a close developmental relationship between ECs and HSCs^{19,20} that has led to the suggestion that perivascular progenitors give rise to HSCs during embryonic development which then can associate with blood vessels in the placenta^{3,23,27}.

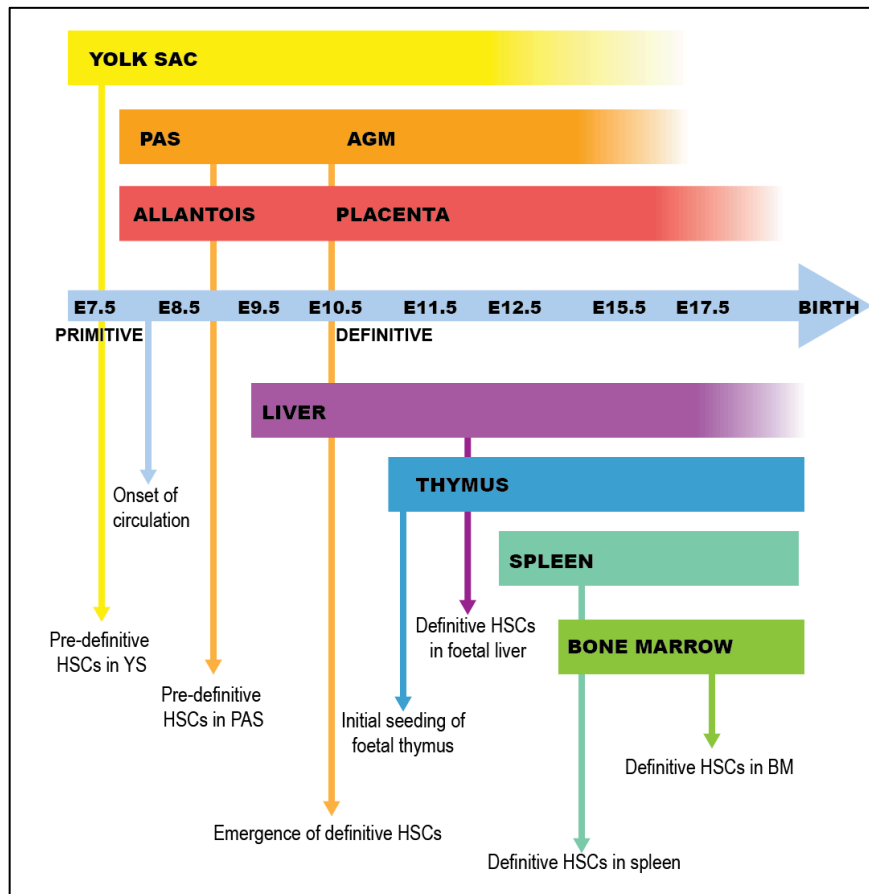


Figure 1.1 Timeline of emergence and development of haematopoiesis in the mouse embryo

First primitive (pre-definitive) haematopoietic cells emerge around day E7.5 in the yolk sac (YS) and E8.5 in the para-aortic splanchnopleura (PAS). Definitive haematopoiesis is initiated at E10.5 when the first definitive HSCs emerge in the AGM. Bars represent the presence of haematopoietic activity in the respective embryonic tissues. The arrows indicate the time of initial emergence/generation of definitive HSCs (unless otherwise indicated) and the initial time-point for colonisation of the secondary haematopoietic tissues. Key: Embryonic day (E), aorta-gonad-mesonephros (AGM), bone marrow (BM). Figure adapted from Dzierzak and Speck, 2008.

1.4 The fate of haematopoietic stem cells

In addition to their defining characteristics of self-renewal and multi-lineage differentiation, HSCs must undergo cell fate decisions such as cell division or apoptosis. They can also be released into the blood circulation (via mobilisation and migration)²⁸. HSCs must maintain the multipotent pool and also differentiate into progressively more mature haematopoietic progenitor cells (HPCs), pre-cursor cells

and mature, circulating blood cells. In order to ensure the haematopoietic system maintains the correct proportion of cells, the size of the HSC pool must be regulated by ensuring the correct balance between HSC differentiation and self-renewal. HSCs can also undergo migration and apoptosis⁴.

Self-renewal can be via symmetrical cell division, which generates two identical HSCs to help expand the stem cell pool or via asymmetrical cell division to generate one HSC and one non-HSC daughter cell to help maintain the stem cell pool.

Differentiation can also proceed via symmetrical cell division to give rise to two identical daughter cells or asymmetrically, to generate two different daughter cells²⁹.

Haematopoietic stem cell fate decisions are summarised in **Figure 1.2**.

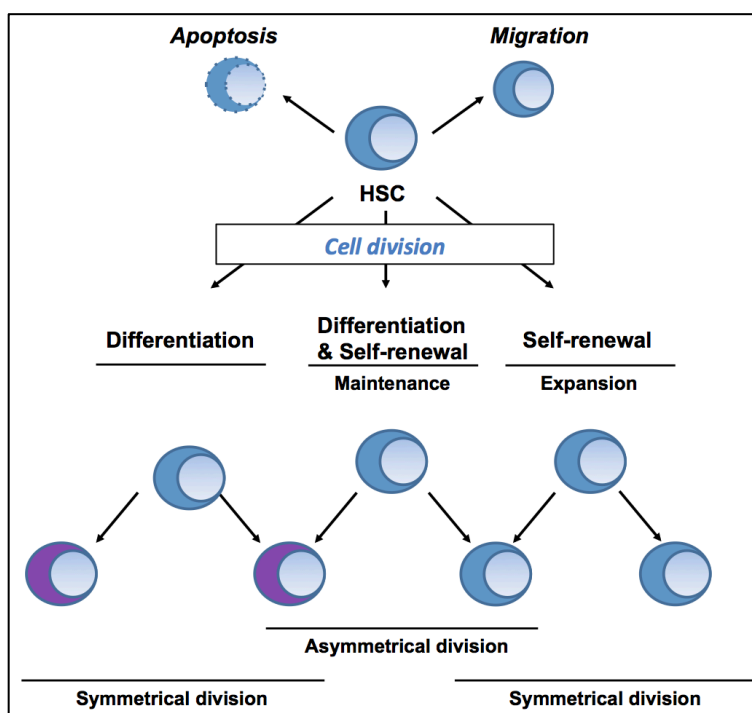


Figure 1.2 Haematopoietic stem cell fate decisions

HSCs have the choice of undergoing cell division, migration or apoptosis. HSCs can undergo symmetrical or asymmetrical cell divisions in their self-renewal or differentiation processes.

1.4.1 Haematopoietic lineage commitment

As stem cells have the ability to form all differentiated blood cells, they are at the top of the haematopoietic progenitor hierarchy. There are currently two main models outlining the steps in lineage commitment: the 'classical model' and the 'alternative model'.

1.4.1.1 The 'classical' model

The 'classical' model proposes that haematopoiesis originates from a homogeneous population of long term self-renewing stem cells (LT-HSC)³⁰ which give rise to short term stem cells (ST-HSC) and in turn generate multi-potent progenitors (MPPs). MPPs retain all lineage potentials but have lost their self-renewal capacity³¹. All three populations (LT-HSC, ST-HSC and MPP) contribute to the lineage marker negative (Lin⁻), Sca-1⁺ and c-Kit⁺ (LSK) population of BM cells. HSCs (LT- and ST-HSCs) are further characterised today based on a series of other markers including Cluster of Differentiation (CD)48, CD150 and CD34 (see **Section 1.5**).

From the MPPs, there is a divergence between myeloid or lymphoid development via the common myeloid progenitor (CMP)³² and common lymphoid progenitor (CLP)³³. CMPs have been defined as Lin⁻ Interleukin-7 receptor (IL-7R)⁻Thy1⁻Sca1⁻c-Kit⁺ CD34⁺FcyR^{lo} with the capacity to generate all cells of the myeloid lineage such as monocytes, granulocytes, erythrocytes, megakaryocytes (Mk) and platelets but they cannot give rise to lymphoid cells³². These myeloid lineages arise after first differentiation of CMPs into i) the granulocyte/monocyte progenitor (GMP; defined as Lin⁻IL-7R⁻Sca1⁻c-Kit⁺ CD34⁻ FcyR^{hi}) which give rise to monocytes and granulocytes or ii) the megakaryocyte/erythroid progenitor (MEP; defined as Lin⁻IL-7R⁻Sca1⁻c-Kit⁺CD34⁻FcyR^{lo}) which generate Mks and erythroid cells³². CLPs defined as Lin⁻IL-

$7R^+Thy1^-Sca1^{lo}c-Kit^{lo}$ generate the lymphoid lineage cells such as natural killer (NK), T and B cells but are unable to give rise to myeloid cells such as erythrocytes or Mk cells³³. However, there has been some debate as to whether dendritic cells are derived from a common precursor or later $CD8\alpha^+$ lymphoid- or $CD8\alpha^-$ myeloid-derived precursors. Although Martín *et al.* seem to favour that dendritic cells derive from a lymphoid-committed precursor³⁴ (**Figure 1.3**).

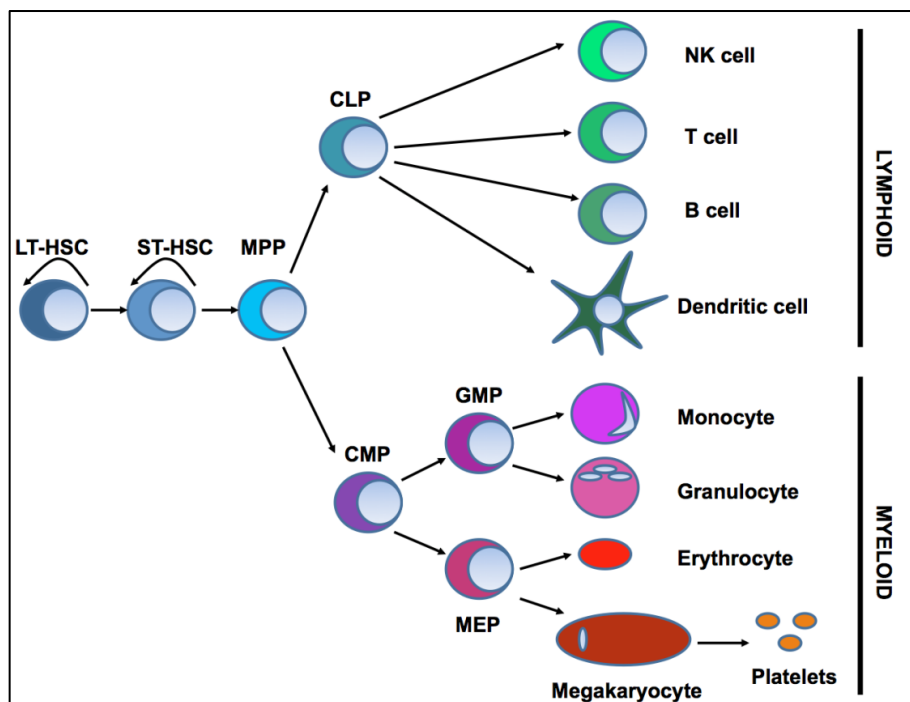


Figure 1.3 'Classical' model of murine haematopoietic cell hierarchy

The 'classical' model demonstrates the sequential restriction of HSCs through fate restriction decisions to form all the differentiated blood cells of distinct lineages with a strict separation of myeloid and lymphoid lineages via the common lymphoid progenitor (CLP) or common myeloid progenitor (CMP). Key: Long-term haematopoietic stem cell (LT-HSC), short-term haematopoietic stem cell (ST-HSC), multi-potent progenitor (MPP), natural killer (NK), granulocyte/macrophage progenitor (GMP), megakaryocyte/erythroid progenitor (MEP). Curved arrows indicate self-renewal potential.

1.4.1.2 The 'alternative' model

Over the last 20 years, further studies by a number of different groups have challenged the classical haematopoietic model; leading to the development of the

‘alternative model’ (**Figure 1.4**). Kawamoto and colleagues presented a ‘myeloid-based’ model arising from the results of a series of their previously published studies. They had developed an *in vitro* method, which enabled the concomitant differentiation of B, T and myeloid cells. Using this assay, they proposed the existence of T/myeloid and B/myeloid progenitors deriving from a myeloid/B/T MPP that lacks erythroid potential³⁵⁻³⁷. More recently, other groups have demonstrated the existence of a lymphoid-primed multi-potent progenitor (LMPP) in both the foetal liver³⁸ and adult BM³⁹. These LMPPs express high levels of Flt3, retain myelo-monocytic capacity but lose megakaryocyte/erythroid-forming ability³⁹.

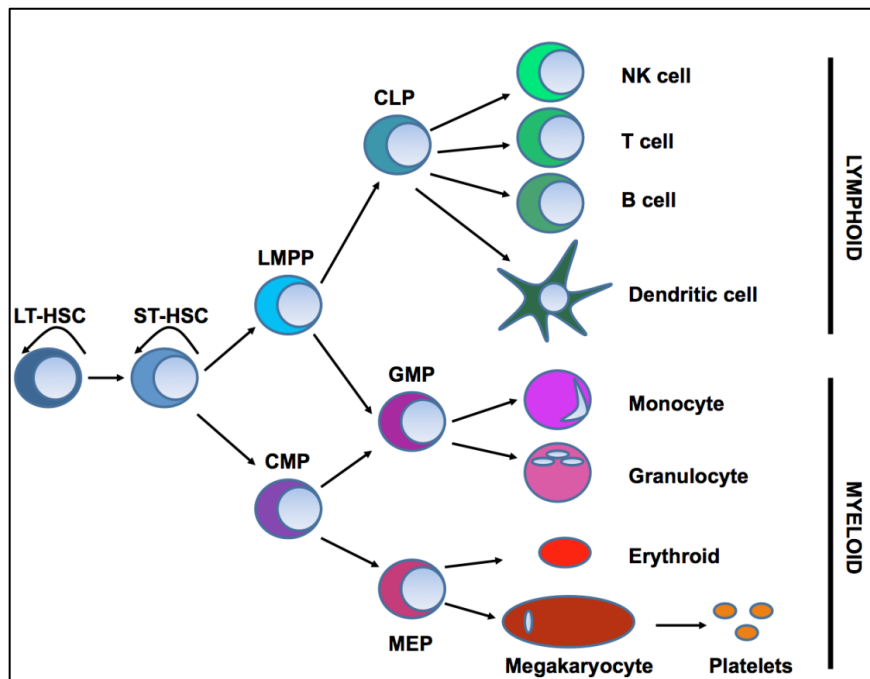


Figure 1.4 ‘Alternative’ model of murine haematopoietic cell hierarchy

The ‘alternative’ model also demonstrates the sequential restriction of HSCs through fate restriction decisions to form all the differentiated blood cells of distinct lineages. However in this model, there is no definitive divergence decision at the HSC/MPP stage and HSCs can give rise to CMPs or primitive LMPPs. The CMP is able to generate all myeloid/megakaryocyte/erythroid cells and LMPPs retain lymphoid/myeloid potentials. Curved arrows indicate self-renewal potential.

1.4.2 Migration and homing

Under homeostatic conditions, most HSCs are located within the BM, however a small proportion is continuously released into the circulation. The trafficking of HSCs into the blood stream is a tightly regulated process, triggered by signals such as light, which contribute to circadian oscillations⁴⁰. This is detected by the central nervous system (CNS) which then directs signals via B3 adrenergic receptors in BM stromal cells to decrease CXC-chemokine ligand 12 (CXCL12, also known as SDF1⁴¹) expression in the BM microenvironment. Loss of interaction between CXCL12-expressing cells and its cognate receptor CXC-chemokine receptor 4 (CXCR4) expressing HSCs, leads to the release of HSCs into the circulation^{40,42}.

During haematopoietic stress, the haematopoietic system responds by mobilising HSCs from the BM. Bacterial infection is a common form of stress which can directly influence HSPCs. Burberry *et al.* demonstrated that systemic infection with the Gram-negative bacterium *E. coli* was able to induce the mobilisation and accumulation of LT-HSCs in the spleen along with a moderate reduction of HSCs in the BM⁴³. Stress to the system can also be caused by BM ablation due to irradiation, changes in cytokine levels or as a response to clinical treatments with agents such as cyclophosphamide or granulocyte colony-stimulating factor (G-CSF)⁴⁴⁻⁴⁶. Mobilised HSCs can then migrate to the liver and spleen where they can give rise to extra-medullary haematopoiesis⁴⁷. Circulating HSCs are capable of homing back to the BM, which is dependent on migration and adhesion pathways such as hyaluronic acid/CD44, CXCL12/CXCR4 and vascular cell adhesion molecule 1 (VCAM-1)/very late antigen 4 (VLA4)⁴⁸.

1.4.3 Apoptosis

Apoptosis is the process of programmed cell death, which helps to maintain haematopoietic homeostasis by regulating HSC numbers and can be activated by two pathways. The first is signalling via death receptors, which become activated upon binding their cognate ligands to initiate cytotoxic and non-cytotoxic signalling cascades⁴⁹⁻⁵¹. Alternatively, it can occur through the interactions of pro- and anti-apoptotic proteins from the B cell lymphoma 2 (BCL-2) family, which is triggered by various signals such as cytotoxic factors, developmental cues or the absence of specific cytokines⁵².

The anti-apoptotic protein, BCL-2, has been implicated in helping to regulate HSC numbers. Over-expression of *Bcl-2* in mouse models leads to a significant increase in HSCs. These cells were then shown to possess an advantage during the competitive repopulation of irradiated recipients compared to wild type (WT) cells^{53,54}. However, BCL-2 deficient mice maintain normal haematopoiesis⁵⁵, indicating a potentially redundant role for this protein. Conversely, myeloid cell leukaemia 1 (MCL-1), another member of the BCL-2 family, has been shown to be essential for haematopoietic stem and progenitor cells (HSPCs) maintenance and survival⁵⁶.

1.5 Analysis and purification of haematopoietic stem and progenitor cells

Analysis of HSCs can be challenging, given the rarity of these cells within haematopoietic organs. No antigen has been identified which is exclusively expressed on HSCs and therefore, a combination of cell markers are used to identify and isolate these cells for functional studies or clinical transplantation.

Early attempts to isolate HSCs from mouse BM involved cell separation based on monoclonal antibodies (MAbs) against mature lineage markers, including those for B cells (B220), T cells (CD4 and CD8), granulocytes (Gr-1), myeloid cells (Mac1) and erythrocytes (Ter119) to ensure the exclusion of mature cells. As previously indicated, cells which lack these markers are defined as Lin⁻. Cells which are Lin⁻ and are positively selected after staining with antibodies against c-Kit and Sca-1 further define them as LSK cells^{11,57}, a multipotent HSC population within the BM⁵⁸. The LSK fraction is a heterogeneous pool, containing both haematopoietic stem and progenitor cells. Additional markers can be used to increase the prospective purification of 'true' stem cells, which include fms-related tyrosine kinase 3 (Flt3; CD135)⁵⁹ and CD34⁶⁰. Analysis by flow cytometry after antibody staining against these cell surface markers (**Section 1.6**) has indicated that LT- HSCs are Flt3⁻ CD34⁻ whilst ST-HSCs are Flt3⁻ CD34⁺ and more differentiated MPPs are known to be Flt3⁺ CD34⁺^{47,61}.

Additionally, HSCs are further defined by low staining by selected dyes. Most adult HSCs are quiescent and express a high proportion of membrane transport pumps at their cell surface. These pumps belong to the ATP-binding cassette (ABC) transporter family which are responsible for drug efflux from cells. Differences in the activity of these pumps can be used to isolate adult HSCs by staining with the DNA binding dye Hoechst 33342⁶² or the mitochondrial binding dye Rhodamine-123 (Rh)⁶³. These dyes are retained in non-quiescent and cycling cells whereas quiescent cells are capable of efficiently pumping out these dyes and therefore LT-HSCs are found in the Hoechst^{lo} or Rh^{lo} fraction^{64,65}.

More recently, an additional set of antigens, known as signalling lymphocyte

activation molecules (SLAM) including CD150, CD48 and CD244, have been found to be differentially expressed between stem and progenitors at different stages of haematopoiesis³. These transmembrane receptors, which are also expressed in a variety of immune cells such as B, T, dendritic cells and macrophages^{66,67} can be used to highly purify HSC preparations, as they facilitate the differentiation of stem and progenitors and can further define murine LSK HSCs as CD150⁺ (SLAMf1⁺) CD244⁻ (SLAMf4⁻) CD48⁻ (SLAMf2⁻)⁶⁸.

1.6 Flow cytometry and FACS analysis

Flow cytometry and fluorescence activated cell sorting (FACS) are key tools for investigating haematopoiesis, allowing distinct subsets of HSCs and their progenitors to be evaluated and sorted for downstream analysis/experimentation. The technique permits the identification of phenotypically distinct cell populations, which can be identified via labelling with small molecule dyes or fluorochrome-conjugated antibodies against the specific cell surface markers discussed in **Section 1.5**. Cells can also be identified by their expression of fluorescent reporters such as green fluorescent protein (GFP). Most surface markers are expressed on more than one cell type, but by using multiple markers, separate populations of cells can be determined.

As a sample enters the flow cytometer, it undergoes a process called hydrodynamic focusing which separates the sample, to ensure that each cell passes through the laser beam individually. As a cell passes through this focused laser beam, light will scatter in different directions according to the size (forward scatter; FSC) and granularity (side scatter; SSC) of the cell. These data are captured by a detector and recorded by a system computer for subsequent analysis.

Labelling cells with MAbs conjugated with different fluorochromes / dyes facilitates the identification of different cell surface antigens. When these labelled cells pass through the laser beam, excitation of the fluorochrome leads to emission of light as fluorescence. The light of specific wavelengths emitted by the different fluorochromes is then captured using a system of mirrors and filters by the detector and data recorded. The system registers the presence of one or more markers on each individual cell but also with the appropriate calibration, permits the expression level of each specific marker to be determined from the intensity of the emitted fluorescence from the corresponding fluorochrome/ fluorescent reporter⁶⁹.

With an ever-increasing number of fluorochromes to which antibodies can be conjugated and advancement in instrumentation, it is now possible to detect more than ten parameters simultaneously. This allows for the identification of phenotypically distinct cell populations based on multiple markers. There is, however, a limitation to the number of markers that can be precisely detected simultaneously. This is because fluorochromes often have broad, overlapping emission spectra which have to be subtracted from each other, through the application of compensation between channels. If multiple fluorochromes are used simultaneously, multiple compensations have to be applied which can result in a loss of resolution and therefore each experiment needs to be evaluated and optimised carefully.

In addition to using multiple markers simultaneously to identify distinct subsets of cells for the purposes of analysis, they can also be used to sort purified subsets of these cells using FACS (**Figure 1.5**). Once the fluorescently labelled cells have passed through the laser beam to measure their fluorescence properties, energy is applied to the stream to split it into highly identical droplets. When a cell is detected

that meets the sorting criteria (correct size, cell density, expressing and lacking specific antigens), an electrical charge is applied to the stream just as the droplet containing that particle breaks off. The charged droplet passes between two charged deflection plates and is deflected into a collection tube⁷⁰. FACS enables the purification of cells with specific combinations of markers down to the single cell level and the sorted cells can be used for subsequent *in vivo* and *in vitro* analyses⁷¹. Obtaining single cells is important to be able to detect potential rare events or anomalies, which may otherwise be masked if performed on the wider, heterogeneous, cell population.

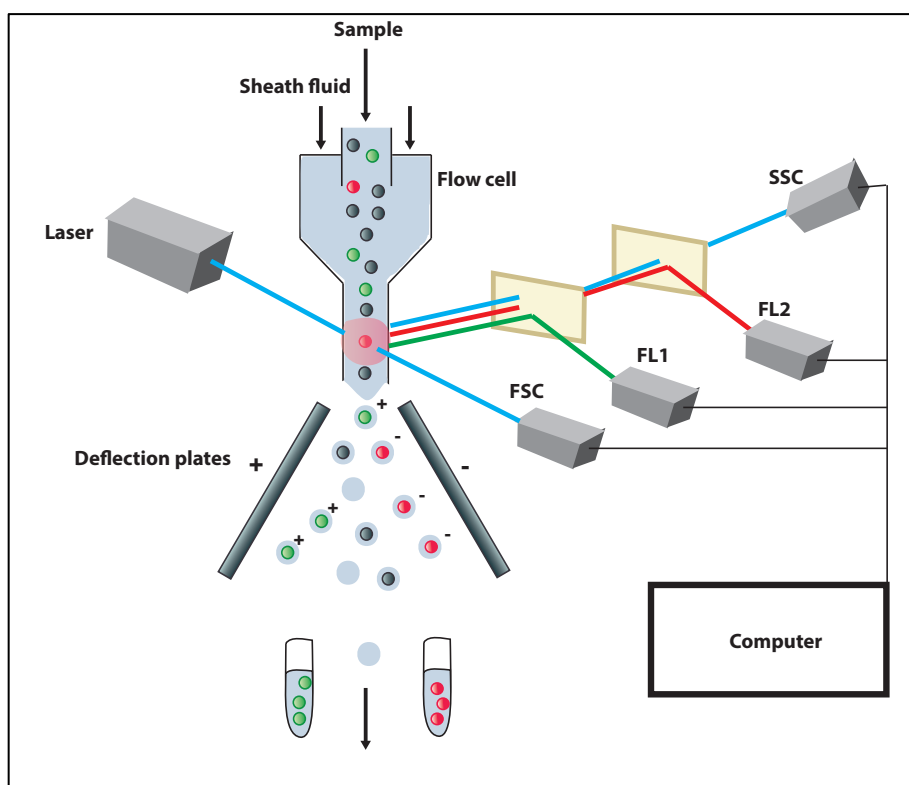


Figure 1.5 Schematic representation of flow cytometry analysis and fluorescent activated cell sorting (FACS)

In a flow cytometer, cells are aspirated from the sample-tube and pass to the flow cell via the sample line. Cells are separated by hydrodynamic focusing and then individually pass through a laser beam. Fluorescence emission is detected by photomultiplier tubes (PMT, FL1, FL2). Side scatter (SSC) determines cell density and forward scatter (FSC) measures cell size. During FACS, cells pass through a nozzle, which separates cells into individual droplets. Cells which have the required properties become charged and are deflected into collection tubes.

1.7 Stem cell niches

1.7.1 Haematopoietic stem cell niche

A stem cell niche is a specialised 'microenvironment' capable of maintaining stem cells²⁶. In 1978, Schofield published a seminal paper which proposed the concept of a haematopoietic niche, putting forward the idea that HSCs must reside in a defined microenvironment created by specific supportive cells within haematopoietic tissues to be able to survive and self-renew. He postulated that niche cells were capable of blocking HSC differentiation to promote self-renewal and HSC division. He based this on the observation that HSC daughter cells must remain located in the BM to retain their stem cell capability or alternatively after migration to other tissues, they differentiate into more lineage-restricted colony-forming cells¹³.

Initial niche studies were carried out in invertebrate systems by investigating the gonadal tissue from *Drosophila melanogaster*⁷² and *Caenorhabditis elegans*⁷³. This work led to the first definition and identification of ancillary niche cells²⁶. Physical cell-cell interactions and activation of key molecular pathways within these niches influenced the interactions between stem and progenitor cells and their local environment^{73,74}.

Haematopoietic niches were subsequently identified in mammals using targeted genetic manipulations of mice, with two independent groups led by respectively Li and Scadden, proposing in 2003 an OB-centred microenvironment which has become known as the osteoblastic (endosteal) niche^{75,76}, building on the work of Nilsson *et al.* who had determined the spatial localisation of HSCs within mouse femoral BM following transplantation⁷⁷. A third group (Kiel *et al.*) in 2005 using improved identification of HSCs based on SLAM receptors and their imaging of

mouse femur BM sections, revealed a majority of HSCs associated with ECs of sinusoidal vessels leading to the proposal of a vascular (perivascular) niche⁶⁸.

These early studies seemed to suggest the existence of two niches, the osteoblastic and the vascular niches (**Figure 1.6**). Additional investigations reported in **Sections 1.7.2 - 1.8** indicate the complexity and the close proximity of the stromal cell populations in these niches, which must interact to maintain HSCs. The cell populations implicated in the haematopoietic niche are summarised in **Table 1.1**.

These studies have also revealed the involvement of the sympathetic nervous system (SNS) and as indicated above, a complex array of cytokines (**Section 1.8.3.2**) implicated in HSC maintenance, migration, proliferation, differentiation and quiescence of HSPCs^{3,6,10,13,78,79}. Furthermore, stem and progenitor cells have been shown to be differentially distributed within the niche depending on their stage of maturation and are arranged in a spatial hierarchy in relation to ECs and mesenchymal and osteoblastic precursors^{80,81}.

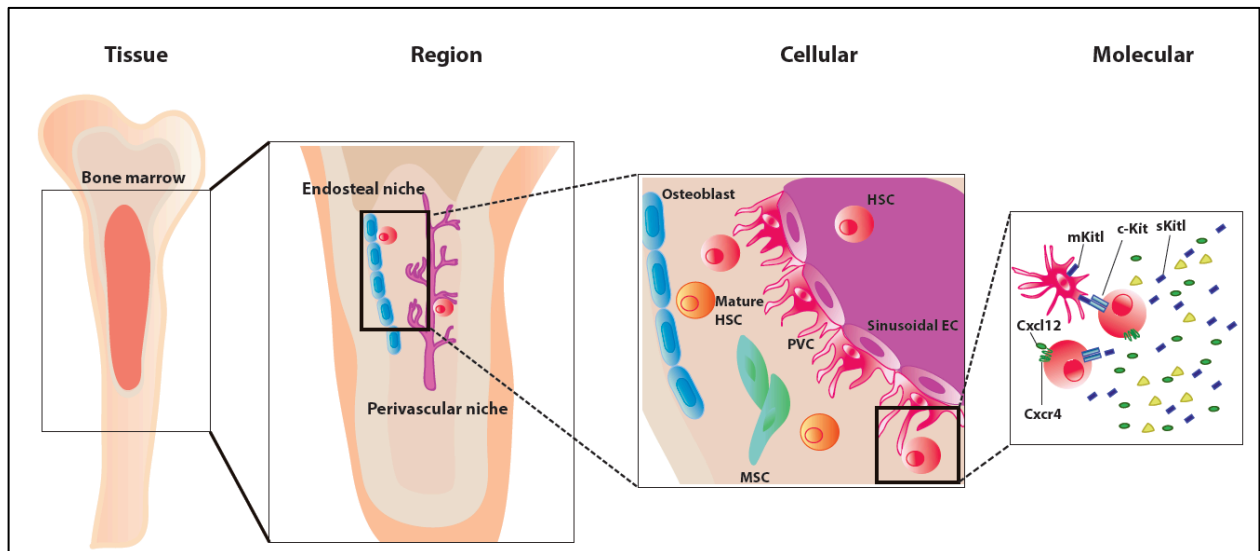


Figure 1.6 Schematic of the BM HSC niche at increasing resolution

The majority of functional adult HSCs reside in the BM. Within the BM, HSCs are located in perivascular and endosteal regions. These regions contain different cell types such as osteoblasts, mesenchymal stem cells (MSCs), endothelial cells (ECs) and perivascular cells (PVCs), which control HSCs via both direct and indirect mechanisms. Specific cell types are capable of expressing and/or secreting factors required for haematopoietic homeostasis such as the cytokines CXCL12 and Kit ligand (Kitl). CXCL12 interacts with CXCR4 expressed on HSCs. Kitl can be expressed as both a membrane-bound and soluble protein to interact with the c-Kit receptor on HSCs. Given the complexity of the cytokines involved in haematopoiesis, only CXCL12 and Kitl are shown. Adapted from Ugarte and Forsberg, 2013.

Table 1.1 Summary of key cellular components of the haematopoietic niche

Cell population	Identity/ Markers⁸²	Model(s)	Effects
Osteoblasts	Osteocalcin Osteopontin N-Cadherin	Stimulation of PTH1R in mice	Increased HSPCs in BM and induction of HSPC-active factors ^{76,83}
		Loss of <i>Ang</i> in mice	Defects in post-natal HSCs ⁸⁴
		Hyperparathyroidism in humans	Increased mobilisation of HSPCs and endothelial progenitors ⁸⁵
Osteoclasts	Osterix RANK	Inhibition of osteoclasts	Altered HSPC mobilisation, reduced HSPC homing and poorer recovery following haematological injury ^{86,87}
Perivascular stromal cells	CXCL12 ^{hi} Kitl ^{hi} NG2 PDGFR α PDGFR β CD146 Sca-1 VCAM1	Immunofluorescent studies of the BM	HSPCs located near vascular-associated reticular and endosteal cells ^{41,83,88}
		Conditional deletion of <i>Kitl</i> from BM cell types	Impaired HSPC maintenance without Kitl in Tie2 ⁺ ECs or LepR ⁺ PVCs ⁸⁹
Endothelial cells	CD31 Endomucin VE-cadherin VCAM1 Laminin Sca-1 MECA-32 Endoglin	Pharmacologic inhibition of VEGFR2 during recovery from BM injury	Mice exhibit BM failure ⁴¹
Endosteal monocytes and macrophages	Gr-1 CD169 CD11b F4/80	Depletion of monocyte/macrophage populations	Reduction in HSPC-active factors in the BM and induction of HSPC mobilisation ^{90,91}
Sympathetic nervous system neurons		Inhibition of adrenergic neurotransmission	Lack of HSPC mobilisation in response to G-CSF ⁹²
		Pharmacologic stimulation of β 2 adrenergic receptors	Enhanced HSPC mobilisation ⁹²
Glial (non-myelinating Schwann) cells	Active TGF β GFAP Nestin	Immunofluorescent analysis of HSPC niche factors and cell types	GFAP ⁺ glial cells were in contact with HSPCs and produce TGF β which appears to regulate HSPC quiescence ⁹³

		Denervation of sympathetic nerves to reduce BM glial cells	HSPC loss ⁹³
Mesenchymal stem cells	CXCL12 Nestin	Depletion of Nestin ⁺ MSCs	Reduction of BM HSPCs ⁸³
CAR cells	CXCL12	Conditional deletion of <i>CXCR4</i>	Severe reduction in HSC numbers ⁴¹
Adipocytes	FABP4 Adiponectin Perilipin A-ZIP/F1	A-ZIP/F1 'fatless' mice	Increased HSCs in the absence of BM adipocytes ⁹⁴ (Negative regulators of haematopoiesis)

Key: Angiopoietin (Ang), lipotrophic 'fatless' A-ZIP/F1 (A-ZIP/F1), bone marrow (BM), CXCL12 abundant reticular (CAR), cluster of differentiation (CD), C-X-C motif chemokine 12 (CXCL12), C-X-C chemokine receptor type 4 (CXCR4), endothelial cell (EC), fatty acid binding protein 4 (FABP4), granulocyte colony-stimulating factor (G-CSF), glial fibrillary acidic protein (GFAP), haematopoietic stem cell (HSC), haematopoietic stem and progenitor cells (HPSCs), kit ligand (Kitl), mesenchymal stem cell (MSC), NG2 glia (NG2), platelet-derived growth factor receptor (PDGFR), parathyroid hormone 1 receptor (PTH1R), perivascular cell (PVC), receptor activator of nuclear factor kappa-B (RANK), transforming growth factor β (TGF β), vascular endothelial growth factor receptor 2 (VEGFR2).

As a result of the interactions between HSCs, stromal cells and soluble and membrane bound cytokines, the number of haematopoietic cells in the niche is tightly regulated. In mammalian systems, HSCs are conserved in number between animals of varying size, for example cats and mice both possess approximately 1.1×10^4 HSCs per animal²⁶.

1.7.2 The osteoblastic (endosteal) niche

The endosteum forms the inner surface of the bone, which lies at the interface of the bone and BM. The endosteal surface is comprised of bone-lining cells (BLCs), bone forming OBs and bone-resorbing osteoclasts (OCs). The activities of these cells create an equilibrium which helps to maintain bone within the niche and also to regulate HSCs⁹⁵. The osteoblastic niche is generally accepted as a region of hypoxia, with low levels of oxygen⁷⁹. During the process of bone formation, bones are initially infiltrated by vessels and phagocytic cells which target cartilage known as

chondroclasts²⁶. OBs then arrive, which permits the mineralisation of the extracellular matrix. This process in turn acts as a signal for HSCs to migrate to the endosteum⁷⁹.

As early as the 1970s, it was demonstrated that CFU-S cells in the central BM were enriched close to the endosteum. These cells were found to be three times more abundant in proximity to the endosteal bone surface compared to the central axial region of the BM⁹⁶. This and subsequent studies led to initial characterisation of the haematopoietic niche, largely focusing on the mechanisms occurring at the endosteum. Following transplantation of phenotypic HSCs into lethally and non-lethally irradiated mice, these cells were found to preferentially home to sites in the BM less than 3 cell radii (<20 µm) from endosteal bone surfaces. This endosteal localisation of HSCs was demonstrated by using primitive stem cells labelled *ex vivo* and then tracked (down to the single cell level) *in vivo*^{77,81}. Other groups have reported that more mature, lineage-restricted progenitors lodge further from the endosteum^{97,98} whilst there also exist specific osteoblastic niches for LMPPs^{99,100}.

Key signalling pathways within the osteoblastic niche have been shown to be important for HSC maintenance. This has been demonstrated in studies where there has been selective loss, for example of angiopoietin (*Ang*) from OBs or its receptor, *Tie2*, expressed on HSCs. This led to defects in post-natal HSCs, confirming that this ligand/receptor interaction and its downstream signalling pathway seem to maintain HSCs^{84,101}.

OBs have been reported to regulate niche size through the expression of bone morphogenic protein signalling receptor type IA (BMPRII)⁷⁵. Furthermore, parathyroid hormone stimulation of OBs (via parathyroid hormone (PTH)/parathyroid

hormone-related peptide receptors (PPRs)) caused them to become increased in number, produce high levels of the Notch ligand Jagged-1 and led to an increase in the number of HSCs *in vivo*⁷⁶.

The osteoblastic niche also helps maintain HSCs in a quiescent state (either slowly cycling or in G₀ of the cell cycle) with 75 % of HSCs found to be quiescent during steady state haematopoiesis in adult BM^{79,102,103}. Endosteal cells such as OBs have been shown to express and/or secrete factors such as N-cadherin, Ang1, Thpo and CXCL12, which bind to receptors on HSCs (N-cadherin, Tie-2, Mpl and CXCR4 respectively), maintaining them in a quiescent state^{26,46,84,104,105}. Knockout (KO) mice for CXCL12 or its cognate receptor CXCR4, exhibit defective BM colonisation by HSCs in addition to a reduction in HSC numbers and reduced activity after transplantation⁴¹.

In addition to OBs maintaining HSCs, haematopoietic cells also contribute to keeping the balance within bones, as OCs can be derived from multi-potent HSCs in a process known as osteoclastogenesis. OBs are able to induce the differentiation of HSCs into OCs via macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL). M-CSF stimulates the proliferation and prevents the apoptosis of early osteoclast progenitors. RANKL is a membrane-bound protein present on osteoblasts and their precursors, which activates the RANK receptor on osteoclast precursors. Deletions of the RANKL (*Tnfrsf11*) or RANK (*Tnfrsf11a*) genes results in the absence of osteoclasts due to arrested differentiation of M-CSF expanded osteoclast progenitor cells¹⁰⁶.

1.7.3 The vascular niche

The BM vascular niche consists of a network of thin-walled and fenestrated specialised sinusoidal vessels, which possess a single layer of ECs and no connective tissue, making them highly permeable^{107,108}. The vascular niche also includes some PVCs³. Evidence for the importance of the vascular niche has been suggested by findings, which indicate that 60 % of HSCs in the BM reside adjacent to sinusoidal blood vessels, with almost all HSCs within 5 cell diameters of a sinusoid^{68,109}. Further refinements have shown that highly purified HSCs (as determined using SLAM family markers) localise to sinusoids in tissue sections. Furthermore, *Gp130 Tie2Cre* mice generated to specifically KO the cytokine receptor subunit GP130 (part of the receptor which binds to the IL-6 family of cytokines) in ECs and HSCs, displayed reduced cellularity around sinusoids whilst maintaining cellularity at the endosteum and developed a fatal anaemia within 1 year¹¹⁰.

Additional evidence for the involvement of ECs comes from *in vitro* studies in which ECs from a range of tissues can maintain HSC cultures. As an example, semi-immortalised transfected human umbilical endothelial cells (HUVECs) with constitutively active v-akt murine thymoma viral oncogene homolog 1 (AKT1) were able to maintain and support LT-HSCs via Notch and Ang1 signalling¹¹¹.

Furthermore, ablation of murine ECs *in vivo* via the administration of an anti-VE Cadherin antibody resulted in haematopoietic failure¹¹². Normal EC function has therefore been shown to be important within the niche.

In addition to ECs, the vascular niche may also be comprised of MSCs, a multi-potent stromal cell population that can give rise to OBs and other cells, which have been shown to be mostly perivascular in BM¹¹³. These cells have also been claimed

to provide microenvironmental support for HSCs⁸⁸. Ablation of Nestin-positive MSCs (Nes⁺ MSCs; expressing high levels of CXCL12 and kit ligand, Kitl) in *Nes-cre^{ERT2}/iDTR* mice, caused a 50 % reduction in phenotypic MPPs and LT-HSCs, with increased mobilisation of the remaining HSPCs to the spleen⁹⁴. Homing of WT HSCs to the BM of these mice was reduced by 90 % following irradiation and competitive transplantation⁸³. Furthermore, 60 % of Nes⁺ MSCs were found to co-localise with Lin⁻ LSK CD48⁻CD150⁺ HSCs⁸³, providing further indication for the requirement of these cells within what the authors described as a new MSC-HSC niche⁸³.

However, as indicated above, others have speculated that MSCs are a component of the perivascular niche⁸⁸ or both the vascular and endosteal niches in adult BM⁴¹ and not as considered by Mendez-Ferrer *et al.* to be a separate niche⁸³. Furthermore, the Sugiyama *et al.* study revealed most HSCs to be in contact with a subset of perivascular reticular cells, expressing very high levels of CXCL12 known as CXCL12-abundant reticular (CAR) cells⁴¹, which have since been described as MSC progeny¹¹⁴. These CAR cells were shown to surround sinusoidal ECs or were located near the endosteum. Induced deletion of CXCR4, the receptor to CXCL12, results in severe reduction of HSC numbers and increased sensitivity to myelotoxic injury in mice, although it did not impair expansion of more mature progenitors. CAR cells appear to be important in both vascular and endosteal niches in adult BM, as CXCL12-CXCR4 signalling is required for maintenance of HSCs⁴¹.

Laser scanning cytometry in combination with confocal imaging has been used to define the three dimensional (3D) vascular architecture within the BM. This work highlighted the extent of the vascularisation within the BM microenvironment, particularly at the proximal regions of the bone. This study also quantified the location

and distribution of haematopoietic cells, which demonstrated that HSPCs were enriched in PV areas of bone-proximal regions¹¹⁵. Work by the same group showed that hypoxic HSPCs are located throughout the BM, contrary to previous reports, which had confined the areas of hypoxia to the endosteum^{6,82,116}. A more recent study by Spencer *et al.* also investigated oxygen concentrations by performing direct *in vivo* measurements of the local oxygen tension (pO_2) in the BM of live mice. The lowest pO_2 was detected in peri-sinusoidal regions, whereas the endosteal region was found to be less hypoxic owing to the presence of small arteries¹¹⁷.

The vascular niche also plays a role in providing a differentiation microenvironment to enable the mobilisation and proliferation of activated stem and progenitor cells through specific secreted factors^{41,118}. Levesque *et al.* demonstrated that releasing G-CSF impairs CXCL12/CXCR4 signalling and results in HPC mobilisation from the BM niche and migration across the BM sinusoidal endothelium. The mobilisation of HPCs coincides with the decreased expression of both CXCL12 and CXCR4 in the BM, as result of increased cleavage of both the ligand and the receptor⁴⁵.

As HSCs have been shown to be located close to both osteoblastic and vascular regions^{10,119}, debate has ensued whether the osteoblastic and vascular niches are physically and functionally distinct or whether they interact to collectively constitute the haematopoietic niche. OBs were thought to be key for HSC quiescence whilst the sinusoidal endothelium promoted proliferation and differentiation, leading eventually to the release of mature blood cells into the circulation^{108,120}.

The latest 3D study from Nombela-Arrieta *et al.* discussed above, has also indicated that the endosteal surface is highly vascularised and hypoxic HSPCs are not

confined to the osteoblastic areas within the niche. These hypoxic HSPCs can be associated with the vasculature and the level of HSPC hypoxia appeared independent of cell cycle status¹¹⁵. These findings in combination with the high and specific vascularisation within the BM endosteum suggest much more complex interplay between the cellular niche components than originally conceived, requiring close interaction between the osteoblastic and vascular niches.

The concept of fixed spatial niches is potentially further undermined by cell-specific KO studies which deleted CXCL12 from osterix-expressing cells, PVCs and OBs, where effects were only seen in the former two models. The loss of CXCL12 from the different cell populations impacted only B-lymphoid or HSCs respectively^{99,100}. It could therefore be postulated that cytokine expression by different stromal cell populations helps to define niches which are comprised of multiple cell types found in close proximity within the microenvironment, with the capacity to support different haematopoietic populations. If this were to be the case, studies which investigate the impact of loss of cytokines in a cell specific manner, such as those reported in this work, will be highly informative to understand HSC regulation within the BM niche.

1.8 Niche regulation of HSCs

Initially, it was proposed that HSCs merely required input from the cellular microenvironment and were purely dependent on cell-cell contact with the stromal cells which if lost would lead them to differentiate¹⁰. From this initial concept, the stem cell niche has been shown to regulate haematopoiesis using a range of different mechanisms via both cell intrinsic (e.g. molecular regulators of transcription/translation; micro ribonucleic acid (RNA); epigenetic factors) and cell

extrinsic factors (e.g. growth factors including cytokines, ion gradients, oxygen tension and blood flow) and is subject to hormonal and CNS involvement.

1.8.1 Age, physiochemical and biomechanical factors affecting the niche

The ageing process and the role of physical and mechanical inputs, in addition to non-proteinaceous factors, are becoming better defined and it is increasingly evident that they can influence both the HSCs and the stromal cell populations.

1.8.1.1 Shear stress

Shear stress is generated by blood flow and has been shown to influence EC proliferation, acting as a mechanical stimulus to trigger responses which can lead to changes in cell function, morphology and gene expression¹²¹. It has also been shown to increase the EC-production of several cytokines which are known to have important roles within haematopoiesis such as platelet-derived growth factor (PDGF)^{122,123}, IL-6¹²⁴ and granulocyte/macrophage colony stimulating factor (GM-CSF)¹²⁵.

In addition to influencing these niche cells, shear stress can also directly regulate HSCs. Adamo *et al.* demonstrated that fluid shear stress led to an increase in *Runt-related transcription factor 1 (Runx1)* expression (a master regulator of haematopoiesis¹²⁶) in CD41⁺c-Kit⁺ HPCs and in the colony-forming potential of mouse embryonic stem (ES) cells differentiated *in vitro*. It also increased the expression of haematopoietic markers in the PAS and AGM regions of mouse embryos. Removal of nitric oxide, which is known to mediate shear stress-induced signalling, led to reduced haematopoietic potential *in vitro* and *in vivo*⁷. These data

demonstrate an evident role for biomechanical forces in haematopoietic development and regulation.

1.8.1.2 Calcium ions

The gradient of calcium ions within the BM niche is also capable of influencing HSCs. Extracellular calcium ions are recognised by 7-transmembrane (TM) calcium-sensing receptors, which have been identified on HSCs^{127,128}. Mice which lack calcium-sensor receptors display a reduction in BM cellularity and a lower proportion of HSCs, in addition to increased HPC mobilisation to the circulation and spleen^{26,128}. These findings indicate that the ability of HSCs to sense and respond to the increased calcium concentrations at the endosteal surfaces helps to create a stromal niche cell-HSC interaction, required for haematopoiesis.

1.8.1.3 Oxygen tension and hypoxia

Oxygen tension (which is dependent on oxygenation and hypoxia) has also been shown to be an important physiochemical control factor within the niche. Hypoxia can influence the cell transcriptome via hypoxia-inducible transcription factors (HIFs). When the oxygen concentration is greater than 2 %, HIFs are degraded by the proteasome after they are synthesised and therefore cannot activate transcription¹²⁹.

HIFs have been demonstrated to be important during embryogenesis, tumourigenesis^{80,130} and in the regulation of HSCs^{131,132}. HIFs have also been shown to be constitutively present at the endosteum, a hypoxic region associated with LT-reconstitution potential HSCs within the BM during steady state^{129,132}. HIF-1 α , the O₂-labile subunit of HIF-1 has been shown to be essential for HSC self-renewal.

Conditional deletion of the gene encoding HIF-1 α did not affect HSC survival and the

cells were able to reconstitute primary recipients. However, HSCs from *HIF-1α*^{Δ/Δ} mice were incapable of reconstituting secondary recipients and had reduced survival rates in response to cytotoxic challenge with 5-fluorouracil compared to WT controls¹³³.

HIF-1α is degraded by a protein complex which includes the von Hippel-Lindau tumour suppressor (VHL), elongin B, elongin C, cullin-2, and ubiquitin ligase E3. Conditional deletion of *Vhl*, results in the stabilisation of HIF-1α even in normoxic conditions, with the HSCs in these mice showing reduced homing to the BM⁸⁰. These findings strongly suggest the role of hypoxia via HIF-1α within the niche.

1.8.1.4 Ageing

The ageing process leads to the decline in the functioning of cells and tissues within the body and is known to result in haematopoietic changes. Several studies have demonstrated that haematopoiesis is affected by the aging of the haematopoietic niche microenvironment¹³⁴. HSCs are less efficient at homing to their niches upon transplantation in old versus young recipients^{135,136}. Evidence would suggest that adhesive interaction between the niche and HSCs changes with age, as treatment with G-CSF in aged mice leads to increased HSC mobilisation compared to young mice¹³⁷. The microenvironment has also been shown to be affected by telomere dysfunction, which is associated with ageing. Telomerase deficient mice exhibit impaired B-lymphopoiesis and increased myelopoiesis. Telomere dysfunction and the associated ageing in these mice restricted the engraftment of WT HSCs¹³⁸. These findings indicate that the fitness of the stem cell niche changes with age.

HSC intrinsic changes occur with age. Notably, there is an accumulation and expansion of phenotypic HSCs with decreased self-renewal potential^{139,140}.

Furthermore, ageing HSCs have less lymphoid differentiation potential, thereby losing the ability to produce lymphoid cells (B, T and NK cells), which contributes to a decline in adaptive immunity¹⁴¹. There is a bias towards myelopoiesis, which results in the increased production of myeloid cells including macrophages and granulocytes in mice¹⁴². In addition, in humans, it has been shown that anaemia increases with age and is prevalent among the elderly, which may be a result of HSCs becoming increasingly resistant to EPO with age¹⁴³.

1.8.2 HSC intrinsic factors

Molecular programming at both the transcriptional and translational level contributes to cell intrinsic regulation, in addition to micro RNA, cell cycle associated factors and epigenetic regulators. There are several classes of cell intrinsic genes that play a role in the correct regulation of haematopoiesis¹⁴⁴, four of these, along with representative genes of each class are shown in **Table 1.2**.

Table 1.2 Classes of intrinsic genes which play a role in haematopoiesis

Gene group	Examples
HSPC-specific	LMO2, GATA1, PU.1
Homeobox	Hox, MEIS1
Polycomb	BMI-1, EZH2
Cell cycle regulators	CIP/ KIP, INK4A
Key: LIM domain only 2 (LMO2), globin transcription factor 1 (GATA1), transcription factor PU.1 (PU.1), homeobox (Hox), Meis homeobox 1 (MEIS1), Bmi1 polycomb ring finger oncogene (BMI-1), Enhancer of Zeste Homolog 2 (EZH2), CDK-interacting protein (CIP), kinase inhibitory protein (KIP) and Inhibitors of CDK4 (INK4).	

Of these intrinsic HSC genes, one of the most studied is the chromatin-associated factor, Bmi1 polycomb ring finger oncogene (BMI-1) which has specifically been

shown to influence cell function *in vivo*¹⁴⁵. BMI-1 controls the cell cycle status of HSCs by altering the expression of *Ink4a* (*p16*) and *Arf* (*p19*). BMI-1 null (*Bmi-1*^{-/-}) mice are born with HSCs, which quickly become exhausted and exhibit increased levels of the cell cycle regulator Ink4a, indicating that BMI-1 is responsible for the repression of the encoded gene^{146,147}. The self-renewal defect in *Bmi1*^{-/-} mice can be partially rescued by crossing with mice which lack *Ink4a* or *Arf*^{148,149}. Equally, *Bmi-1* over-expression increases HSC self-renewal. These findings demonstrate a direct mechanism by which a chromatin-associated factor can modulate the HSC cell-cycle status and self-renewal.

Another intrinsic regulatory mechanism shown to be important is gene (de)methylation. The *DNA methyltransferases* (DNMT) DNMT3A and DNMT3B which are expressed in HSCs have been linked to their self-renewal. A competitive haematopoietic transplantation system was used to investigate the role of these two factors during haematopoiesis. Individual deletion of the gene encoding each of these *methyltransferases* in murine (CD34⁻LSK) HSCs did not impact upon differentiation or self-renewal, however, HSCs which had deletion of both factors were unable to compete with WT counterparts in re-population assays although there was no impact on differentiation¹⁵⁰. These results indicate that methylation is important for self-renewal and that both gene products are required together for DNA methylation in HSCs. These epigenetic changes which are capable of modulating self-renewal can be inherited by daughter cells¹⁵¹ and can therefore alter the long-term fate of HSCs.

1.8.3 Cell extrinsic factors

1.8.3.1 Sympathetic nervous system (SNS) and hormones

As indicated above, the trafficking of HSCs into the blood stream, triggered by signals such as light, contribute to circadian oscillations (**Section 1.4.2**). Given the innervation of the BM¹⁵², a number of other studies have revealed the role of the SNS in HSC niche regulation. The cyclical release of HSCs in mice is regulated by the circadian secretion of neurotransmitter norepinephrine^{42,153}. Severing the femoral nerve in mice decreases BM cellularity with significant mobilisation of progenitor cells¹⁵⁴. As indicated previously (**Section 1.7.2**) osteoblasts produce haematopoietic growth factors and are activated by parathyroid hormone (PTH) or the locally produced PTH-related protein (PTHrP), through the PTH/PTHrP receptor (PPR)⁷⁶.

1.8.3.2 Cytokine regulation of HSCs

Within the haematopoietic system, cytokines provide both positive and negative signals to influence the different physiological functions of HSPCs with most cytokines acting upon cells from different lineages and most haematopoietic lineages being controlled by multiple cytokines¹⁵⁵. Despite the involvement of cytokines for HSC regulation, it is still undetermined whether they are ‘deterministic’ or ‘permissive’¹⁵⁶, as two alternative models have been proposed. In the ‘deterministic model’, based on the work of Rieger *et al.*¹⁵⁷ and Sarrazin *et al.*¹⁵⁸, cytokines bind to their receptors and instruct progenitor cells to lineage commit. While in the ‘permissive’ model, cell-autonomous processes drive commitment to distinct blood cell lineages. In this latter model, cytokines are needed to sustain the survival of committed progenitor cells. In both models, the action of cytokines results in a pool of specific mature cell types.

Insight into cytokine control of HSCs was initially gained from *in vitro* studies using purified HSCs from humans and mice. Analysis of KO mouse models and naturally occurring mutants which lack specific cytokines or their receptors, has confirmed the specific requirement for cytokines during haematopoiesis⁴. A summary of cytokines involved in haematopoiesis and their target cells are given in **Table 1.3**.

Most cytokines implicated in haematopoiesis are part of a sub-group which have a 4- α -helix bundle structure. Most receptors for these cytokines belong to the haematopoietin or tyrosine kinase receptor (TKR) subclasses^{4,155}. Ligand binding to a TKR leads to ligand-induced aggregation, which causes activation of the receptor's intrinsic tyrosine kinase activity. The phosphorylation of one or several tyrosine residues triggers the downstream signalling pathway¹⁵⁹. This generates cascades of protein activators. Cells integrate the signals generated by these activators, which acts as the cue to alter their gene expression and exert a suitable biological response⁴.

Table 1.3 Cytokines involved in haematopoiesis and their target cells

Regulator	Receptor Class	Responding Cells
G-CSF	Haematopoietin	HSC, G, M
GM-CSF	Haematopoietin	HSC, Eo, G, M, Mk
M-CSF	Tyrosine kinase	HSC, G, M
IL-3	Haematopoietin	G, M, Eo, Ery, HSC, Mast, Mk
EPO	Haematopoietin	HSC, Ery, Mk
THPO	Haematopoietin	HSC, Mk
IL-1 β	Immunoglobulin-like	T, B, HSC
IL-2	Haematopoietin	T, B
IL-4	Haematopoietin	T, B, G, M, Mast
IL-5	Haematopoietin	Eo, B
IL-6	Haematopoietin	B, G, HSC, Mk
IL-7	Haematopoietin	B, T, M
IL-9	Haematopoietin	T, B, Ery, Mast
IL-10	Interferon-like	B, Ery, Mk, HSC
IL-11	Haematopoietin	Mk, B, HSC, Ery
IL-12	Haematopoietin	NK, T
IL-13	Haematopoietin	B, M
IL-15	Haematopoietin	T, B
Kitl	Tyrosine kinase	HSC, Ery, G, B, T, Mk, Mast
LIF	Haematopoietin	HSC, Mk, M
OSM	Haematopoietin	Mk
Flt3l	Tyrosine kinase	HSC, G, M, B
CXCL12	GPCR	HSC, B

Key: Granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF), erythropoietin (EPO), thrombopoietin (THPO), interleukin (IL), kit ligand (Kitl), leukaemia inhibitory factor (LIF), osteostatin M (OSM), flt3 ligand (Flt3l), G protein coupled receptor (GPCR). Granulocytes (G), macrophages (M), eosinophils (Eo), megakaryocytes (Mk), erythroid cells (Ery), mast cells (Mast), haematopoietic stem cells (HSC), B lymphocytes (B), T lymphocytes (T), natural killer cells (NK).

Table adapted from Alexander, 1998 and Alexander, 2000 ^{4,155}.

The majority of cytokines are found as soluble proteins, however there are also many membrane-bound factors. HSCs form cell-cell contacts with BM stromal cells via membrane bound growth factors under steady-state conditions^{160,161}. Several growth factors such as Kitl, colony stimulating factor-1 (CSF-1 = M-CSF) and Flt3 ligand (Flt3l) known to be involved in haematopoiesis are synthesised as transmembrane precursors which can be proteolytically cleaved to produce biologically active, soluble factors^{162,163}. The proteolytic cleavage process that converts membrane mCSF-1, mFlt3l and mKitl to their soluble forms is highly regulated by agents which increase

levels of cytosolic calcium¹⁶⁴ or activate protein kinase C¹⁶⁵ (PKC; via the PKC pathway) to cause the immediate cleavage and release of these factors¹⁶⁶. The cleavage of these transmembrane proteins and subsequent release of their ectodomains also affects other proteins such as the receptors for these cytokines. For example, the receptors for CSF-1 and tumour necrosis factor α (TNF α) become rapidly released in response to cleavage of their ligands following activation by PKC^{167,168}. In addition to the cell-anchored cytokine receptors, receptors for several growth factors also exist in soluble forms¹⁶⁹.

1.8.4 Key haematopoietic cytokines

In addition to those mentioned above, a number of other cytokines and molecules have been tentatively claimed to have some potential role in HSC quiescence and proliferation¹⁷⁰. Osteopontin and other proteins involved in Wnt and Notch pathways have also been implicated¹⁷¹. However, four key cytokines in mice and humans appear to be EPO, Thpo, Flt3 ligand (Flt3l) and Kitl, which have been shown to have potent effects on HSC survival, maintenance and expansion both *in vitro* and *in vivo*. In addition, they seem to have a greater influence when they act in combination^{163,172,173}. CXCL12 has additionally been shown in murine studies to be important. Similarly, IL-3, G-CSF and those which act via the GP130 receptor (IL-6, IL-11) have also been shown to affect HSCs *in vitro*, especially if the key cytokines Thpo, Flt3l and/or Kitl are also present^{4,174}.

1.8.4.1 Erythropoietin

During homeostasis EPO is primarily produced at low levels by the peritubular capillary ECs in the kidney and is then released into the blood stream to target cells in the BM¹⁷⁵. Its role is to control RBC production by regulating the differentiation and

proliferation of erythroid progenitor cells in the BM (burst-forming-unit erythroid (BFU-E) cells, colony-forming-unit erythroid (CFU-E) cells and erythroblasts are EPO-responsive¹⁷⁶). Mice lacking a functional *Epo* gene are embryonically lethal¹⁷⁷.

1.8.4.2 Thrombopoietin

Thpo is a ligand mainly produced by the liver¹⁷⁸, which interacts with the thrombopoietin receptor, c-MPL, expressed on all HSCs¹⁷⁹⁻¹⁸¹. It is also synthesised in BM stroma by OBs and in the kidneys, which can be transported into the BM via the circulation^{3,182}. It is the primary regulator of megakaryopoiesis and thrombopoiesis, which helps to control the production and maturation of megakaryocytes to ensure the correct number of platelets is maintained^{183,184}. *Thpo* or *c-Mpl* deficient mice display defects in megakaryopoiesis, are thrombocytopenic and also display a large reduction in HSCs¹⁸⁵. The requirement of Thpo for HSC maintenance was shown directly by analysing *c-Mpl*^{-/-} mice, which had 10 times fewer CFU-S cells in their BM, compared to WT^{186,187}.

1.8.4.3 Fms-like tyrosine kinase 3 ligand

Flt3l, also called foetal liver kinase-2 (flk-2) is the ligand for Flt3. It is a growth factor for haematopoietic progenitors and induces HSPCs cell mobilisation *in vivo*. It has structural similarity to both Kitl and CSF-1 and like these cytokines, exists in both membrane and soluble forms. KO mice for both the ligand and receptor have demonstrated the importance of this Flt3l/Flt3 signalling in HSPC regulation¹⁶³. Mice lacking Flt3 possess normal numbers of myeloid progenitor cells (MPCs) and CFU-S however, they have significant HSC deficiencies upon competitive transplantation, with a 5-fold reduction in the repopulating potential from the BM of KO mice compared to WT⁴, however later studies have not confirmed this¹⁸⁸. *Flt3l*^{-/-} mice have

impaired haematopoiesis with reduced leukocyte cellularity in the BM, spleen, peripheral blood (PB) and lymph nodes. They also exhibit a two-fold reduction in the number of progenitor cells and a 40 % reduction in the number of CFU-S compared to WT¹⁸⁹.

1.8.4.4 CXCL12

As previously described (**Section 1.7**), CXCL12 has been implicated in HSC maintenance in mice, in part via its expression on CAR cells⁴¹. Selective ablation of these cells causes a reduction in CXCL12 and Kitl protein expression, resulting in an impairment of BM cell differentiation to the adipo-osteogenic lineages and significant reduction in the number of cycling erythroid and lymphoid progenitors¹⁹⁰. As HSCs are found in close proximity to CAR cells in both perivascular and endosteal regions of the BM which highly express CXCL12, it suggests that both the endosteal and vascular niche expression of CXCL12 is important for haematopoiesis.

1.9 c-Kit and Kit ligand

As indicated above, another important cytokine with impact upon haematopoiesis is Kitl via interaction with its receptor c-Kit.

1.9.1 c-Kit receptor

The *c-Kit* gene is located at the dominant *white spotting* locus (*W*) on murine chromosome 5 and mapped to the long arm of human chromosome 4 (4q11-4q12)¹⁹¹. It was originally identified as the oncogene *v-Kit* found in Hardy-Zuckerman 4-feline sarcoma virus (HZ4-FeSV)¹⁹². c-Kit is a transmembrane protein and a class III TKR, in the same group as platelet-derived growth factor receptors PDGFR α and PDGFR β , Flt3 and granulocyte-macrophage colony-stimulating factor receptor,

GM-CSFR. These receptors exhibit structural similarities in their extracellular domains and 2-part (split) intracellular TK domains¹⁹³.

1.9.1.1 c-Kit expression, function and regulation

c-Kit primarily functions as a cell surface receptor¹⁹⁴, with an extracellular domain comprised of 5 immunoglobulin-like loops, the second of which contains the high affinity ligand-binding site¹⁹⁵. Ligand binding links 2 c-Kit molecules into a dimer, which causes cross-phosphorylation at specific tyrosine residues. This phosphorylation leads to a signalling cascade activating downstream protein targets such as mitogen-activated protein kinase (MAPK)¹⁹⁶. Final targets include TFs such as signal transducers and activation of transcription (STAT) proteins, microphthalmia TF in melanocytes and Slug in haematopoietic and germ cells¹⁹⁷.

The receptor is widely expressed throughout the body on neurons, germ cells, interstitial cells of Cajal (ICCs) and melanocytes^{172,198,199}. Within the haematopoietic system its expression is largely restricted to HSCs and HPCs and becomes down regulated upon maturation of all haematopoietic lineages, with mast cells being the only mature cell populations to express the receptor¹⁹⁸. A small proportion of both human and murine c-Kit⁺ BM mononuclear cells (MNCs) expressing c-Kit have been shown to co-express lymphoid markers²⁰⁰.

c-Kit signalling is associated with varied biological responses in different cell types, with its activation implicated in the regulation of cell proliferation, differentiation, adhesion, chemotaxis and apoptosis¹⁹⁶. Several physiological regulators such as GM-CSF²⁰¹, IL-4²⁰², TGFβ²⁰³, TNFα²⁰⁴ and interferon-gamma (IFN-γ)²⁰⁵ modulate the level of *c-Kit* mRNA and the protein. Protein turnover also regulates the level of

receptor cell surface expression, as binding of Kitl can lead to kinase-dependent receptor endocytosis and subsequent ubiquitination and degradation²⁰⁶⁻²⁰⁸.

Alternatively, activation of the receptor can result in the cleavage of the extracellular domain, releasing a soluble form of the receptor^{206,209}.

Gain-of-function mutations in *c-Kit* in humans are associated with disease phenotypes including different types of cancer². Mutations resulting in aberrant and constitutive c-Kit activation and signalling cause neoplasms resulting in germ cell seminomas and gastrointestinal stromal tumours (GISTs), which derive from ICCs^{196,210}. Mutations are also common in haematological malignancies such as acute myeloid leukaemias (AML)¹⁹⁶ and mastocytosis which can lead to mast cell leukaemias²¹¹. In addition, myelodysplasias and blast transformations of chronic myeloid leukaemia (CML) are often c-Kit⁺, whereas B- and T-lymphoid leukaemias are predominantly c-Kit⁻, with only a small proportion (<5 %) of childhood T-lymphoid leukaemias being c-Kit⁺^{196,212}.

1.9.2 Kit ligand

The ligand for c-Kit, Kitl^{213,214} (also known as stem cell factor (SCF)²¹⁵, steel factor²¹⁶ and mast cell growth factor²¹⁷) is encoded at the *Steel* (*Sl*) locus on murine chromosome 10 and mapped to human chromosome 12 (12q22-12q24)^{2,218}. The protein is expressed on neurons, stromal, endothelial, Sertoli and smooth muscle cells^{89,199,219}.

1.9.2.1 Kitl isoforms

The production of membrane Kitl (mKitl) and soluble Kitl (sKitl) is regulated at both the mRNA level and the protein level. Kitl exists as two alternative biologically active

splice forms, one soluble and one membrane-bound isoform^{164,217}. These isoforms differ by the presence or absence of exon 6, which contains the primary proteolytic cleavage site²¹⁸. Both of these splice forms encode a membrane-bound protein with an extracellular domain, transmembrane domain and intracellular domain². The primary translation products from both *Kitl* mRNAs contain an N-terminal 22 amino acid (aa) signal sequence, which is absent in the mature proteins^{216,220}. The longer splice form contains exon 6 (+E6, also called KL1), encoding a 248 aa TM precursor that produces the 165 aa soluble isoform (sKitl) when proteolytically cleaved at the site within exon 6. The *Kitl* mRNA which lacks exon 6 (-E6, also termed KL2), encodes a 220 aa TM protein which lacks the primary cleavage site and is predominantly membrane bound (mKitl)¹⁶⁴.

This is summarised by the schematic in **Figure 1.7**. In total, alternative splicing can give rise to six alternative transcripts of *Kitl* in humans, of which four encode protein. In the mouse, only four transcripts have been found, which all code for protein. However, the two above-mentioned alternative transcripts (KL1 and KL2) are the ones mainly expressed, and most literature reports these transcripts.

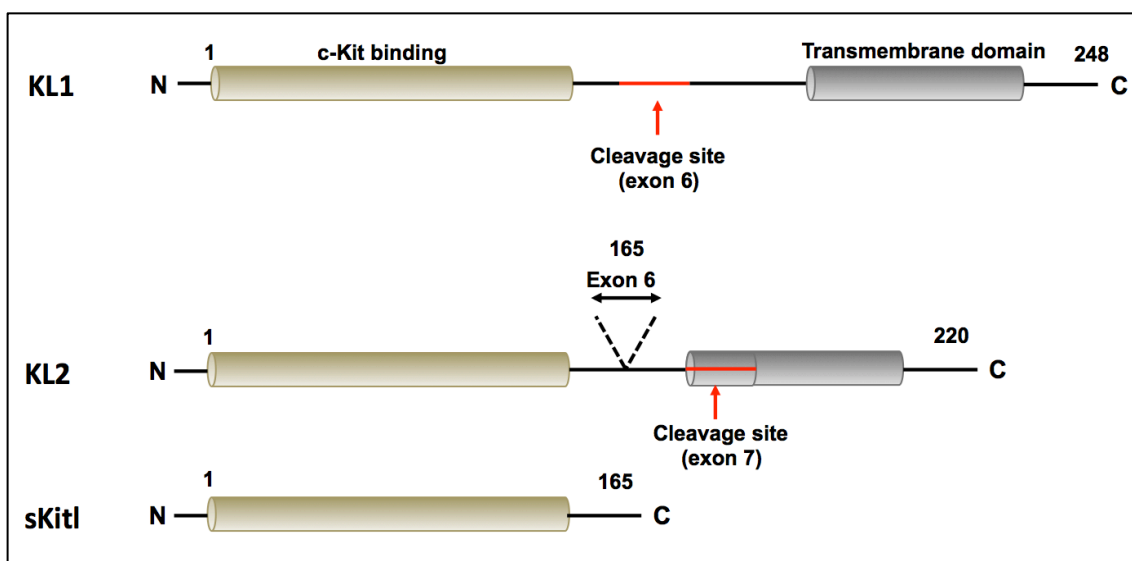


Figure 1.7 Schematic representation of mouse *Kitl* splice forms and protein processing

Kitl protein is produced as two transmembrane forms due to alternative splicing at exon 6, KL1 (Kitl²⁴⁸) and KL2 (Kitl²²⁰). In KL1, exon 6 is maintained and encodes a proteolytic cleavage site, generating the soluble Kitl¹⁶⁵. A secondary proteolytic cleavage site is located in exon 7. Figure adapted from Lennartsson and Rönstrand, 2012.

A secondary cleavage site is present in murine Kitl encoded within exon 7, which can be utilised if the primary site in exon 6 is absent and will lead to the release of a different sKitl isoform²²¹. Cleavage of Kitl occurs in a region of the protein comprised of small, apolar residues such as alanine and valine²²⁰. Different proteases are involved, such as matrix metalloprotease 9 (MMP9)¹⁶¹, chymase-1²²² and a disintegrin and metalloproteases (ADAMs) family members including ADAM17 and ADAM33²²³. As indicated previously, PKC or factors which increase the cytosolic level of calcium ions can induce cleavage of Kitl from the cell surface¹⁷².

The importance of post-translational cleavage of mKitl is emphasised from studies using MMP9 known to promote the release of extracellular matrix-bound or cell-surface bound cytokines. Under stress conditions MMP9 becomes upregulated, promoting the cleavage of mKitl to release sKitl which then helps to recruit cKit⁺ HSPCs²²⁴. In MMP9^{-/-} mice, this release of sKitl is diminished which reduces HSC motility and prevents BM repopulating cells from migrating towards the vascular niche to reconstitute the stem and progenitor cell pools¹⁶¹.

Both mKitl and sKitl are post-translationally glycosylated at both *N*- and *O*-linked sites and form non-covalently linked dimers²²⁵⁻²²⁷. Both proteins have been shown to be biologically active *in vitro*, however the two isotypes can have markedly different effects. It has been shown that mKitl expressed by a stromal cell line (SI/SI4) maintains haematopoiesis for a longer period of time than sKitl expressed by the same stromal cells when tested on M07E cells. The same study showed that mKitl

maintained c-Kit activation for longer, as receptor activation by sKitl is more transient²²⁸. In addition, the soluble form of the ligand causes a greater down-regulation of c-Kit cell surface expression than mKitl^{172,228}.

It is still relatively unknown how the sequence and structure of Kitl is important for its function. There have only been a limited number of studies which have investigated the structure/function relationship using cultured cells *in vitro*. Nishikawa *et al.* constructed several C-terminal deletions of *Kitl* and demonstrated that only the first 142 aa were required for HPC growth *in vitro*²²⁹. Mantous *et al.* suggested the importance of 3 of the 4 α -helical domains of Kitl by generating mouse-human chimeras of sKitl. Substitution of 4 aa from the 4th α -helical domain prevented c-Kit binding and subsequently led to a loss of biological activity *in vitro*²³⁰. These studies provided information about the structural requirements for Kitl function *in vitro* but it should be noted that the assays used were unable to replicate the correct *in vivo* environment.

1.9.2.2 Kitl function

During embryogenesis, Kitl and c-Kit have been shown to be important for the migration of germ cells, melanocytes and haematopoietic cells, as RNA for both the ligand and receptor is expressed in cells along migratory pathways and at the homing sites of these cell types^{231,232}. In adults, Kitl/c-Kit signalling continues to be important for HSC maintenance, melanogenesis and spermatogenesis. Furthermore, it is also required for erythroid progenitor proliferation and mast cell function²³³.

Within haematopoiesis, the interaction between Kitl/c-Kit is able to promote different responses including cell survival/preventing HSC apoptosis, proliferation,

differentiation, adhesion, homing, migration and functional activation^{161,171,198,234}. A recent study in mice which investigated the expression of Kitl within the BM niche and its role in haematopoiesis, revealed the ligand to be primarily expressed in ECs and PVCs associated with the vasculature. Deletion of *Kitl* from ECs or from Leptin-receptor (LepR)-expressing PVCs led to a depletion of phenotypic and functional HSCs and this effect was greater when deleted from both cell types simultaneously⁸⁹.

As indicated above, Kitl is particularly important in erythroid lineages, however it also impacts multi-potent HSPCs, megakaryocytes and a subset of lymphoid progenitors²³⁵. In erythropoiesis, Kitl binding to c-Kit activates phosphoinositide 3-kinase (PI3K). The importance of Kitl to the erythroid lineage is demonstrated by the inhibition of PI3K, which results in decreased numbers of BFU-Es and CFU-Es *in vivo* and reduced erythroblast proliferation *in vitro*²³³.

In vitro and *in vivo* experiments have thus demonstrated that Kitl is a potent growth factor in haematopoiesis, however it often acts synergistically with other cytokines. The combination of erythropoietin (EPO), Flt3l and Kitl can induce HSC proliferation^{163,172,198,236}. The synergistic interaction between Kitl and EPO has also been found to impact erythroid cell production *in vitro* and *in vivo*. *In vitro* cultures have shown significant synergistic effects of Kitl and EPO on the production of erythroid colony-forming cells, early and late erythroblasts and reticulocytes. This synergistic erythroid cell amplification appears to be conserved across cultures of PB, FL, BM and umbilical cord blood stem cells, as well as in cultures of multi-potent cell lines²³⁷.

1.9.3 Role of Kitl/c-Kit *in vivo*

Allelic mutations have proven vital in understanding the gene function of both *Kitl* and *c-Kit*. Mutations at either the *S/* or *W* locus cause similar phenotypic defects. Neural-crest derived melanocytes are unable to develop normally which results in a lack of pigmentation in both the skin and the coat hairs^{238,239}. Mice also have reduced fertility owing to defective primordial germ cell development²⁴⁰. The sterility phenotype associated with several *W* mutations is due to intrinsic defects in the proliferation and migration of primordial germ cells as they move from the hindgut region to the germinal ridge during gestation²⁴⁰.

S/ and *W* mutations also have a variety of haematological consequences which include macrocytic anaemia that cannot be alleviated by administration of EPO²⁴¹, in addition to reduced granulocytopoiesis²⁴² and a deficiency of mast cells^{243,244}.

Despite the similarity between phenotypes, mutations at the *W* locus cause cell intrinsic defects and therefore affect the number and function of melanoblasts, germ cells, mast cells and HSCs; whereas mutations at the *S/* locus are primarily cell extrinsic and affect the microenvironment which supports these different c-Kit⁺ cell lineages^{214,238,245}.

1.9.3.1 *c-Kit* mutations in mice

The *W* locus in mice was first described in the 1900s and pigmented coats and the presence of a white spot on stomachs originally identified mice with mutations at this locus^{239,241}. Over twenty naturally-occurring *c-Kit* mutants exist which exhibit phenotypes of varying severities²⁴¹. Most of the mutant alleles occur due to point mutations in the cytoplasmic domain, which produce TKRs with decreased tyrosine phosphorylation ability. Some mutations are not structural and instead cause

regulatory changes, which results in reduced expression of the receptor¹⁶³.

Homozygous *c-Kit* null *white spotting* mice (*c-Kit*^{W/W}) lack the coding region for the *c-Kit* gene which results in perinatal lethality due to a severe anaemia²⁴⁶.

Heterozygous *c-Kit*^{W/+} mice have a milder phenotype, as they are viable yet exhibit defective gametogenesis and pigmentation and are anaemic^{247,248}. Transgenic over-expression of EPO in *c-Kit*^{W/W} mice (Wepo mice) is able to rescue the lethal phenotype and maintain long-term myelopoeisis, however mice show a continuous decline in B and T cell numbers with age²⁴⁹.

The *W41* mutation in *c-Kit*^{W41/W41} mice encodes a point mutation in the kinase domain, which leads to the expression of a receptor with reduced kinase activity. These mice are capable of normal foetal HSC expansion, indicating this process is c-Kit-independent or that this mutation does not affect this function of c-Kit¹¹. However, these mice are unable to maintain quiescent HSCs in steady-state adult haematopoiesis or sustain the adult HSC pool post-transplantation²⁵⁰.

The embryonic lethality of *W/W* (*c-Kit* null) mice had previously limited the investigation of c-Kit's overall function during adult haematopoiesis until Kimura *et al.* published findings using a c-Kit conditional KO GFP-reporter mouse²⁵¹. *Cre* recombination following tamoxifen treatment resulted in the loss of cell surface expression of c-Kit. These mice were shown to have markedly reduced total BM and splenic cellularity, fewer phenotypic HSCs and their c-Kit deficient HSPCs were unable to reconstitute haematopoiesis in irradiated recipients. Their findings also demonstrated that the interaction between c-Kit and Kitl enhanced the interaction of HSPCs with niche cells, permitting the correct functional positioning of re-populating HSPCs²⁵¹. Overall their findings reinforced the requirement of c-Kit in the BM and

spleen during adult steady-state haematopoiesis and in haematopoietic recovery following BM depletion.

1.9.3.2 *Kitl* mutations in mice

There are over 80 different *Kitl*^{Sl} mutant alleles, which possess a variety of molecular alterations. The extent of the alteration to the *Kitl* gene associated with an individual mutation determines the severity of the resulting phenotype. The majority of *Sl* mutations are loss-of-function, with the most common *Kitl*^{Sl} mutations eliminating the entire *Kitl* coding region, with deletions ranging in size from 120 kilobases (kb) to some which are large enough to be cytogenetically visible^{252,253}. *Sl/Sl* homozygotes are *Kitl* null and consequently die *in utero* with severe anaemia²⁴⁸, indicating that *Kitl* is essential for survival.

Some *Sl* mutations are non-deletion alleles such as *Steel Panda* (*Sl*^{pan}) and *Steel-17H* (*Sl*^{17H}), which have a less severe phenotype. *Sl*^{pan}/*Sl*^{pan} mice have a regulatory mutation, reducing the *Kitl* mRNA level by 80 %, which leads to loss of pigmentation, mild macrocytic anaemia and sterility in females²⁵⁴. The *Sl*^{17H} allele contains a point mutation at the end of intron 7, which results in the loss of exon 8 and causes a frame-shift, altering the mRNA structure, which in turn affects splicing. The resultant protein has a shorter cytoplasmic tail with a novel sequence. The number of germ cells in *Sl*^{17H}/*Sl*^{17H} mice embryos is reduced by 94 %. Post-natally, the number of oocytes in female mice is able to recover slightly, however there is a progressive decline in male germ cells which leads to the absence of spermatogenesis causing sterility in male mice²⁵⁵. It has since been demonstrated that instead of being localised to the baso-lateral compartment within polarised cells in the embryo, the *Kitl* encoded by *Sl*^{17H} is mis-localised to the apical compartment. This results in disrupted

melanocyte development and post-natal coat pigmentation²⁵⁶. These findings demonstrate that Kitl is important for germ cell and melanocyte development and that the cytoplasmic domain is key to the correct functioning of the ligand.

Studies have also identified intragenic mutations which affect the *Kitl* coding region. *Kitl*^{SI1Neu}, *Kitl*^{SI2Neu} and *Kitl*^{SI3Neu} all contain varying sizes of deletion and exhibit varying levels of effects on fertility, fur colour and erythropoiesis. *Kitl*^{SI1Neu} lacking 25 aa of signal sequence and 26 aa from the N terminus; *Kitl*^{SI2Neu} has a loss of 69 aa plus the addition of 2 novel aa and *Kitl*^{SI3Neu} contains a missense mutation which leads to the substitution of asparagine 97 in mature sKitl and mKitl^{257,258}.

1.9.3.2.1 Steel dickie

One of the most extensively studied *Sl* mutations is the naturally-occurring *Steel dickie* (*Sl^d*) mutation, which causes a 4 kb intragenic deletion of sequence which encodes the transmembrane and intracellular domains, truncating the *Sl* coding sequence²⁵⁹. This results in a loss of 242 base pair (bp) in the coding region of the gene in addition to insertion of 67 bp of novel sequence. The overall net loss of 174 aa results in an encoded protein which is a mutant truncated form of sKitl, retaining the N-terminal signal sequence and a majority of the extracellular domain but lacking the transmembrane and cytoplasmic domains²¹⁷. Compound heterozygote *Sl/Sl^d* mice (with one *Sl* allele and one *Sl^d* allele) show major haematopoietic defects including severe macrocytic anaemia and reductions in HPC numbers²¹⁷.

Several studies have been undertaken to investigate the *Sl^d Kitl* gene and the encoded, truncated protein. Flanagan *et al.* used Southern blot analysis to confirm

that the deletion in the S^{f^d} *Kitl* RNA resulted from a deletion of genomic DNA (gDNA). Using densitometry in combination with RNase protection assays, they found that the levels of mRNA expression from the S^{f^d} allele in adult tissues were comparable to WT²¹⁷, indicating that despite its altered sequence, it was still present at normal levels *in vivo*.

Brannan *et al.* isolated RNA from different tissues in Sl/S^{f^d} mice and conducted Northern blot analysis using *Kitl* cDNA probes to demonstrate that *Kitl* mRNA isolated from the brain and lung of Sl/S^{f^d} mice is slightly smaller in size than WT *Kitl* mRNA but is present at comparable levels²⁵⁹, correlating to a previous study which had shown a similar finding in Sl/S^{f^d} spleen²¹⁴. These results implied that the S^{f^d} deletion led to a loss of exon sequences but as the mRNA levels were similar to WT mice, the deletion does not affect transcription. Having confirmed the sequence of the encoded mutation, the group then went on to test the biological activity of the S^{f^d} Kitl protein. Recombinant S^{f^d} *Kitl* cDNA was first constructed and expressed in yeast. The recombinant S^{f^d} Kitl protein obtained was then purified and used to culture MC-6 cells, a murine mast cell line which expresses c-Kit. The purified protein was found to be mitogenic to MC-6 cells and have equivalent activity to WT sKitl²⁵⁹, demonstrating that S^{f^d} Kitl is biologically active when expressed *in vitro*.

1.9.4 Role of mKitl

Investigation of Sl/S^{f^d} mice provided the first indication that the membrane-bound isoform of Kitl may be specifically required. The mutation in these compound heterozygotes results in lack of expression of mKitl as they only express a soluble truncated form of the ligand²¹⁷. These mice are viable potentially indicating a role for sKitl (albeit the truncated form) yet they demonstrate almost as severe a phenotype

as *Sl/Sl* mice (that have complete deletion of the *Kitl* gene), which includes lack of pigmentation, sterility, macrocytic anaemia and decreased numbers of HSPCs and mast cells^{213,214,239,241}. These data indicated the potential role of mKitl and sKitl.

In fact, injection of pharmacologic doses of recombinant sKitl into *Sl/Sl^d* mice alleviated their phenotype, as mice showed a reduction in their anaemia and mast cells developed at the injection sites²¹⁴. The exogenous sKitl was able to partially compensate for the lack of endogenously produced mKitl, however the mice still retained several phenotypic abnormalities, indicating that sKitl alone is insufficient to fulfil the roles of the ligand and therefore that mKitl may be specifically required *in vivo*.

It remains to be fully elucidated if mKitl and sKitl have particular roles and how they are differentially required within the haematopoietic niche. However, the importance of mKitl can be inferred from the phenotype of *Sl/Sl^d* mice which has provided the most striking evidence so far for the specific role of a membrane-bound form of a growth factor.

Thesis Overview

Haematopoiesis, as indicated above, is a paradigm for other stem cell systems as it has been widely studied since the 1960s¹², with much defined about the processes implicated in HSC maintenance and lineage commitment including the cell intrinsic and extrinsic regulatory factors involved. With the recent discovery of new stromal cell populations implicated in haematopoiesis, the components of the BM niche microenvironment continue to be refined but there remain key gaps in our understanding. At the molecular level, it is still not fully understood whether cell-cell contact between membrane-bound cytokines on stromal cells and their cognate receptors on HSPCs or localised soluble or systemic soluble isoforms of the same cytokine are differentially important for haematopoiesis. For example, the interplay between the membrane-bound and soluble Kitl isoforms in steady-state haematopoiesis has not been elucidated.

The *Sl/Sl^d* mouse, which was investigated by a number of different groups, suggested potential roles for mKitl and sKitl. As discussed previously, these studies indicated that the mice only express a truncated form of sKitl, although no attempt was made to confirm the presence or biological activity of this protein *in vivo*.

Brannan *et al.* appeared to attach much more importance to sKitl and postulated that the severe deficiencies in germ and blood cells seen in *Sl/Sl^d* mice might be attributable to an inability to produce or secrete sKitl protein in other than low levels *in vivo*²⁵⁹. Brannan *et al.* also speculated that mKitl in addition to sKitl might be required during normal development²⁵⁹. Flanagan *et al.* on the other hand, despite studies which showed that injection of high exogenous sKitl could partially reverse the defective haematopoietic phenotype²¹⁴, hypothesised that the truncated sKitl produced by the *Sl^d* allele had little ability to fulfil its normal activity *in vivo*²¹⁷. They

speculated that mKitl was key to maintaining this cytokine at high concentrations in close proximity to c-Kit⁺ cells and therefore might regulate migration and homing of these cells, by acting as a source of localised sKitl within the BM niche²¹⁷. From the *Sl/Sl^d* mice, there remain deficiencies in our understanding of the precise role of mKitl and sKitl but on balance, as indicated in **Section 1.9.4**, the data seem to indicate an important role for mKitl. More recent work by the Morrison group indicated the importance of perivascular niche cell expression of Kitl for HSC maintenance⁸⁹, however the study did not determine the individual protein expression of mKitl and sKitl *in vivo* nor attempted to distinguish between the roles of mKitl and sKitl. Furthermore, hemizygous models were used (with one allele null for *Kitl*) and there was no investigation of the levels of systemic sKitl. Accordingly the aim of this project was to better define the function of the membrane-bound and soluble isoforms in murine haematopoiesis.

Despite clear evidence that niche cells express Kitl^{89,251}, it remains to be shown using cell-specific KO approaches that localised expression of the membrane-bound and soluble forms of this cytokine by niche cells is critical for their interaction with stem or progenitor cells both within and outside of the haematopoietic system. Using the *Sl/Sl^d* mutant as a starting point, I sought to progressively develop and study different, novel mouse models in which there was specific loss of *mKitl* from all tissues (germline deleted *Kitl^{Δex7/Δex7}* and conditional inducible *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice), loss of *mKitl* from specific stromal cell populations (*Tie2Cre⁺;Kitl^{Δex7/Δex7}*, *LepRCre⁺;Kitl^{Δex7/Δex7}* and *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* mice) loss of total *Kitl* from specific stromal cell populations (*Tie2Cre⁺;Scf^{fl/fl}*, *LepRCre⁺;Scf^{fl/fl}* and *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* mice) and loss of total or *mKitl* from BM in the presence of unaffected systemic sKitl in kidney subcapsular explant (KSE)

studies. Given that the haematopoietic niche microenvironment and haematopoiesis are affected by ageing^{134,141,142}, another aim of this work was to compare the role of mKitl in young and aged (*Tie2Cre⁺;Kitl^{Δex7/Δex7}*, *LepRCre⁺;Kitl^{Δex7/Δex7}* and *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}*) mice.

Additionally, studies to delete *mKitl* in the Sertoli cells within the spermatogonial niche of *AmhCre⁺;Kitl^{Δex7/Δex7}* mice were undertaken, to further elucidate the importance of this membrane-bound isoform in niche biology and to also potentially provide further insights into the localised role of Kitl within the BM niche.

Declaration of Work

Unless otherwise stated, all scientific data was collected and analysed by Raffaella Facchini (See **Addendum**). The scope of my project was originally the development and evaluation of a c-Kit KO mouse. Unfortunately, a paper by Kimura *et al.* (2011)²⁵¹ describing a c-Kit KO reporter model resulted in the premature termination of my project and I was therefore obliged to transition into an on-going Kitl haematopoiesis project (working in close collaboration with Dr Sahoko Matsuoka), a project which I subsequently took the lead and forms the majority of this thesis. The testes work was led by and undertaken in close collaboration with Dr Matsuoka.

2 Materials and Methods

2.1 Mice

2.1.1 Animal husbandry

The animals used in this study were bred and maintained in pathogen-free animal facilities at University of Oxford Biomedical Services (BMS). All procedures and animal handling were approved and licensed by the Home Office and performed under the UK Animals Scientific Procedures Act (1986).

2.1.2 Mouse strains

Sl/+, *Sl^d/+*, *RosaCre^{ERT2}*, *Tie2Cre*, *LepRCre* and *AmhCre* transgenic mice^{248,260-264} were purchased from the Jackson Laboratory, USA. *CMV-Cre²⁶⁵* transgenic mice were obtained from EMMA, Germany. *Rosa26-YFP* mice²⁶⁶ were engineered in Frank Costantini's laboratory (Columbia University Medical Center, USA), obtained with agreement from Shoumo Bhattacharya (The Wellcome Trust Centre for Human Genetics, United Kingdom) and crossed with *RosaCre^{ERT2}* mice to generate *RosaCre^{ERT2};Rosa26-YFP* mice. *LepRCre⁺* mice were crossed with *Rosa26-YFP⁺* mice to generate *LepRCre⁺;Rosa26-YFP⁺* mice. *Scf^{fl/fl}* mice were obtained from Sean Morrison (University of Texas, USA)⁸⁹ and crossed with *Tie2Cre* and *LepRCre* mice to generate *Tie2Cre;Scf^{fl/fl}*, *LepRCre;Scf^{fl/fl}* and *Tie2Cre;LepRCre;Scf^{fl/fl}* mice. They were backcrossed to a C57BL/6 background (obtained from the Oxford BMS) and maintained in homozygous breeding for SCF. *Sl/Sl^d* mice were generated by crossing *Sl/+* and *Sl^d/+* mice. All strains are on a C57BL/6 background except *Sl/+*, which are on a WB/ReJ background.

2.1.3 Generation of membrane *Kitl* conditional KO mice

The *Kitl*^{ex7fl/ex7fl} mouse model was generated in collaboration with the Nerlov lab (Section 3.2.3) as follows: a *mKitl* conditional targeting vector was designed by recombineering as previously described²⁶⁷. *LoxP* sites were added in *cis* arrangement (orientated in the same direction) on either side of exon 7, encoding the transmembrane domain of *Kitl*. The targeting vector was linearised and electroporated into 129/Ola-derived IB 10/C ES cells. Gentamicin 418-resistant ES cell clones were selected, genomic DNA (gDNA) was isolated and then restriction digested using *BamHI* (New England BioLabs, NEB). Clone DNA was screened by Southern blotting and the correctly targeted ES cell clones were injected into C57BL/6 blastocysts which were then transferred to pseudopregnant C57/B6 females to generate chimeras. Correctly targeted male chimeric offspring were crossed with C57BL/6 females to verify germline transmission. Offspring were mated with *FLP1*-expressing females to generate mice with a *Kitl* exon 7 floxed allele without the neomycin resistance cassette. *Kitl* exon 7 floxed (*Kitl*^{ex7fl/ex7fl}) mice were backcrossed for 10 generations onto a C57BL/6 background for use in experiments. *Cre recombinase* activity mediated the deletion of the floxed segment.

I subsequently crossed these *Kitl*^{ex7fl/ex7fl} mice to generate germline *Kitl*^{Δex7/Δex7}, *Tie2Cre;Kitl*^{Δex7/Δex7}, *LepRCre;Kitl*^{Δex7/Δex7}, *RosaCre*^{ERT2};*Rosa26YFP; Kitl*^{Δex7/Δex7} (*RosaCre*^{ERT2};*Kitl*^{Δex7/Δex7}) and *AmhCre;Kitl*^{Δex7/Δex7} by crossing with *CMV-Cre*, *Tie2Cre*, *LepRCre*, *RosaCre*^{ERT2};*Rosa26YFP* and *AmhCre* mice respectively. Mice were bred for a minimum of two generations for use in experiments. Genotypes were confirmed by PCR (Section 2.2), except for *Sl/Sl^d* and *Kitl*^{Δex7/Δex7} mice, which were genotyped according to coat colour.

2.1.4 Generation of embryos

Embryos for experiments were generated by timed mating overnight (O/N). The next morning mating was confirmed by detection of the vaginal plug. The morning of vaginal plug detection was set as embryonic day 0.5.

2.1.5 Isolation of peripheral blood

Mice were sacrificed using CO₂. PB was collected by cardiac puncture using a 25 gauge (G) needle.

For analysis of blood cell parameters, ~100 µl of blood was transferred to ethylene-diamine-tetraacetic acid (EDTA) tubes (Sarstedt). Blood was determined for haematocrit (HCT), haemoglobin (Hgb), mean cell volume (MCV) and mean cell haemoglobin (MCH), and cell numbers counted for white blood cells (WBCs), red blood cells (RBCs) and platelets (Plts) using a Sysmex haemocytometer (KX-21N, Sysmex).

For analysis of Kitl levels in serum, ~500 µl of blood was collected into 1.5 ml collection tubes (Starlab). Blood was kept for 2 hr at RT before centrifugation for 20 min at 2000 x g (5417C centrifuge, Eppendorf). Serum samples were transferred to separate 1.5 ml collection tubes and then stored at -20°C until assayed (**Section 2.6.1**).

2.2 Genotyping

2.2.1 DNA isolation

All mice used in experiments were genotyped by polymerase chain reaction (PCR) analysis using gDNA extracted from ear or tail biopsies using the E.Z.N.A.[®] Tissue

DNA Kit (Omega Biotek) according to manufacturer's instructions. 1- 2 µl of each gDNA preparation was used directly for the PCR reaction.

2.2.2 Polymerase chain reaction

Specifically optimised PCR reactions were run for individual genotypes. 2 µl pre-diluted gDNA per sample were added to a total reaction volume of 20 µl containing a combination of forward and reverse primers (final concentration 0.2-0.5 µM), "10x Buffer" [200 mM Tris HCl (pH 8.4), 500 mM KCl], deoxyribonucleotide triphosphate (dNTP) mix [8 mM], magnesium chloride (MgCl₂) [50 mM], 0.2-0.4 µl (1.0-2.5 U) *Taq* polymerase (all Life Technologies) and deionised water. The final concentration of dNTPs and MgCl₂ was optimised for each reaction by addition of specific volumes of the stock solutions indicated in square brackets above.

PCR reactions were performed using a thermal cycler (Tetrad II 96-Well Alpha Unit, Biorad). All genotyping PCRs were carried out using primers from Life Technologies, which are listed in **Table 2.1**. Specific PCR protocols are listed in **Table 2.2**.

Table 2.1 List of primers for genotyping

Allele	Primer name	Sequence (5'-3')
<i>AmhCre</i>	Cre_Foreward	CGTTTTCTGAGCATACCTGGA
	Cre_Reverse	ATTCTCCCACCGTCAGTACG
<i>Kitl</i>^{Δex7/Δex7}	Kitl_Foreward	GGGCCTCAAAGAAATTCAAA
	Kitl_Reverse	TCATCATGATTTGGCACCTT
<i>LepRCre</i>	OLD434	CATTGTATGGGATCTGATCTGG
	OLD435	GGCAAATTTTGGTGTACGGTC
<i>RosaCre</i>^{ERT2}	Rosa26_1	AAAGTCGCTCTGAGTTGTTAT
	Rosa 26_2	GCGAAGAGTTTGTCTCAACC
	Rosa 26_3	GGAGCGGGAGAAATGGATATG
<i>Scf</i>^{fl/fl}	SCF_Foreward	CGA GGTAGGGGAAAAGAACC
	SCF_Reverse	GGATCTCCCAGAGGTTGGA
<i>Tie2Cre</i>	OLD435	GGCAAATTTTGGTGTACGGTC
	OLD438	CCTGTGCTCAGACAGAAATG

Table 2.2 List of PCR protocols for genotyping

Allele	PCR Protocol
<i>AmhCre</i>	1) 5 min at 98°C 2) 5 s at 98°C 3) 58°C for 5 s 4) 72°C for 20 s (<i>Back to 2</i>) - 30 cycles total) 5) 72°C for 1 min
<i>Kitl</i>^{Δex7/Δex7}	1) 3 min at 94°C 2) 30 s at 94°C 3) 60°C for 30 s 4) 72°C for 50 s (<i>Back to 2</i>) - 35 cycles total) 5) 72°C for 1 min
<i>LepRCre</i>	1) 3 min at 94°C 2) 30 s at 94°C 3) 63.8°C for 30 s 4) 72°C for 50 s (<i>Back to 2</i>) - 35 cycles total) 5) 72°C for 10 min
<i>RosaCre</i>^{ERT2}	1) 3 min at 94°C 2) 30 s at 94°C 3) 54°C for 30 s 4) 72°C for 40 s (<i>Back to 2</i>) - 35 cycles total) 5) 72°C for 5 min
<i>Scf</i>^{fl/fl}	1) 3 min at 94°C 2) 30 s at 94°C 3) 58°C for 1 min 4) 72°C for 1 min (<i>Back to 2</i>) - 35 cycles total) 5) 72°C for 10 min
<i>Tie2Cre</i>	1) 3 min at 94°C 2) 30 s at 94°C 3) 67.1°C for 30 s 4) 72°C for 50 s (<i>Back to 2</i>) - 35 cycles total) 5) 72°C for 10 min

2.2.3 Agarose gel electrophoresis

PCR products were separated by electrophoresis on 2 % agarose gels, prepared with agarose powder (Invitrogen) dissolved in 1x Tris-Acetate EDTA (TAE) buffer

prepared in-house. Ethidium bromide (2 drops per 100 ml of agarose solution; Dutscher Scientific) was added (to give a final concentration in gel of 0.5 µg/ml). Gels were allowed to cool. 5 µl of loading dye, prepared in-house [glycerol and bromophenol blue] (both Sigma) was added to each PCR reaction product and 10 µl of each sample was added to individual wells. As a control, a DNA ladder was added in a separate lane (O'Gene Ruler 50 bp DNA ladder, Thermo Scientific). PCR products were separated by electrophoresis (Power Station 300, Labnet), run at 100 V for 25 minutes. Products were visualised by ultra-violet (UV) trans illumination (Gel Doc XR+, Biorad).

2.3 Flow cytometric analysis and cell sorting

2.3.1 Isolation and processing of mouse tissues

2.3.1.1 Preparation of single cell suspensions from BM for haematopoietic cells

Femurs and tibiae were harvested, cleaned to remove excess muscle tissue and placed into phosphate buffered saline pH7.4 (PBS; Life Technologies) supplemented with 5 % foetal calf serum (FCS; Thermo Fisher Scientific). Bones were crushed in a mortar in PBS/5%FCS and the BM tissue obtained was disaggregated by pipetting and then passed through a 70 µm cell strainer (CellTrics, Partec) into a 15 ml tube (Falcon). Cells were re-suspended in a defined volume (10 ml for adult BM, 5ml for foetal BM) of PBS/5%FCS to give a single cell suspension and then counted using a Sysmex haemocytometer. Once processed, cells were kept on ice at 4°C.

2.3.1.2 Preparation of single cell suspensions from spleen and thymus

Spleens and thymii were harvested, placed in PBS/5%FCS and fat tissue and blood removed. Tissues were homogenised and passed through a 70 µm cell strainer into a 15 ml tube. The cells obtained were re-suspended in 5 ml of PBS/5%FCS to give a

single cell suspension and then counted using the Sysmex haemocytometer.

2.3.1.3 Preparation of single cell suspensions of stromal BLCs

Femurs and tibiae were harvested, cleaned of excess muscle tissue and placed into PBS/5%FCS. Bones were crushed in a mortar and the BM removed. The fragmented bones were cut into very small pieces with scissors and transferred to a 15 ml tube. The bone pieces were spun down (500 rcf, 5 min, 4°C - Multifuge 3 S-R, Heraeus) and the supernatant removed. Cells were obtained by incubating at 37°C with 5 ml PBS/5%FCS containing type I collagenase (3 mg/ml; Worthington) for 45 min with continuous rotation (SB3 rotator, Stuart). The cell suspension obtained was subsequently passed through a 70 µm cell strainer and the filtrate collected in a 15 ml tube. The residual bone fragments were subject to a second treatment with 5 ml PBS/5%FCS/type I collagenase, which were incubated for a further 45 min at 37°C. Cells were collected as indicated above and filtrates from the two collagenase treatments combined. Cells were then spun down (500 rcf, 5 min, 4°C) and the supernatant aspirated. Cells were re-suspended/washed in 10 ml PBS/5%FCS containing 2 mM EDTA to reduce clumping. Cells were again centrifuged as before, the supernatant aspirated and re-suspended in 10 ml PBS/5%FCS/2mM EDTA, from which an aliquot was taken for cell counting in a Neubauer chamber after live/dead staining, using Trypan blue (Sigma-Aldrich) supplemented with Zap-OGLOBIN II (Beckman Coulter).

2.3.1.4 Preparation of single cell suspension of central bone marrow (CBM) cells

Cells were prepared according to **Section 2.3.1.1**, centrifuged (500 rcf, 5 min, 4°C) and the supernatant aspirated. The cell pellet was re-suspended in 5 ml Dulbecco's

Modified Eagle Medium (DMEM, Life Technologies)/10%FCS containing type IV collagenase (2 mg/ml, Sigma-Aldrich) and *Dnase I* (200 U/ml, 3.24 µg/ml, Merck) and incubated at 37°C for 20 min with continuous rotation. Cells were passed through a 70 µm cell strainer, washed with 10 ml PBS/5%FCS and centrifuged (500 rcf, 5 min, 4°C). The supernatant was aspirated and cells were re-suspended in 5 ml of ammonium chloride solution (Stem Cell Technologies) for 10 min at room temperature (RT) to lyse RBCs. A 100 µl aliquot was taken for cell counting using the Symex haemocytometer. Cells were washed with the addition of 10 ml PBS/5%FCS, centrifuged (rcf, 5 min, 4°C), the supernatant was removed and the cell pellet was re-suspended in PBS/5%FCS to achieve 1×10^7 cells per 50 µl.

To deplete these samples of CD45⁺ cells, they were stained with CD45-PE antibody (1:2 dilution) for 15 min at 4°C. Cells were washed in 10 ml PBS/5%FCS, centrifuged (500 rcf, 5 min, 4°C), the supernatant removed and cells re-suspended in 80 µl PBS/5%FCS per 10×10^6 cells. 20 µl per 10×10^6 cells of anti-PE MicroBeads (Miltenyi Biotec) were added to cells, which were then agitated and incubated for 15 min at 4°C in darkness. Cells were washed in 10 ml PBS/5%FCS, centrifuged again (500 rcf, 5 minutes, 4°C), the supernatant aspirated and cells re-suspended in 1 ml PBS/5%FCS.

Separate magnetic LS columns for each sample were placed onto magnets (MACS[®] Separator, both Miltenyi Biotec). Columns were equilibrated by washing through with 3 ml of PBS/5%FCS, which was collected and discarded. 1 ml cell suspensions were added to the columns and washed through with 9 ml of PBS/5%FCS in order to obtain the CD45-PE negative cells. Eluates from the

columns were collected into 15 ml tubes. Cells were counted after live/dead staining using Trypan blue in a Neubauer chamber.

2.3.1.5 Preparation of single cell suspensions from testes

Testes were harvested and placed into PBS/5% FCS. The tunica albuginea was removed and the testes were transferred into 5 ml DMEM/2%FCS with Collagenase IV (1 mg/ml) and *DNase* I (200 U/ml, 3.24 µg/ml). The testes were incubated at 37°C for 20 min with continuous rotation. Cells were washed with the addition of 10 ml PBS/2%FCS, centrifuged (500 rcf, 5 min, 4°C), the supernatant aspirated, cells re-suspended in trypsin (0.25 %, PAA labs) and *DNase* I and then incubated at 37°C for 10 min with continuous rotation. Cells were again washed with the addition of 10 ml PBS/2%FCS, passed through 70 µm nylon mesh and then counted after live/dead staining with Trypan blue in a Neubauer chamber.

2.3.2 Antibody staining for flow cytometric analysis and sorting

For flow cytometric analysis and cell sorting, the compensation set up of the flow cytometer was performed using single stained CompBeads (Beckton, Dickinson and Company). 1 µl of the respective antibody was mixed with one drop of species-specific and one drop negative control CompBeads and incubated for 15 min at 4°C. After one washing step with 1 ml PBS/5%FCS, beads were re-suspended in 200 µl PBS/5%FCS for analysis.

Cells for fluorescence minus one (FMO) controls were re-suspended in 25 µl PBS/5%FCS containing a 1:200 dilution of purified anti-Fc gamma receptor (FcγR antibody) (in house production) and incubated for 10 min at 4°C (except for the myeloid progenitor stain – at this stage, cells were instead re-suspended in

PBS/5%FCS). FMO controls were typically stained in a total volume of 25-50 μ l. Test samples for analysis and sorting were typically stained in a total volume of 50-100 μ l. Accordingly, test sample cells were typically re-suspended in half the total staining volume of PBS/5%FCS/anti-Fc γ R and incubated for 10 min at 4°C.

Antibody cocktails (**Table 2.3**) were prepared at 2x concentration and half the total staining volume. For staining FMOs or samples, cells were mixed with 2x antibody cocktails and incubated for 15 min at 4°C then washed in 1 ml PBS/5%FCS and spun down (500 rcf, 5 min, 4°C) using a microcentrifuge (5415, Eppendorf). For staining panels involving a secondary antibody or biotin/streptavidin staining step, FMO and sample cells were washed 1 ml PBS/5%FCS, centrifuged (500 rcf, 5 min, 4°C) and the supernatant aspirated. Cells were subsequently re-suspended in a 1x dilution of the respective secondary antibody or streptavidin conjugate (prepared in PBS/5%FCS) followed by 15 min incubation at 4°C. Cells were washed with the addition of 1 ml PBS/5%FCS, centrifuged and the supernatant aspirated. Stained test cells were typically re-suspended in 200-800 μ l PBS/5%FCS for analysis. For sorting, cells were re-suspended at a level of ≤ 10 million cells per ml.

For both flow cytometric analysis and cell sorting, dead cells were excluded by 4,6-diamidino-2-phenylindole (DAPI, Invitrogen) or 7-amino-actinomycin D (7-AAD, Sigma-Aldrich) staining. Flow cytometry analyses were performed on an LSRII analyser (**Table 2.4**). Cell sorts were performed on a FACSAriaII cell sorter (**Table 2.4**, both special order research products, Beckton, Dickinson and Company, BD). Sort purity as determined by test sorting and re-analysis prior to and after the sample sort, was typically above 95 %. Flow cytometry data analysis was performed using FACSDiva (BD) or FlowJo (TreeStar).

2.3.2.1 Cell cycle analysis

BM haematopoietic cells were prepared and counted according to **Section 2.3.1.1**, 60×10^6 cells were aliquoted to be used for FMOs. Cells were re-suspended in FcyR according to **Section 2.3.2**. Cells were centrifuged (500 rcf, 5 min, 4°C), supernatant aspirated, cells then re-suspended in fixation/permeabilisation buffer (0.5×10^6 cells/100 µl, BD) and kept on ice for 30 min. Cells were washed twice in 10 ml of the supplied wash buffer (BD), centrifuged (500 rcf, 5 min, 4°C) and supernatant aspirated. Cell samples and FMOs were stained with Ki-67 Ab, transferred to 1.5 ml tubes and kept O/N with agitation at 4°C in darkness. Thereafter, cells were washed using 1 ml wash buffer, centrifuged (500 rcf, 5 min, 4°C), the supernatant aspirated, then cells were re-suspended in DAPI solution (0.5×10^6 cells / 100 µl) and kept on ice for 1 hr. Samples were washed in 1 ml wash buffer, centrifuged (500 rcf, 5 min, 4°C) and supernatant aspirated. Cell samples and FMOs were re-suspended in 200 and 150 µl of wash buffer respectively, which were then analysed using the LSRII (**Section 2.3.2**).

Table 2.3 List of antibodies for flow cytometry

Antigen	Conjugate	Clone	Supplier	Application
7-AAD (7 – aminoactinomycin)			Sigma-Aldrich	Viability dye for BLC, BM, CBM, HSC, MPC staining
ALCAM	Biotin	AAC06342	R&D Systems	BLC staining
B220	PE-Cy5	RA3-6B2	Biolegend	Lineage cocktail for CC, HSC and MPC staining
B220	APC	RA3-6B2	Biolegend	Lineage cocktail for T-cell progenitor staining
CD105 (Endoglin)	Biotin	MJ7/18	Biolegend	MPC staining
CD11c	APC	N418	eBioscience	Lineage cocktail for T-cell progenitor staining
CD150	APC	TC15-12F12.2	Biolegend	MPC staining
CD150	PE-Cy7	TC15-12F12.2	Biolegend	CC and HSC staining
CD19	APC	1D3	BD	Lineage cocktail for T-cell progenitor staining
CD25	PerCPCy5.5	PC61	Biolegend	T-cell progenitor staining
CD3	APC	145-2C11	eBioscience	Lineage cocktail for T-cell progenitor staining

CD4	AlexaFluor700	RM4-5	eBioscience	T-cell progenitor staining
CD4	PE-Cy5	RM4-5	Biolegend	Lineage cocktail for CC, HSC and MPC staining
CD31	APC	390	eBioscience	BLC staining
CD31	PE-Cy7	390	eBioscience	CBM staining
CD41	PE-Cy7	MWReg30	eBioscience	MPC staining
CD45	PE	30-F11	eBioscience	BLC and BM staining
CD45	PE-Cy7	30-F11	eBioscience	SSC staining
CD48	APC	HM48-1	Biolegend	HSC staining
CD5	PE-Cy5	53-7.3	Biolegend	Lineage cocktail for CC and HSC staining
CD51	PE	RMV-7	Biolegend	SSC staining
CD8	PE-Cy5	53-6.7	Biolegend	Lineage cocktail for CC, HSC and MPC staining
CD8	PE-Cy7	53-6.7	Biolegend	T-cell progenitor staining
CD9	Biotin			SSC staining
c-Kit	APC-eFluor780	2B8	Biolegend	CC, HSC, MPC, T-cell progenitor staining;
DAPI (4', 6-diamino-2-phenylindole)			Invitrogen	CC staining, viability staining
Flt3	PE	A2F10.1	BD	T-cell progenitor staining
Gfra-1	Purified	-	R&D Systems	SSC staining
Gr-1	APC	RB6-8C5	Biolegend	Lineage cocktail for T-cell progenitor staining
Ki67	PE	B56	BD	CC staining
Kitl	Biotin	PMP23	Bio-Rad	293T cell staining
LeptinR	Biotin	-	R&D Systems	BM staining
Mac-1 (CD11b)	PE-Cy5	M1/70	Biolegend	Lineage cocktail for CC, HSC and MPC staining
NK1.1	APC	PK136	eBioscience	Lineage cocktail for T-cell progenitor staining
Sca-1	FITC	E13-161.7	Biolegend	CC staining
Sca-1	PacificBlue	E13-161.7	Biolegend	HSC, MPC staining
Sca-1	PE-Cy7	E13-161.7	Biolegend	BLC staining
Streptavidin	PE-TexasRed	-	BD	MPC staining
Streptavidin	BrilliantViolet 421	-	Biolegend	BLC, CBM and SSC staining
Streptavidin	BrilliantViolet 450	-	Biolegend	CBM staining
Ter119	BrilliantViolet 650	TER-119	Biolegend	MPC staining
Ter119	FITC	TER-119	eBioscience	BLC, CBM and MPC staining
Ter119	PE-Cy5	TER-119		CBM staining
Ter119	PerCP-Cy5.5	TER-119	eBioscience	MPC staining
TCRγδ	APC	eBioGL3	eBioscience	Lineage cocktail for T-cell progenitor staining
TCRβ	APC	H57-597	eBioscience	Lineage cocktail for T-cell progenitor staining
Thy1 (CD90)	APC	30-H12	BD	SSC staining
-	AlexaFluor488	-	Invitrogen	SSC staining

Table 2.4 Instrument configurations of BD FACSAriaII SORP and BD FACS LSRII SORP

Instrument	Laser wavelength	Fluorophore	Emission filter
<i>BD FACS AriaII</i>	Violet 407 nm	Brilliant Violet 421 (BV421)	450/50
	Blue 488 nm	SSC	488/10
	Green 532 nm	PE	575/25
		PE-Cy5	685/35
		PerCPCy5.5	710/20
		PE-Cy7	780/60
	Red 640 nm	APC	670/14
<i>BD FACS LSRII</i>	Violet 405 nm	Pacific Blue (PBL)	450/50
		Brilliant Violet 421 (BV421)	450/50
		Pacific Orange (PO)	525/50
	Blue 488 nm	SSC	488/10
		FITC	525/50
		Alexa Fluor 488 (AF488)	525/50
	Green 532 nm	PE	575/25
		PE-Texas Red	610/20
		PE-Cy5	685/35
		PerCP-Cy5.5	710/40
		PE-Cy7	780/60
	Red 640 nm	APC	670/14
		Alexa Fluor 700	730/45
		APC-eFluor 780	780/60

2.4 *In vivo* assays

All *in vivo* procedures were performed under sterile conditions and in a laminar flow hood.

2.4.1 Kidney subcapsular explantation (KSE) assays

Embryos were collected from pregnant mice at E15.5 and foetal femurs dissected under a stereomicroscope. Tail biopsies were taken for genotyping. Both femurs from each embryo were implanted under the left kidney capsule of 8-12 wk old female WT C57BL/6 mice. Bones were dissected from the kidney 3 weeks after the explant date.

2.4.2 Tamoxifen treatment

Tamoxifen was dissolved completely in 100 % ethanol (60 mg/ml; w/v), mixed in a 1:1 ratio with Cremophor (all Sigma-Aldrich) and then double the volume of PBS was added and mixed thoroughly. A dose of 3 mg was given in a 200 µl subcutaneous

injection on days 1 and 3 to *RosaCre^{ER2};Kitl^{Δex7/Δex7}* mice. Each mouse received 2 doses in total.

2.5 Immunohistochemistry

2.5.1 Preparation of tissues

2.5.1.1 Preparation of femurs

Dissected femurs were fixed in 4 % (w/v) paraformaldehyde (PFA, Electron Microscopy Science) at 4°C and then placed in decalcifying solution (Sigma-Aldrich) for 48 hr. Specimens were dehydrated at RT using an ethanol (Sigma-Aldrich) gradient from 70-100 % in 4 steps for 1 hr at each concentration and then 3 hr in Histoclear (Fisher Scientific). Samples were subsequently put in a 1:1 Histoclear:paraffin solution (Sigma-Aldrich) for 1 hr at 65°C and then paraffin-embedded.

2.5.1.2 Preparation of testes

Dissected testes were prepared using two separate methods; one testicle from each animal being fixed-frozen and the other fresh frozen. Those testes to be fixed-frozen were placed in 4 % PFA for 4 hr at 4°C and then placed into individual histocette embedding cassettes (Simport). The cassettes were immersed in a 10 % sucrose (Sigma-Aldrich) aqueous solution for 2 hr, then in a 20 % solution for 2 hr and finally a 30 % solution O/N, all at 4°C. Histomolds (Leica) were filled with optimal cutting temperature compound (OCT, Sakura) and placed on ice to pre-cool for 2-3 min. The moulds were then removed and the testes samples placed in the centre of the OCT using tweezers. The sample was then kept on dry ice until the OCT compound turned white. Samples were placed at -80°C until ready to be sectioned.

For samples to be fresh-frozen, testes following their dissection and rinse with PBS were placed directly into OCT compound and frozen according to the protocol given above.

2.5.2 Sectioning of tissues

Paraffin-embedded sections (8-10 μm) were cut using a microtome (Leica RM 2165 microtome) onto poly-lysine coated slides (VWR), then dried on a hot plate (Leica HI1220 flattening table) to straighten the slides O/N. Slides were then placed in Histoclear for 30 min, followed by 100 % ethanol for 30 minutes before staining.

Frozen sections were cut using a cryostat (Leica CM 3050 S Cryostat), placed onto poly-lysine coated slides (Matsunami), left to dry at RT for several hours and if not used immediately, slides were stored at -80°C . Before blocking and staining, slides were washed for 15 min with PBS at RT.

2.5.3 Staining of tissue sections

Sections were incubated with target antigen retrieval buffer and proteinase K (both Dako) for antigen retrieval. Sections were blocked with 5 % foetal bovine serum (FBS) (v/v) and incubated O/N at 4°C with primary antibodies (**Table 2.5**). Sections were incubated with secondary antibodies for 2 hr at RT (**Table 2.6**). Sections were mounted with coverslips using ProLong Gold Antifade Reagent (Life Technologies). All immunofluorescence data were obtained using an Olympus FV1000 confocal microscope.

Table 2.5 List of primary antibodies for immunohistochemistry

Antigen	Host/ Isotype	Supplier	Application
CD150	Purified	eBioscience	HSC staining
Lineage	Rabbit	R&D Systems	HSC staining
CD48	Rabbit	Abcam	HSC staining
c-Kit	Goat	R&D Systems	HSC/HPC staining
c-Kit	Rat	Millipore	HSC/HPC/SPC staining
DAPI (4', 6-diamino-2-phenylindole)	-	Life Technologies	Nuclear staining
EPOR	Rabbit	R&D Systems	Erythroid progenitor staining
Gfra-1	Purified	R&D Systems	SSC staining
Kitl (extracellular)	Goat	R&D Systems	Endothelial staining
Kitl (intracellular)	Rabbit	Besmer lab	Endothelial staining (membrane bound)
MECA-32	Rat	Abcam	Endothelial staining
TOPRO-3	-	Life Technologies	Nuclear staining
VE-Cadherin	Goat	R&D Systems	Endothelial staining
VE-Cadherin	Rat	R&D Systems	Endothelial staining
VEGFR3	Rat	R&D Systems	Endothelial staining

Table 2.6 List of secondary antibodies for immunohistochemistry

Antibody	Supplier	Application
Donkey anti-goat IgG 488	Life Technologies	SSC/ Endothelial staining
Donkey anti-rabbit IgG 488	Life Technologies	Endothelial staining
Donkey anti-rat 488	Life Technologies	HSC/HPC/SPC staining
Donkey anti-goat IgG Cy3	Strattech (Jackson ImmunoResearch Laboratories)	Endothelial/ Erythroid progenitor staining
Donkey anti-rabbit IgG Cy3	Strattech (Jackson ImmunoResearch Laboratories)	Endothelial staining
Donkey anti-rat IgG Cy5	Strattech (Jackson ImmunoResearch Laboratories)	HSC/ Endothelial staining
Donkey anti-rat IgG Dylight 549	Strattech (Jackson ImmunoResearch Laboratories)	Endothelial staining

2.6 *In vitro* assays and procedures

2.6.1 Murine SCF Immunoassay: ELISA

Levels of sKitl were determined using a mouse SCF Quantikine® Immunoassay ELISA kit (R&D systems) according to the manufacturer's instructions. A 2-fold dilution series was made using a known concentration standard of recombinant

mouse SCF in the supplied calibration buffer to generate the standard curve. Test samples were diluted 1 in 2 in calibration buffer. 50 µl of calibration buffer was added to each well. 50 µl of each standard curve SCF sample and test samples were added in duplicate to wells (coated with a polyclonal antibody specific for mouse SCF) in a 96-well polystyrene plate.

Samples were incubated for 2 hr at RT then washed using the supplied wash buffer. 100 µl mouse SCF conjugate was added to each well, incubated at RT for 2 hr then washed again. 100 µl of colour substrate solution made using hydrogen peroxide and chromogen (tetramethylbenzidine) was added to each well and the plate kept in the dark for 30 min at RT. This resulted in a colour change to blue, which with the addition of 100 µl of the supplied stop solution, turned samples yellow. Optical density measurements of each plate were determined within 30 min, using a microplate reader (Molecular Devices-SpectraMaxM2e) set to 450 nm with wavelength correction set to 570 nm.

2.6.2 Production of Sl^d Kitl and sKitl sequences *in vitro*

2.6.2.1 Isolation of RNA

Mouse cerebellum was isolated from $Sl^d/+$ and $Kitl^{\Delta ex7/+}$ mice, gently homogenised and mixed vigorously with 160 µl chloroform (Sigma-Aldrich) and 800 µl TRIzol (Life Technologies) to achieve an emulsion. Samples were kept at RT for 2-3 min before centrifugation (12,000 g, 15 min at 4°C). The aqueous phase was transferred to a new 1.5 ml tube and then 1 µl linear polyacrylamide (LPA, 25 µg/µl, Sigma-Aldrich) and an equivalent volume of isopropanol was added. This was thoroughly mixed and after 10 min at RT, samples were centrifuged (16,000 g, 10 min at RT) and the supernatant aspirated. To the pellet, 900 µl 70% ethanol was added, the sample was

thoroughly mixed, centrifuged (16,000 g, 5 min at RT) and the supernatant removed. Pellets were air-dried at RT and then re-suspended in 14 µl nuclease-free water.

2.6.2.2 *Sl^d Kitl* and *sKitl* sequence translation and amplification

Kitl^{Δex7/+} RNA was used for direct *reverse transcriptase specific target amplification* PCR (RT-STA PCR) using the primers in **Table 2.7** and conditions in **Table 2.8**. *Sl^d/+* RNA was converted to cDNA and then targeted gene amplification was carried out as indicated below (**Table 2.9**). *Kitl* PCR products were cloned using TOPO Cloning®Kit PCR®II-TOPO®-vector and transformed into One Shot®TOP10 Chemically Competent Cells (both Life Technologies). Correct insertion of *mKitl*, *sKitl* and *Sl^d Kitl* sequences into the TOPO vector were confirmed by digestion with *BamH*I /*Not*I (both Fermentas). Samples were sequenced using primers in **Table 2.10**.

Table 2.7 List of primers for sequence amplification

Reaction	Primer name	Sequence (5'-3')
<i>Kitl^{Δex7}</i> RT-STA PCR	Kitl2F	TCCTCCTCCTCCTCTTCCTC
	Kitl2R	AAGCAGGGCTTTCTGTTCAA
<i>Sl^dKitl</i> -PCR	Kitl3F	AGAGGCCAGAACTAGATCC
	Kitl3R	AGAGGCCAGAACTAGATCC

Table 2.8 RT-STA PCR protocol

PCR Protocol	
<i>Kitl^{Δex7/+}</i> RT-STA PCR	1) 50°C for 5 min 2) 95°C for 2 min 3) 95°C for 20 s 4) 60°C for 30 s (<i>Back to 2</i>) - 42 cycles total) 5) 72°C for 3 min

Table 2.9 Production from *Sl^d Kitl* sequence

	Protocol
<i>Production of cDNA</i>	1µl random hexamer primers (50 ng/µl) (Fermentas) 1 µl dNTPs (10mM) (Fermentas) 10 µl RNA - 65°C for 10 min - Put immediately on ice 4 µl 5x First Strand buffer (MMLV-RT kit) 2 µl 0.1 M DTT (MMLV-RT kit) 1µl RNase out RNase inhibitor (Life Technologies) 1 µl Superscript II Reverse Transcriptase (SSII) (Life Technologies) - 25°C for 10 min - 42°C for 50 min - 70°C for 15 min
<i>Sl^d Kitl-PCR</i>	1) 98°C for 30s 2) 98°C for 10 s 3) 60°C for 30 s 4) 72°C for 10 s (<i>Back to 2</i>) - 35 cycles total) 5) 72°C for 10 min

Table 2.10 Sequencing-primer sequences

Primer name	Sequence (5'-3')
M13R	CAGGAAACAGCTATGACC
KitLR	GCCACTGTGCGAAGGTAAC

2.6.3 Subcloning of *Kitl* sequences into viral vectors

Soluble and membrane-bound *Kitl* cDNA were inserted into pMSCV-iRES-GFP plasmids by digesting with *Hpa* I/*Xho* I and *Pvu* I/*Xho* I (Fermentas/ NEB), ligated using T4 DNA ligase and transformed into TOP10 *E. coli* cells (both Life Technologies) according to the manufacturer's protocol. *Sl^d Kitl* was subcloned into *mKitl*-pMSCV-iRES-GFP using *Bam*H I, *Bgl* II and *Nsi* I (Fermentas). Correct ligation of *mKitl*, *sKitl* and *Sl^d Kitl* into pMSCV-iRES-GFP vectors was confirmed by digestion with *Spe* I (NEB). Samples were sequenced using primers given in **Table 2.10**.

2.6.4 Assessment of biological activity of Kitl *in vitro*

2.6.4.1 Transient transfection of 293T cells

Human embryonic kidney (HEK) 293T/17 cells (293T, ATCC® Number: CRL-11268™) plated at a density of 1.5×10^6 cells /well in a 6-well dish (Costar) were cultured in DMEM/10%FCS/1% Penicillin Streptomycin (PS) (PAA labs) (v/v) at 37°C in a humidified atmosphere in 5 % CO₂ for 24 hr (Sanyo CO₂ incubator). Each transfection reaction (with vectors containing different isoforms of *Kitl*) contained 570 µl DMEM/1%PS (serum free), 6 µg of DNA Kitl-pMSCV-iRES-GFP vector DNA and 24 µl FuGene transfection reagent (Promega). These transfection solutions were mixed and incubated for 15 min at RT and then added in a drop-wise manner to the cells. Transfected 293T cells were incubated in a humidified atmosphere in 5 % CO₂ at 37°C for 48 hr. The supernatants from the transfected cells were collected and then evaluated for the level of sKitl by ELISA as described previously (**Section 2.6.1**).

2.6.4.2 Proliferation assay

MC/9 cells (ATCC-CRL-8306™) were plated at 250,000 cells/well in 96-well plates (Costar). 100 µl of the above 293T supernatants were added to each well and incubated in a humidified atmosphere in 5 % CO₂ at 37°C for 48 hr. After this time, 2 ml CellTiter96 AQueous Non-Radioactive Cell Proliferation Assay MTS reagent was added to 75 µl PMS reagent (both Promega) and thoroughly mixed. 20 µl of this MTS/ PMS solution was added to each well. The cells were then incubated for a further 4 hr and absorbance determined at 490 nm (Molecular Devices-SpectraMaxM2e plate reader). Absorbance values were used to calculate percentage of maximum proliferation of cells (in relation to cell proliferation - in the

recommended optimal medium: DMEM high glucose/10 % FCS/10 % rat T-STIM (v/v)/0.05 mM β -mercaptoethanol (v/v)).

2.7 Gene expression analysis

2.7.1 Sample preparation and multiplex quantitative PCR

For multiplex quantitative gene expression analysis using the BioMark 48.48 dynamic array (Fluidigm), 100 cells were FACS sorted into 0.2 ml reaction tubes containing a pre-amplification mix in a total volume of 10 μ l. Reverse transcription and amplification of target transcripts was performed on a thermal cycler (Tetrad II 96-Well Alpha Unit, Biorad) using the protocol given in **Table 2.11**.

Table 2.11 Fluidigm quantitative gene expression protocols

	Protocol
<i>Pre-amplification mix</i>	5 μ l CellsDirect 2x reaction mix (Invitrogen), 0.1 μ l SUPERase 12-In RNase inhibitor (Ambion) 1.2 μ l TE buffer (Sigma) 1.2 μ l CellsDirect RT/Taq mix (Invitrogen) 2.5 μ l 0.2x gene specific TaqMan assay mix (Applied Biosystems)
<i>Pre-amplification reaction</i>	RT reaction: 50°C for 15 min Inactivation of reverse transcriptase: 95°C for 2 min Target gene amplification (22 cycles): 95°C for 2 min 60°C for 4 min
<i>Quantitative PCR reaction</i>	1) 95°C for 10 min 2) 95°C for 15s 3) 60°C for 60 s (<i>40 cycles in total</i>)

Pre-amplified cDNA samples were diluted 1:5 in TE buffer (Sigma) to a total volume of 50 μ l. Murine Taqman assays (**Table 2.12**) and sample mixes were prepared according to manufacturer's instruction and loaded onto a primed 48.48 dynamic array chip (Fluidigm). The quantitative polymerase chain reaction (qPCR) reaction was performed according to the protocol given in **Table 2.11**. The data were analysed using the BioMark Real-Time PCR Analysis software v2.0 (Fluidigm) and

cycle threshold (ΔC_t) values were calculated by normalising to housekeeping genes such as *Hprt* or *Gapdh*, which were always included in the assay mix.

Table 2.12 List of Taqman assays

Gene symbol	Gene name	Assay ID	Gene aliases
<i>Actb</i>	B-actin	Mm00607939_s1	
<i>Alcam</i>	Activated leukocyte cell adhesion molecule	Mm00711623_m1	
<i>Ang1</i>	Angiopoietin 1	Mm00456503_m1	
<i>Ang2</i>	Angiopoietin 2	Mm00545822_m1	
<i>Angptl1</i>	Angiopontin-like protein 1	Mm00472259_m1	
<i>Angptl2</i>	Angiopontin-like protein 2	Mm00507897_m1	
<i>Angptl 4</i>	Angiopontin-like protein 4	Mm00507897_m1	
<i>Cd31</i>	CD31 antigen	Mm00476702_m1	
<i>Cd34</i>	CD34 antigen	Mm00519283_m1	
<i>Cd44</i>	CD44 antigen	Mm00507897_m1	
<i>Cd45</i>	CD45 antigen	Mm01293577_m1	
<i>Cd90</i>	CD90 antigen	Mm00493681_m1	
<i>Cdh5</i>	Cadherin 5	Mm00486938_m1	VE-Cadherin
<i>Csf1</i>	Colony stimulating factor 1	Mm00432688_m1	
<i>Csf2</i>	Colony stimulating factor 2	Mm00438328_m1	GM-CSF
<i>Cxcl12</i>	Chemokine (C-X-C motif) ligand 12	Mm00445552_m1	SDF1
<i>Cxcr4</i>	Chemokine (C-X-C motif) receptor 4	Mm01292123_m1	
<i>Dll1</i>	Delta-like1	Mm00432841_m1	
<i>Dll4</i>	Delta-like4	Mm00444619_m1	
<i>EfnB2</i>	Ephrin b2	Mm01215897_m1	
<i>Epha4</i>	Eph receptor A4	Mm00433056_m1	
<i>Epo</i>	Erythropoietin	Mm01202755_m1	
<i>Epor</i>	Erythropoietin receptor	Mm00438760_m1	
<i>Flk1</i>	Kinase insert domain protein receptor	Mm01222421_m1	VEGFR2
<i>Flt1</i>	FMS-like tyrosine kinase 1	Mm00438980_m1	VEGFR1

<i>Flt4</i>	FMS-like tyrosine kinase 4	Mm00433333_m1	VEGFR3
<i>Flt3l</i>	FMS-like tyrosine kinase 3 ligand	Mm00442801_m1	
<i>Flt3</i>	FMS-like tyrosine kinase 3	Mm00439016_m1	
<i>Gapdh</i>	Glyceraldehyde-3-phosphate dehydrogenase	Mm99999915_g1	
<i>Gypa</i>	Glycophorin A	Mm00494848_m1	Ter 119
<i>Hprt1</i>	Hypoxanthine guanine phosphoribosyl transferase 1	Mm00446968_m1	
<i>Id1</i>	Inhibitor of DNA binding 1	Mm00775963_g1	
<i>Il3</i>	Interleukin 3	Mm00439631_m1	
<i>Il6</i>	Interleukin 6	Mm00446190_m1	
<i>Il7</i>	Interleukin 7	Mm00434291_m1	
<i>Jag1</i>	Jagged 1	Mm00496902_m1	
<i>Jag2</i>	Jagged 2	Mm01325629_m1	
<i>Kit</i>	Kit oncogene	Mm00445212_m1	
<i>Kitl</i>	Kit ligand	Mm00442972_m1	
<i>mKitl</i>	Membrane kit ligand	Mm01232339_m1	
<i>Mmp7</i>	Matrix metalloproteinase 7	Mm00487724_m1	
<i>Mmp9</i>	Matrix metalloproteinase 9	Mm00442991_m1	
<i>Mpl</i>	Myeloproliferative leukemia virus oncogene	Mm00440310_m1	Tpo
<i>Nes</i>	Nestin	Mm00450205_m1	
<i>Notch1</i>	Notch gene homolog 1	Mm00435249_m1	
<i>Notch2</i>	Notch gene homolog 2	Mm00803077_m1	
<i>Opn</i>	Osteopontin (Secreted phosphoprotein 1)	Mm00436767_m1	Sp1
<i>Osx</i>	Osterix (Sp7 transcription factor 7)	Mm00504574_m1	Sp7
<i>Pdgfa</i>	Platelet derived growth factor, alpha	Mm00435540_m1	
<i>Ly6a</i>	Cell surface antigen	Mm00726565_s1	
<i>Tek</i>	Endothelial specific receptor tyrosine kinase	Mm00443242_m1	Tie2
<i>Timp1</i>	Tissue inhibitor of metalloproteinase 1	Mm00441818_m1	
<i>Timp2</i>	Tissue inhibitor of metalloproteinase 2	Mm00441825_m1	
<i>Vwf</i>	Von Willebrand factor homolog	Mm00550376_m1	

2.7.2 qPCR analysis for deletion efficiency determination

BLC and CBM cells were prepared as per **Section 2.3.1.3** and **Section 2.3.1.4** and then stained and sorted as per **Section 2.3.2** directly in 800 µl TRIzol and RNA was prepared according to **Section 2.6.2.1**.

2.7.2.1 cDNA synthesis

Per sample, 4 µl VILO reaction mix, 2 µl 10x SuperScript Enzyme mix (both Life Technologies) and 1 µl of nuclease-free water were added to 13 µl of RNA in 1.5 ml tubes. A no RT control was set up in parallel, with nuclease-free water added instead of the SuperScript Enzyme mix. Tubes were gently mixed, incubated at 25°C for 10 min, then 42°C for 1 hr and the reaction terminated by incubating at 85°C for 5 min.

2.7.2.2 qPCR reaction

For each sample, 10 µl of CellsDirect 2x Reaction mix, 1 µl Taqman probe, 7 µl nuclease-free water and 2 µl cDNA were mixed (prepared in triplicate). A separate reaction mix was made for each gene to be investigated using the Taqman probes for total *Kitl*, *mKitl* and *Hprt* indicated in **Table 2.12**. Samples were pipetted into a specialised 96-well plate (MicroAmp Fast Optical 96-well Reaction Plate) and run using 7500 Fast Real Time PCR system (both Applied Biosystems).

2.8 Statistical analysis

Values shown represent the mean $\pm$ standard error of the mean (SEM). The statistical significance of differences between groups of samples was assessed using the two-tailed unpaired Student's t-test except for analysis of Mendelian ratios, which used the Chi-squared test.

3 Validation of an Appropriate Model for Loss of *mKitl*

3.1 Introduction

Many models of *Sl* locus mutations have demonstrated the importance of *Kitl* for haematopoiesis *in vivo*. The most extensively studied mutant with altered *Sl* alleles is the *Sl/Sl^d* mouse^{259,268}, which exhibits macrocytic anaemia, decreased numbers of progenitor and mast cells^{217,239,241}, has half the number of CFU-S cells and has a significant reduction in phenotypic HSCs compared to WT littermates^{259,260}. These mice are reported to only express the gene encoding a truncated, soluble form of the cytokine²¹⁷. When this *Sl^d* truncated soluble protein (*Sl^dKitl*) is recombinantly expressed in yeast *in vitro*, it demonstrates comparable levels of activity to the WT sKitl^{217,259}. Administration of pharmacological doses of recombinant sKitl into *Sl/Sl^d* mice, partially reduces the severity of the macrocytic anaemia and locally repairs the mast cell deficiency, but not completely²¹⁴. These data and the observation that *Sl/Sl^d* mice exhibit many of the same phenotypic defects to *Sl/Sl* mice (which lack the *Kitl* gene, resulting in embryonic lethality^{241,248}), indicated that the action of sKitl alone cannot compensate for the absence of mKitl. Therefore the soluble form of the growth factor seems insufficient and the membrane bound form of *Kitl* would also appear to be specifically required for haematopoiesis.

This *Sl/Sl^d* mutant has long been claimed to be the model for loss of mKitl, however, this model has not been thoroughly validated given that there has been no *in vivo* demonstration of the specific loss of mKitl or investigation/confirmation of the expression of sKitl protein in the BM or the circulation. It is also limited by being a non-conditional model.

The understanding of Kitl biology has been further developed by the work of the Morrison group, which highlighted the role of EC and LepR⁺ PVC expression of *Kitl* for HSC maintenance, with selective simultaneous deletion of *Kitl* expression in the EC and LepR⁺ PVC cells of mice leading to a reduction in the absolute number of HSCs in BM to ~5 % of WT levels⁸⁹. However, the studies failed to prove the importance of the localised role of Kitl expression, as there was no investigation of the individual expression of mKitl and sKitl protein in BM or of the latter in the circulation. Therefore, these studies did not investigate whether the soluble and/or membrane forms of Kitl (which have been shown to have varying activities *in vitro*²²⁸) have different roles within the BM niche *in vivo*. In the absence of determination of systemic sKitl levels it is difficult to assign the HSC phenotype seen, solely to a local effect in the BM.

Given the limitations of existing models, starting from the basis of the *Sl/Sl^d* mutant, mouse models were developed which had germline deletion and conditional, inducible deletion of *mKitl* in all tissues, haematopoietic effects were determined in an attempt to better discriminate the roles of mKitl and sKitl.

3.2 Results

3.2.1 *Sl/Sl^d* mice lack expression of both mKitl and sKitl *in vivo*

As indicated above, *Sl/Sl^d* mice are currently considered the ‘proof of principle’, demonstrating a specific requirement of mKitl and it has been hypothesised that the attributed phenotype in the *Sl/Sl^d* mice results from the specific loss of mKitl^{217,259}. Accordingly, these mice were initially chosen to investigate the specific role of mKitl in haematopoiesis within the BM niche. Prior to proceeding with studies in novel

models, one of the key questions in Sl/Sl^d mice, as they are unlikely to express any mKitl protein, is whether they actually express the truncated sKitl *in vivo*?

Femurs from Sl/Sl^d mice and WT littermate controls were processed to obtain tissue sections as indicated in **Section 2.5**. Immunohistochemistry (IHC) analysis of BM femur sections involved staining with antibodies (Abs) against the extracellular (ExC) and intracellular (IC) domains of Kitl in addition to the nuclear stain TO-PRO®-3 (**Figure 3.1**, performed by *Dr. S. Matsuoka, (S.M.)*). BM from both groups showed staining with TO-PRO®-3. As expected, the littermate WT control BM showed staining with both Abs. However the Sl/Sl^d BM sections did not show staining for either mKitl (IC Ab) or sKitl+mKitl (ExC Ab). This was unexpected, as the published data^{217,259} would suggest that these mice would lack the membrane-bound form but express the soluble protein and therefore show staining with the ExC domain-specific Ab.

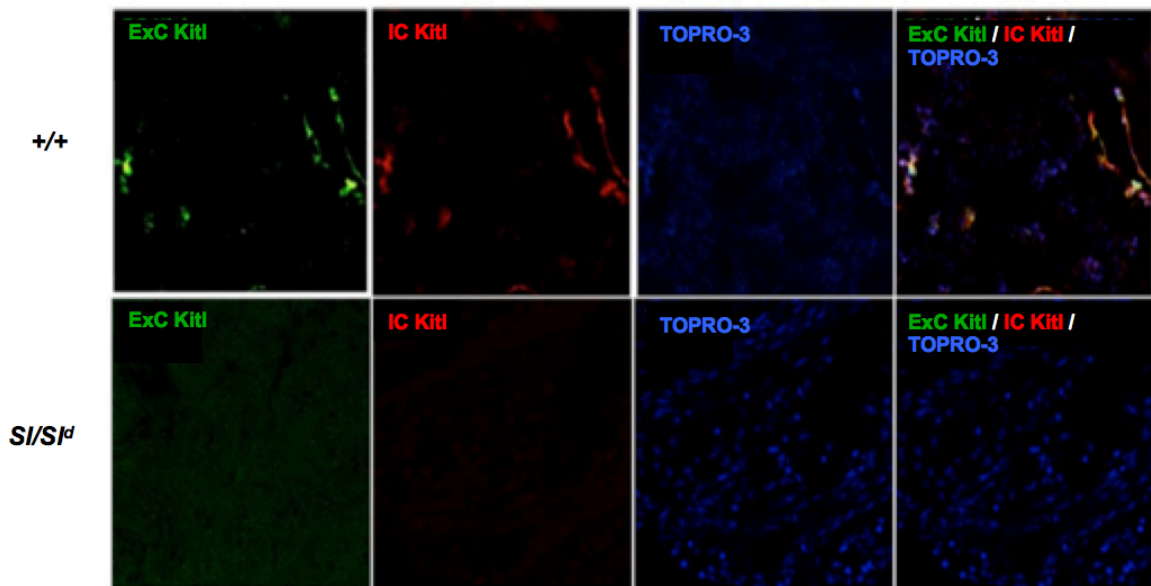


Figure 3.1 Kitl is not expressed in the BM of Sl/Sl^d mice

Immunostaining for extracellular Kitl (ExC Kitl, green: primary + donkey anti-goat IgG 488), intracellular Kitl (IC Kitl, red: primary + donkey anti-rabbit IgG Cy3) with TO-PRO®-3 (blue) in newborn Sl/Sl^d and WT (+/+) littermate control mouse BM. Field of view is $367\mu\text{m}^2$. Representative results of 8 sections from 3 mice of each genotype.

To rule out the possibility that the BM sections used had not contained any Kitl-expressing cells, sections were stained with TO-PRO®-3, Kitl Ab and a VE-Cadherin (VE-Cad) Ab for ECs (S.M). These markers are both known to be expressed on ECs^{89,269}. *Sl/Sl^d* BM sections contained VE-Cad⁺ cells, however there was no staining for Kitl, unlike the littermate WT controls which showed a strong level of co-expression between the two markers (**Figure 3.2**).

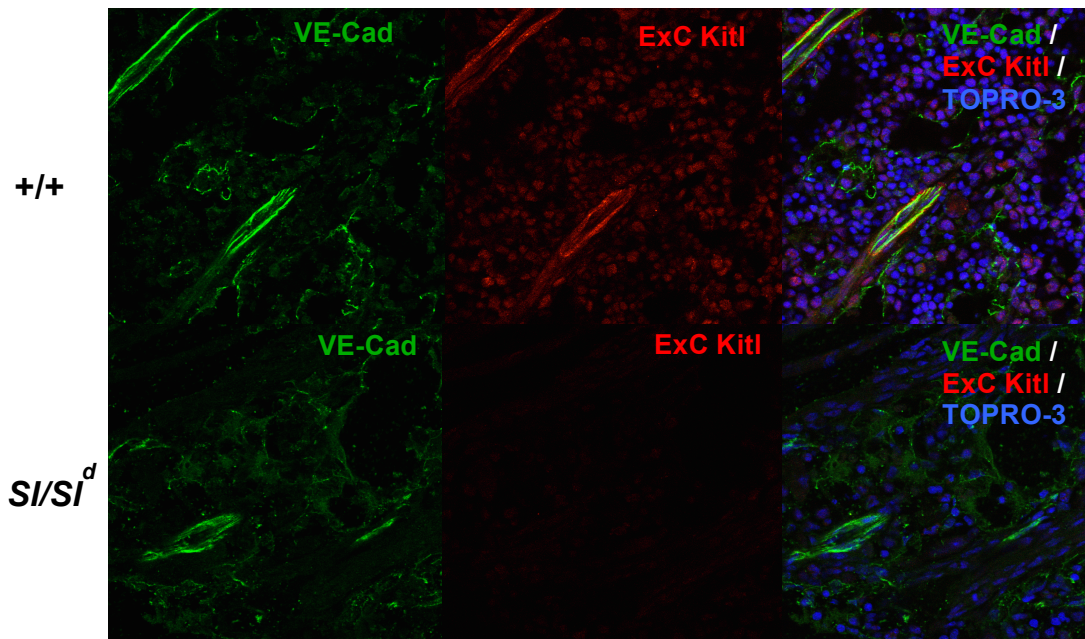


Figure 3.2 VE-Cadherin⁺ endothelial cells express sKitl in WT mice but lack sKitl expression in *Sl/Sl^d* mice

Immunostaining for VE-Cadherin (VE-Cad, green: primary + donkey anti-rat IgG 488), extracellular Kitl (ExC, red: donkey anti-goat IgG Cy3) with TO-PRO®-3 (blue) in newborn *Sl/Sl^d* and WT (+/+) littermate control mouse BM. Field of view is 367µm². Representative results of 8 sections from 3 mice of each genotype.

To confirm if the lack of Kitl was limited to the BM or if it was also systemically absent in *Sl/Sl^d* mice, a second approach was used to determine the presence of sKitl in this strain. Serum was isolated from the PB of groups of 3-week old *Sl/+*, *Sl^d/+*, *Sl/Sl^d* mice and WT littermate controls and then assayed using the sKitl-specific mouse SCF Quantikine ELISA kit (**Section 2.6.1**). This ELISA utilises a murine Kitl Ab which

detects a portion of the ExC domain of the protein which is present in the S^d Kitl and WT sKitl proteins.

Compared to their WT littermate controls, both $Sl/+$ and $S^d/+$ mice showed an ~ 40-50% reduction in the level of systemic sKitl (WT: 165.8 ± 9.1 vs. 72.4 ± 15.5 and WT: 147.5 ± 6.7 vs. 90.0 ± 6.0 pg/ml, respectively, both $p < 0.0001^{****}$), whereas the combination of the Sl and S^d mutations in the compound heterozygote resulted in almost the complete absence of sKitl in the Sl/S^d mice (**Figure 3.3**). In fact, only 1 of 4 of these mice showed a detectable level and the overall mean was only 1.6 ± 1.6 pg/ml compared to their WT littermates which had a value of 154.6 ± 9.9 pg/ml ($p < 0.0001^{****}$). Taken together, these results demonstrate that despite the fact that Sl/S^d encodes a truncated soluble form of Kitl, these mice appear to lack almost complete expression of Kitl protein *in vivo*.

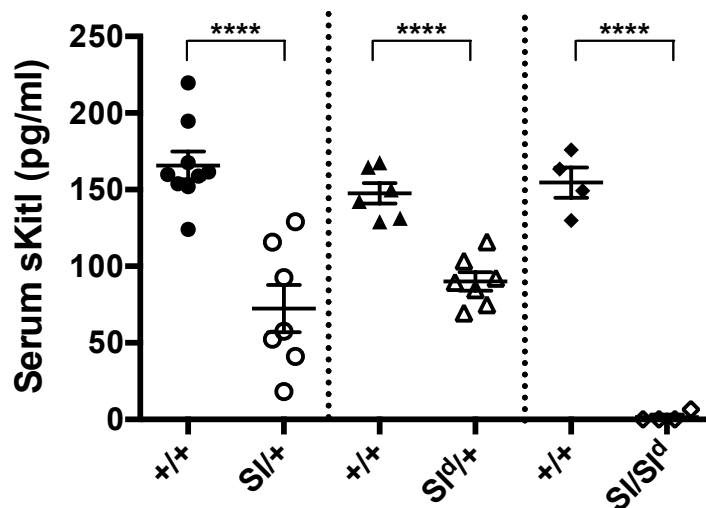


Figure 3.3 Compound heterozygote Sl/S^d mice lack systemic sKitl *in vivo*

Analysis of serum sKitl levels detected by ELISA from 3-week old $Sl/+$, $S^d/+$, Sl/S^d mice and WT (+/+) littermate controls. Bars show the mean values for each genotype (n=4-9 mice per genotype, mean $\pm$ SEM, $p < 0.0001^{****}$, with p values determined using the unpaired Student t-test).

For comparative purposes, it was important to compare and contrast the impact of altered systemic levels of sKitl, notably in *Sl/+* versus *Sl/Sl^d* mice with respect to haematopoiesis. Compared to WT littermate controls, *Sl/Sl^d* mice were confirmed to show significant decreases in their total BM MNC numbers (WT: $84.6 \pm 7.5 \times 10^6$ vs. $31.2 \pm 9.2 \times 10^6$ cells/ 2 femurs and tibiae, $p < 0.005^{**}$) and stem cells (LSK Flt3⁻ CD150⁺ HSCs: WT: $21.5 \pm 3.1 \times 10^3$ vs. $5.6 \pm 1.2 \times 10^3$ cells/ 2 femurs and tibiae, $p < 0.005^{**}$, **Figure 3.4**).

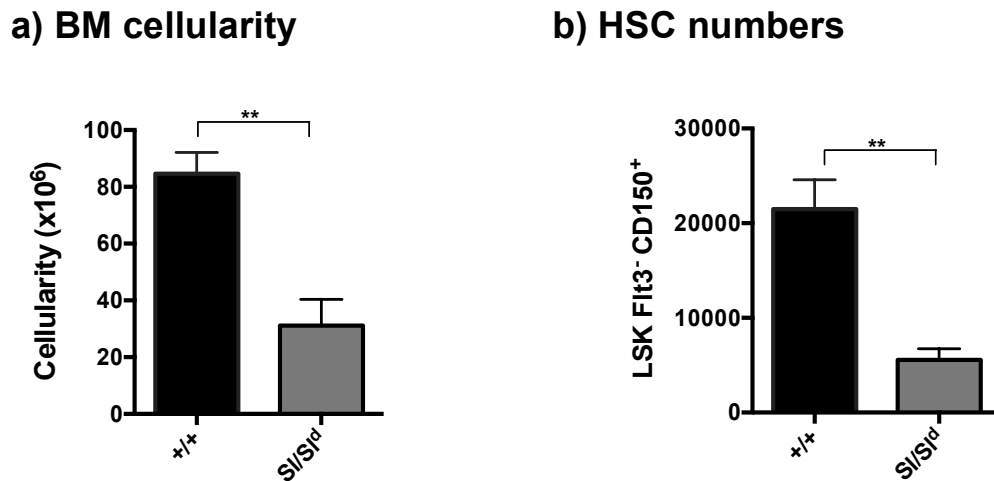


Figure 3.4 *Sl/Sl^d* mice display defective haematopoiesis

Analysis of 4-week old *Sl/Sl^d* and WT (+/+) littermate controls from 2 femurs and 2 tibiae (values shown are the mean $\pm$ SEM, with p values determined using the unpaired Student t-test):

- Total BM MNC numbers (n=6 mice per genotype, $p < 0.005^{**}$).
- Absolute number of phenotypic LSK Flt3⁻CD150⁺ HSCs (n=3 per genotype, $p < 0.005^{**}$).

However compared to WT littermate controls, *Sl/+* mice displayed normal BM cellularity ($70.0 \pm 5.8 \times 10^6$ vs. $60.8 \pm 7.0 \times 10^6$ cells/ 2 femurs and tibiae) and unaltered numbers of stem cells (LSK CD48⁻CD150⁺ HSCs: $10.4 \pm 0.6 \times 10^3$ vs. $10.5 \pm 0.9 \times 10^3$ HSCs/ 2 femurs and tibiae, **Figure 3.5**) as previously reported²⁴¹. This normal cellularity is likely due to the presence of one WT allele producing both mKitl

and sKitl. In the $Sl^d/+$ mouse, which also possesses one WT allele, normal BM cellularity was maintained (data not shown).

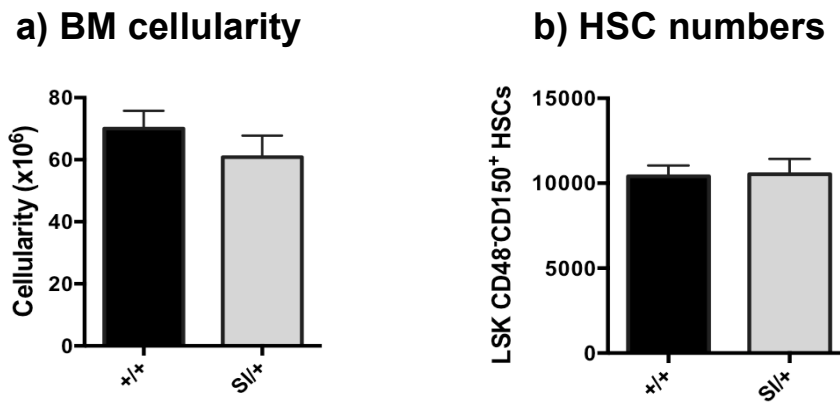


Figure 3.5 $Sl/+$ heterozygotes maintain normal haematopoiesis

Analysis of 4-week old $Sl/+$ and WT (+/+) littermate controls from 2 femurs and 2 tibiae (n=3 mice per genotype, values shown are the mean $\pm$ SEM, statistical analysis undertaken using the unpaired Student t-test):

- Total BM MNC numbers.
- Absolute number of phenotypic LSK CD48⁺CD150⁺ HSCs.

3.2.2 Sl^d *Kitl* expressed *in vitro* demonstrates biological activity

The lack of sKitl expression in the Sl/Sl^d mice may have been due to further mutations in the coding sequence. To establish if additional mutations had arisen, the coding sequence of *Kitl* in these mice was then determined. RNA was isolated, converted to cDNA and cloned as described in **Section 2.6.2**. Sequencing analysis confirmed that the Sl^d *Kitl* sequence from our Sl/Sl^d mice was identical (**Figure 3.6**) to the sequence previously published by Brannan *et al.*²⁵⁹.

Sequencing analysis confirmed that the Sl^d allele encodes 205 aa of the ExC domain that is identical to WT. In addition, there is 67 bp of novel sequence, encoding valine, serine, leucine and a stop codon, which terminates the open reading frame. This results in a loss of the sequence for the TM and IC domains. Overall there is a net

loss of 174 bp of cDNA and the resultant sequence would be predicted to produce a soluble form of *Kitl*. These results confirmed that soluble *Sl^d Kitl* is encoded in our *Sl/Sl^d* mice and no other mutations had arisen.

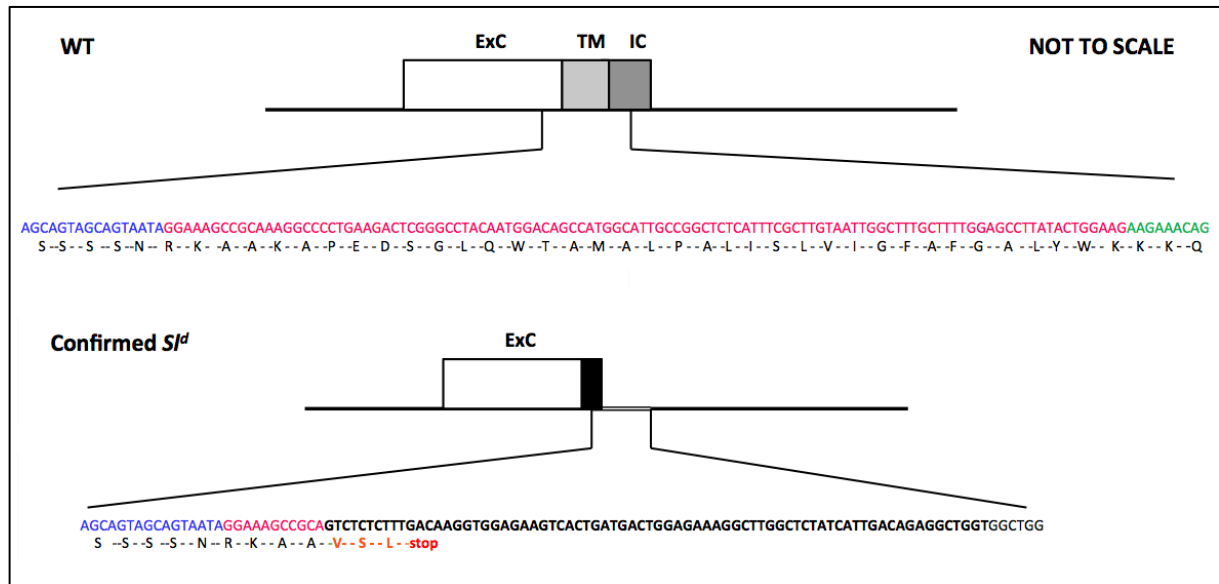


Figure 3.6 *Sl^d* mice retain the sequence for *Sl^d Kitl* when expressed *in vitro*

Schematic representation of part of WT sequence (upper panel) and *Kitl* coding sequence deletion in *Sl^d Kitl* (lower panel) confirmed by sequencing analysis. The *Sl^d* cDNA sequence was confirmed with the published sequence²⁵⁹. The 67 bp of unique *Sl^d* sequence, resulting in the termination of the open reading frame is shown by a black box and white line. Open reading frames are represented by boxes (extracellular domain (ExC, white), transmembrane domain (TM, light grey); intracellular domain (IC, dark grey). Sequencing of domains: EC (blue), TM (pink), IC (green) and altered sequence (orange).

Having confirmed the sequence of the *Sl^d Kitl* encoded in our *Sl/Sl^d* mice, it was then investigated whether sKitl protein could be expressed *in vitro*. The *Sl^d Kitl* sequence was sub-cloned into a pMSCV-iRES-GFP plasmid (to generate a *Sl^d Kitl*-pMSCV-iRES-GFP vector) and transfected into 293T cells. As a control, separate 293T cells were transfected with the control plasmid (pMSCV-iRES-GFP), containing no *Kitl* sequence. Successful transfection was confirmed by identifying green fluorescence protein positive (GFP⁺) 293T cells by microscopy (data not shown).

Supernatant was isolated from both sets of 293T cells and assayed using the sKitl-specific ELISA. Compared to the control plasmid which gave rise to no sKitl, transfection with *Sl^dKitl*-pMSCV-iRES-GFP vector revealed *Sl^dKitl* concentrations of 3.70 ± 0.20 ng/ml ($p < 0.005^{**}$, **Figure 3.7**). This again confirmed that the ELISA kit used could detect *Sl^dKitl* and quantify its levels.

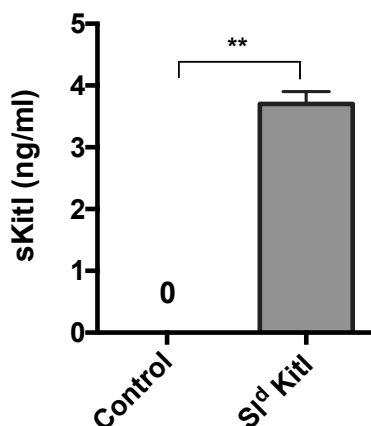


Figure 3.7 *Sl^dKitl* can be expressed *in vitro* and be detected by ELISA

Analysis of sKitl levels detected by ELISA from supernatant harvested from 293T cells transfected with *Sl^dKitl*-pMSCV-iRES-GFP and pMSCV-iRES-GFP control plasmid (values shown are the mean $\pm$ SEM, $p < 0.005^{**}$, with p values determined using the unpaired Student t-test)

These data confirmed that the cloned *Sl^dKitl* gene sequence could produce a sKitl protein *in vitro*. I then wanted to quantitatively demonstrate its biological activity. The *Sl^dKitl*-pMSCV-iRES-GFP vector was again used to transfect 293T cells and the supernatant was collected. An *in vitro* assay was set up whereby Kitl-dependent MC/9 cells were cultured in the supernatant. As controls, MC/9 cells were also grown in their optimal medium or in the presence of supernatants from control-vector (pMSCV-iRES-GFP) transfected- or untransfected-293T cells. After 48 hr incubation, MC/9 cell proliferation was determined using the colorimetric MTS assay (**Section 2.6.4.2**).

Assessment of MC/9 cell growth in the presence of the different supernatants was determined as a percentage, relative to the maximum proliferation obtained in optimal medium. In the presence of S^{fl} Kitl supernatant, MC/9 cells were capable of 57.3 % of the maximum proliferation determined in optimal growth medium, compared to only 7.3 % when these cells were grown in untransfected supernatant or 10.8 % in supernatants from cells transfected with control-vector (**Figure 3.8**). Compared to the control supernatants, these data reveal significant enhancement of the proliferation of MC/9 cells by the S^{fl} Kitl supernatant ($p < 0.0001^{****}$).

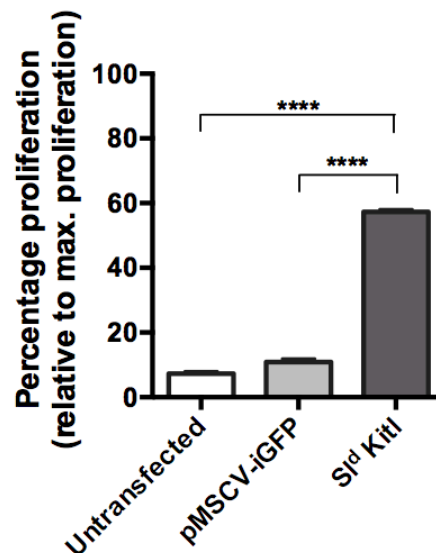


Figure 3.8 S^{fl} Kitl has biological activity *in vitro* which is sufficient to maintain a Kitl-dependent cell line (MC/9)

Proliferation of MC/9 cells after 48 hr when grown in the supernatant of 293T cells transfected with vectors containing S^{fl} Kitl, no Kitl or supernatant from untransfected cells (values shown are the mean $\pm$ SEM, $p < 0.0001^{****}$ with p values determined using the unpaired Student t-test)

These results indicate that the sKitl derived from Sl/Sl^{fl} mouse RNA and expressed *in vitro* is biologically active, as it can promote cell proliferation and maintain cell viability. These data are in agreement with those of Brannan *et al.* and Flanagan *et al.*^{217,259}. However, it must again be emphasised that in Sl/Sl^{fl} mice, the S^{fl} Kitl protein

is detected at essentially negligible concentrations *in vivo* (only 1 in 4 of animals revealed a quantifiable level, **Figure 3.3**).

3.2.3 Development of a KO model specific for *mKitl* which results in phenotypic *Sl* defects

Despite confirming the findings that *Sl^dKitl* can be expressed and is a functional protein *in vitro*, its negligible expression *in vivo* limited the use of the *Sl/Sl^d* mouse as a model to investigate specific loss of mKitl in order to be able to compare the role of mKitl and sKitl. Accordingly the development of novel models to specifically investigate *mKitl* deletion was undertaken.

A mouse with a targeted *Kitl* allele with *LoxP* sites flanking transmembrane exon 7 was generated in collaboration with the Nerlov Laboratory, Edinburgh (**Figure 3.9**). As depicted in **Figure 1.7**, exon 7 forms part of the TM domain. The targeted deletion of exon 7 in this model results in a truncated TM domain and loss of the cytoplasmic domain, resulting in the absence of *mKitl* expression.

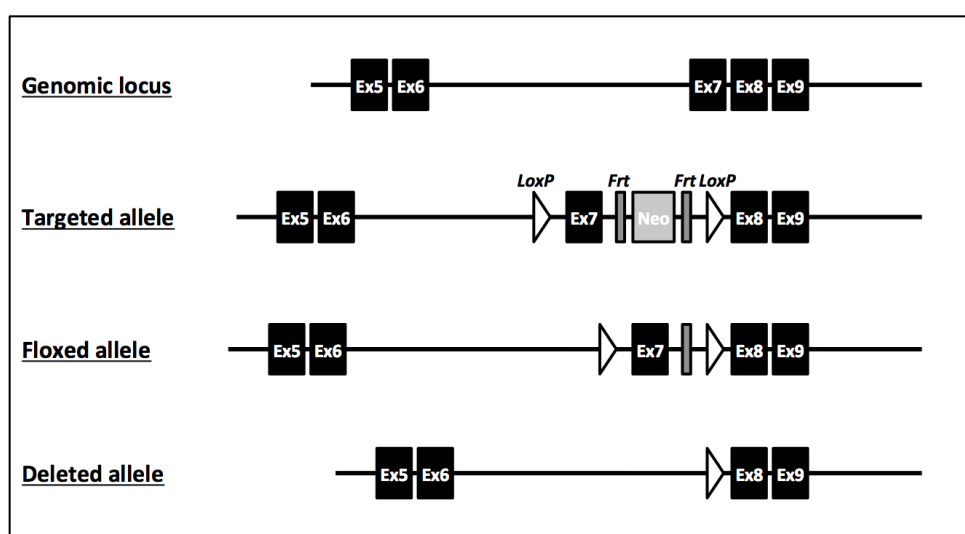


Figure 3.9 Targeting strategy of the *Kitl*^{Δex7} allele

Schematic presentation of exons 5-9 (black) of the *Kitl* genomic locus (top panel); the targeted *Kitl*^{Δex7} allele with *LoxP* sites (white triangles) flanking exon 7 and *Frt* sites (dark grey) flanking the Neo

cassette (light grey, upper middle panel); the floxed *Kitl*^{Δex7} allele with the Neo cassette removed and the *LoxP* sites flanking exon 7 (lower middle panel); the deleted *Kitl*^{Δex7} allele with removal of exon 7 (bottom panel).

These homozygous *Kitl*^{ex7fl/ex7fl} mice were validated by crossing with *CMV-Cre* mice. Though a series of crosses, the *Kitl*^{Δex7/Δex7} and *Kitl*^{Δex7/+} mice were generated. The mice lack *mKitl* as the Cre recombination causes the ubiquitous deletion of the loxP-flanked *mKitl* gene segment *in vivo*²⁷⁰. In the homozygotes (*Kitl*^{Δex7/Δex7}), this results in the germline deletion of *Kitl* exon 7. Confirmation that the *Kitl*^{Δex7/Δex7} mice lack *mKitl* and retain *sKitl* is outlined in **Section 3.2.4**.

Complete loss of *mKitl* led to mice being born at lower frequency than expected due to embryonic lethality around E15. Quantitative assessment of *in utero* mortality was not undertaken. Typically from 11 litters with an average litter size of 8 pups, only 2 *Kitl*^{Δex7/Δex7} mice were born (**Table 3.1**). Of the 90 mice born, Mendelian genetics would predict that 45 would be *Kitl*^{Δex7/+}, and 22.5 would be *Kitl*^{+/+} or *Kitl*^{Δex7/Δex7}. Unsurprisingly, Chi-squared test analysis revealed that the *Kitl*^{Δex7/Δex7} mice were born at significantly reduced levels (p<0.0001****).

Table 3.1 *Kitl*^{Δex7/Δex7} mice are born at a reduced Mendelian ratio

Mendelian ratio of offspring from inter-crosses of *Kitl*^{Δex7/+} mice from 11 litters of mice. Average litter size was 8 pups. Genotypes were determined at birth.

Genotype	Offspring
<i>Kitl</i> ^{+/+}	31
<i>Kitl</i> ^{Δex7/+}	57
<i>Kitl</i> ^{Δex7/Δex7}	2

Viable mice were found to be significantly smaller than littermates at 3 weeks (*S.M.*, **Figure 3.10a**). These germline deleted mice weighed on average 4.6 ± 0.3 g compared to their WT littermates who averaged 11.9 ± 1.4 g ($p < 0.001^{**}$). Despite their C57BL/6 background (which are black), *Kitl* ^{Δ ex7/ Δ ex7} mice had white coats (**Figure 3.10b**). This lack of pigmentation in the fur is a typical phenotype associated with *Sl* mutants²⁴⁸.



Figure 3.10 *Kitl* ^{Δ ex7/ Δ ex7} mice display characteristic *Sl* phenotypic defects

Analysis of 3-week old *Kitl* ^{Δ ex7/ Δ ex7} and WT (+/+) littermate controls (values shown are the mean $\pm$ SEM, with p values determined using the unpaired Student t-test):

- Total body weight (n=3 mice per genotype, $p < 0.001^{**}$).
- Representative appearance of mice demonstrating characteristic white coat colour.

These mice were then analysed at 3 weeks to investigate the specific impact of *mKitl* loss on haematopoiesis. Analysis of PB cell parameters using a Sysmex haemocytometer (**Section 2.1.5**) demonstrated that in the *Kitl* ^{Δ ex7/ Δ ex7} mice, notably WBC and to a lesser degree platelet numbers were raised, but not significantly so, given the high inter-animal variability. In addition, they were anaemic as Hgb levels showed a significant ~2-fold reduction, RBC numbers and the HCT were significantly reduced and MCV and MCH significantly increased compared to WT littermate

controls (**Table 3.2**). In summary, the *Kitl* ^{Δ ex7/ Δ ex7} mice were anaemic with macrocytic RBC indices with no significant changes in WBCs or platelets.

Table 3.2 *Kitl* ^{Δ ex7/ Δ ex7} mice display abnormalities in their peripheral blood counts

Analysis of peripheral blood counts from 3-week old *Kitl* ^{Δ ex7/ Δ ex7} and WT (+/+) littermate controls. (n=4-6 mice per genotype, values shown are the mean $\pm$ SEM, p<0.001**, <0.0005***, p<0.0001**** with p values determined using the unpaired Student t-test). Key: White blood cell (WBC); red blood cell (RBC); haemoglobin (Hgb); haematocrit (HCT); mean cell volume (MCV); mean cell haemoglobin (MCH); platelet (Plt).

	<i>Kitl</i> ^{+/+}	<i>Kitl</i> ^{Δex7/Δex7}
WBC (/ μ l)	4983 $\pm$ 1147	5325 $\pm$ 914
RBC ($\times 10^4$ / μ l)	793 $\pm$ 28	286 $\pm$ 61****
Hgb (g/dl)	12.3 $\pm$ 0.4	6.7 $\pm$ 1.1***
HCT (%)	44.5 $\pm$ 1.7	22.3 $\pm$ 4.6**
MCV (fl)	56.1 $\pm$ 1.3	78.4 $\pm$ 1.8****
MCH (pg)	15.5 $\pm$ 0.4	24.6 $\pm$ 1.8***
Plt ($\times 10^4$ / μ l)	895 $\pm$ 46	993 $\pm$ 143

Overall compared to their WT littermates, the *Kitl* ^{Δ ex7/ Δ ex7} mice had significantly reduced BM cellularity (WT: 86.2 $\pm$ 4.7 $\times 10^6$ vs. 37.9 $\pm$ 2.7 $\times 10^6$ cells / 2 femurs and tibiae, p<0.0001****, **Figure 3.11a**). Phenotypic HSCs were investigated by flow cytometric analysis according to the gating strategy in **Figure 3.11b** and were found to be significantly depleted in *Kitl* ^{Δ ex7/ Δ ex7} mice with a 65 % reduction in their absolute number compared to WT littermates. HSC numbers declined from 32.5 $\pm$ 10.6 $\times 10^3$ in WT to 11.2 $\pm$ 2.4 $\times 10^3$ (p<0.05*, **Figure 3.11c**, experiment conducted by S.M., data analysis by R.F.).

As HSCs sit at the top of the cell hierarchy (**Figures 1.2 & 1.3**), it was necessary to confirm if losing *mKitl* expression impacted upon subsets of myeloid progenitors downstream, by staining cells with fluorochromes for markers characterised by Pronk *et al.*²⁷¹. Investigation of these progenitors using the gating profiles indicated in **Figure 3.11d**, revealed that these mice exhibited significantly decreased numbers of erythroid progenitors (Pre CFU-E and Pro Ery cells; **Figure 3.11e** experiment conducted by S.M., data analysis by R.F). The impact on the myeloid progenitors was however restricted to the erythroid lineage, as granulocyte-macrophage and megakaryocytic cells were unaffected.

These combined data show that the *Kitl* ^{$\Delta_{ex7}/\Delta_{ex7}$} mouse has a similar phenotype to other mouse models with defective Kitl production, including loss of coat colour, anaemia and affected haematopoiesis^{241,248}.

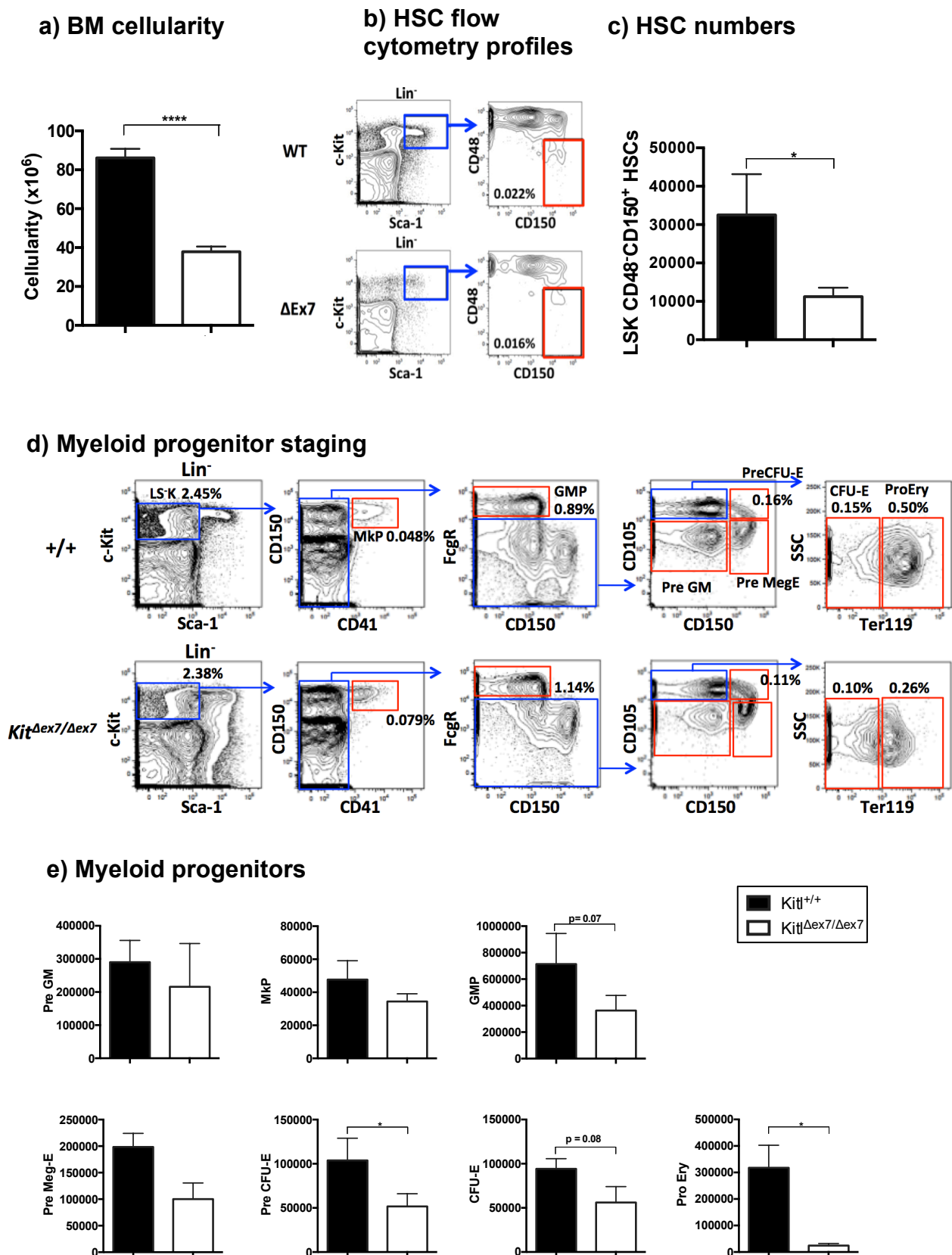


Figure 3.11 Haematopoietic stem/progenitor phenotype of *Kitl*^{ΔEx7/ΔEx7} mice

Analysis of 3-week old *Kitl*^{ΔEx7/ΔEx7} and WT (+/+) littermate control BM (from 2 femurs and 2 tibiae per mouse, n=4-5 mice per genotype, values shown are the mean±SEM, with p values determined using the unpaired Student t-test):

- a. Total BM MNC numbers ($p < 0.0001^{****}$).
- b. Representative FACS profiles of phenotypic LSK CD48⁻CD150⁺ HSCs (red gates). Percentages indicate the mean frequencies of LSK CD48⁻CD150⁺ HSCs among total BM cells. Blue gates indicate gating strategies.
- c. Total LSK CD48⁻CD150⁺ HSC numbers ($p < 0.05^*$).
- d. Representative myeloid progenitor staging. Mean frequencies among total BM cells. Blue gates indicate gating strategies and red gates indicate populations of interest. Key: MkP (Lin⁻Sca1⁻c-Kit⁺CD150⁺CD41⁺), GMP (Lin⁻Sca1⁻c-Kit⁺CD41⁻FcyR⁺), Pre GM (Lin⁻Sca1⁻c-Kit⁺CD41⁻FcyR⁻CD150⁻CD105⁻), Pre MegE (Lin⁻Sca1⁻c-Kit⁺CD41⁻FcyR⁻CD150⁺CD105⁻), Pre CFU-E (Lin⁻Sca1⁻c-Kit⁺CD41⁻FcyR⁻CD150⁺CD105⁺), CFU-E (Lin⁻Sca1⁻c-Kit⁺CD41⁻FcyR⁻CD150⁻CD105⁺Ter119⁻), Pro Ery (Lin⁻Sca1⁻c-Kit⁺CD41⁻FcyR⁻CD150⁻CD105⁺Ter119⁺).
- e. Absolute numbers of myeloid progenitors ($p < 0.05^*$).

3.2.4 sKitl from *Kitl* ^{Δ ex7/ Δ ex7} mice expressed *in vitro* demonstrates

biological activity

In order to demonstrate that the phenotype seen in *Kitl* ^{Δ ex7/ Δ ex7} mice was due to the specific loss of mKitl, I wanted to confirm that first, the sequence of the *Kitl* in the model encoded *sKitl* and second, the soluble form of the protein was expressed in the mice. It was also imperative to confirm that the *sKitl* expressed in these mice gave rise to a biologically active protein.

Using the methods described in Section 2.6.2, by isolating RNA from a *Kitl* ^{Δ ex7/+} mouse, *Kitl* was cloned and sequencing of cDNA confirmed that *Kitl* ^{Δ ex7} mice encode *sKitl*, represented by the schematic in **Figure 3.12**. Sequence analysis revealed that the deletion of exon 7 altered the nucleotide sequence, resulting in the retention of the full-length ExC domain whilst causing a truncated TM domain and the loss of the cytoplasmic domain. This sequence therefore encodes only a soluble protein, confirming that this model retains the ability to encode *sKitl* but no longer encodes *mKitl*, making it a membrane-specific KO of *Kitl*.

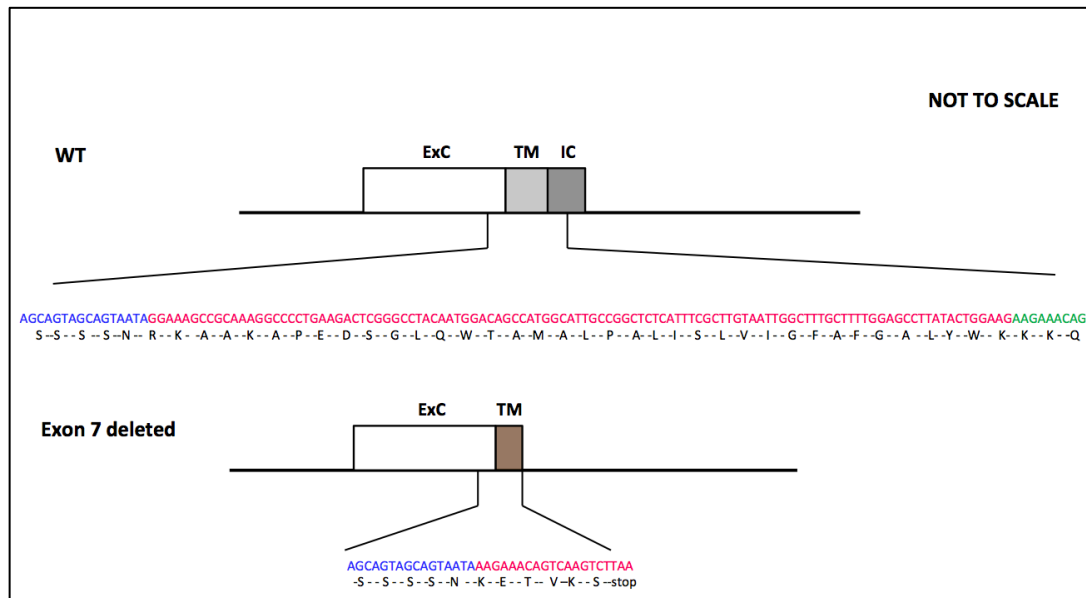


Figure 3.12 *Kitl*^{Δex7} mice retain the sequence for *sKitl* when expressed *in vitro*

Schematic representation of part of WT sequence (upper panel) and the *Kitl* coding sequence deletion in *Kitl*^{Δex7} mice (lower panel) confirmed by sequencing analysis. Open reading frames are represented by boxes ExC domain (white), TM domain (light grey), IC domain (dark grey), truncated TM domain, (brown). Sequencing of domains: EC (blue), TM (pink), IC (green).

The *Kitl*^{Δex7} sequence was sub-cloned into the pMSCV-iRES-GFP vector to produce *sKitl*-pMSCV-iRES-GFP. This plasmid was then used to transfect 293T cells and the supernatant was harvested after 48 hr. This was then assayed using the ELISA kit, which confirmed that the soluble form of the protein could be produced and then detected *in vitro* (1.30 ± 0.08 ng/ml, **Figure 3.13**, p<0.005**).

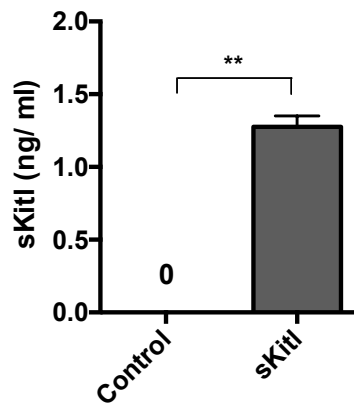


Figure 3.13 *Kitl*^{Δex7} sKitl can be expressed *in vitro* and detected by ELISA

Analysis of sKitl levels detected by ELISA from supernatant harvested from 293T cells transfected with *sKitl*-pMSCV-iRES-GFP or pMSCV-iRES-GFP control plasmid (values shown are the mean±SEM, $p < 0.005^{**}$ with p values determined using the unpaired Student t-test).

The supernatant was also used to culture MC/9 cells as described previously. The encoded protein was able to maintain the cell line, indicating that it was functional and biologically active (**Figure 3.14**). In the presence of the sKitl supernatant, MC/9 cells were capable of 45.4 % of the maximum proliferation determined in optimal growth medium, while values were appreciably lower when cultured in untransfected or control-vector transfected 293T cell supernatant.

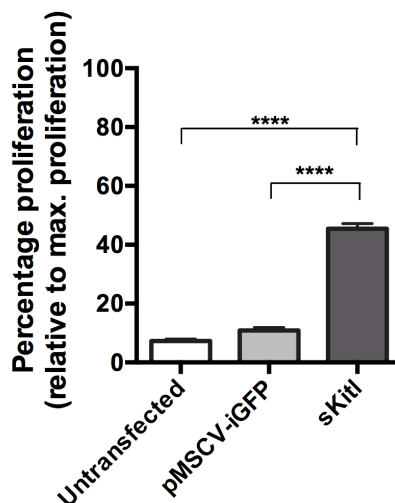


Figure 3.14 *Kitl*^{Δex7} sKitl has biological activity *in vitro* which is sufficient to maintain a Kitl-dependent cell line (MC/9)

Proliferation of MC/9 cells after 48 hr when grown in the supernatant of 293T cells transfected with vectors containing sKitl, no Kitl or supernatant from untransfected 293T cells (values shown are the mean±SEM, $p < 0.0001^{****}$ with p values determined using the unpaired Student t-test).

To verify that the encoded protein was indeed expressed *in vivo*, IHC was performed on *Kitl*^{Δex7/Δex7} femur sections from newborn mice. By staining with Kitl antibodies recognising both IC and ExC portions of the protein, it was demonstrated that *Kitl*^{Δex7/Δex7} BM stained for the soluble protein but lacked staining for the membrane-bound form (**Figure 3.15, S.M**).

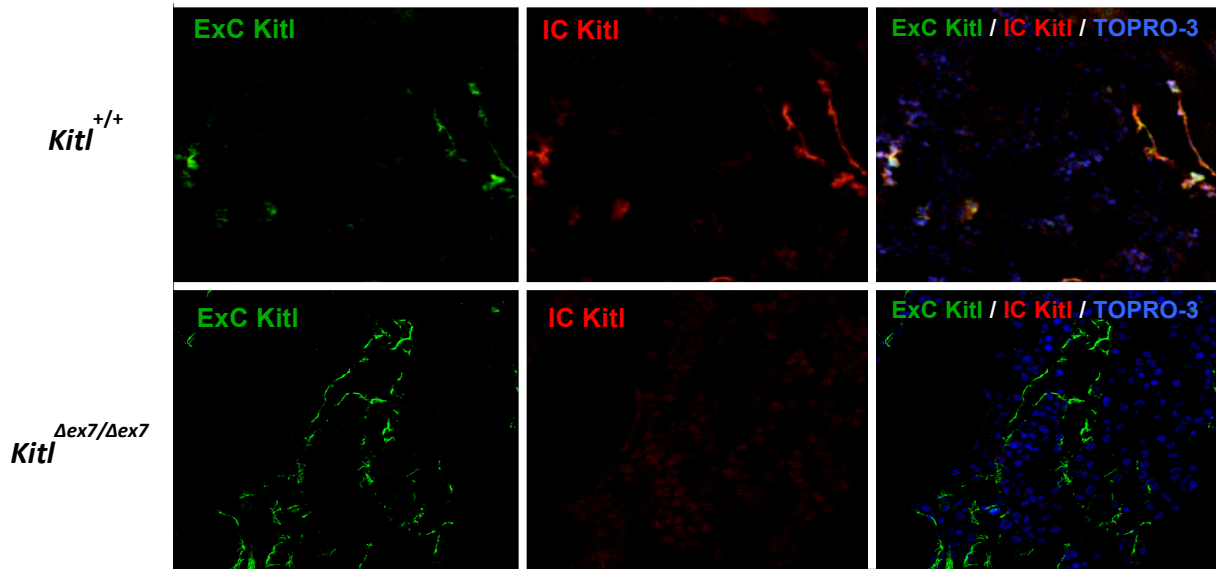


Figure 3.15 *Kitl*^{Δex7/Δex7} mice retain sKitl expression *in vivo*

Immunostaining for extracellular Kitl (ExC Kitl, green: primary + donkey anti-goat IgG 488) and intracellular Kitl (IC Kitl, red: primary + donkey anti-rabbit IgG Cy3) in newborn *Kitl*^{Δex7/Δex7} and WT (+/+) littermate control BM sections. Representative results of 10 sections from 4 mice of each genotype.

Additionally, to further confirm lack of mKitl cell surface expression in our mice, the *sKitl*-pMSCV-iRES-GFP vector was again transfected into 293T cells and the expression of Kitl on their cell surface was compared to cells transfected with full length WT Kitl (*Kitl*-pMSCV-iRES-GFP) or pMSCV-iRES-GFP and also untransfected cells. Surface expression of Kitl was determined by flow cytometry after Ab staining for Kitl.

The data shown in **Figure 3.16** reveal that there is no cell surface expression of Kitl from 293T cells transfected with the *sKitl*-pMSCV-iRES-GFP vector derived from the *Kitl*^{Δex7} mice sequence. Given that only cell-surface expression is detected using this method, this is further evidence that the Kitl sequence in *Kitl*^{Δex7/Δex7} mice does not give rise to mKitl.

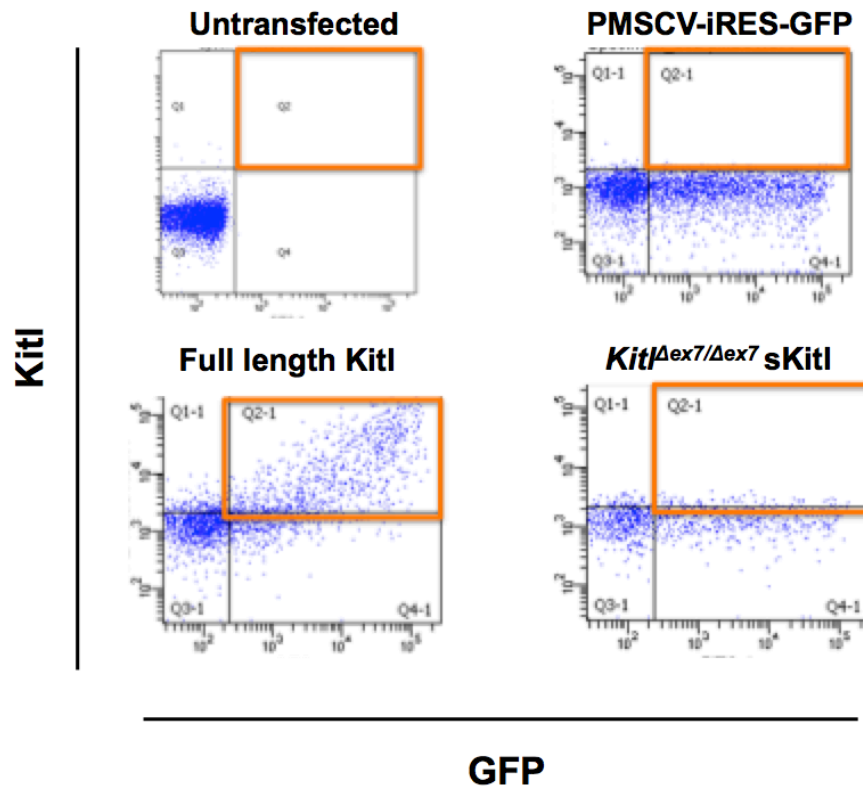


Figure 3.16 *Kitl*^{Δex7} Kitl is not expressed on the surface of 293T cells transfected with *sKitl*-pMSCV-iRES-GFP *in vitro*

Flow cytometry profiles of viable (DAPI⁻) untransfected 293T cells, 293T cells transfected with pMSCV-iRES-GFP, WT *Kitl*-pMSCV-iRES-GFP or *sKitl*-pMSCV-iRES-GFP, stained for Kitl. Orange boxes indicate Kitl⁺ cells.

Having established the presence of *sKitl* and the absence of mKitl, I next quantitatively determined systemic levels of *sKitl* *in vivo*. Serum from 3-week old *Kitl*^{Δex7/Δex7} and WT mice was determined for *sKitl* levels by ELISA. *Kitl*^{Δex7/Δex7} mice had a significant 61.4 % reduction in the level of the circulating ligand, which fell from 160.8 ± 4.6 pg/ml in WT littermates to 62.1 ± 8.7 pg/ml (p<0.0001****, **Figure 3.17**).

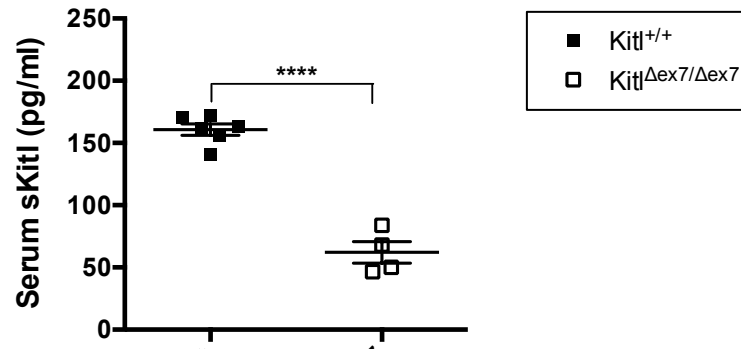


Figure 3.17 *Kitl*^{Δex7/Δex7} mice display reduced levels of circulating sKitl

Analysis of serum sKitl levels determined by ELISA in 3-week old *Kitl*^{Δex7/Δex7} and WT (+/+) littermate controls (n=4-6 mice per genotype, values shown are the mean±SEM, p<0.0001**** with p values determined using the unpaired Student t-test).

3.2.5 Induction of *mKitl*-loss in adult *RosaCre*^{ERT2+};*Kitl*^{Δex7/Δex7} mice leads to characteristic *SI* defects

Having demonstrated the specificity of the *Kitl*^{Δex7} targeting strategy and the phenotype associated with the germline deletion of *Kitl* exon 7, it was necessary to investigate the impact of *mKitl*-loss on adult steady-state haematopoiesis

Given the potential impact of the absence of *mKitl* during development, an inducible model was crossed with our *Kitl*^{Δex7} line to facilitate the study of *mKitl*-deficiency on steady-state adult haematopoiesis. By crossing homozygous *Kitl*^{Δex7} mice, which have *loxP* sites flanking exon 7, with *RosaCre*^{ERT2} mice (which also contain a LoxP-3xStop-LoxP-EYFPm sequence inserted into the *Rosa* locus),

RosaCre^{ERT2};*R26*^{YFP};*Kitl*^{Δex7/Δex7} (*RosaCre*^{ERT2};*Kitl*^{Δex7/Δex7}) mice were generated, which on injection of tamoxifen induces *Cre*-mediated recombination and should result in the deletion of the flanked sequence in all tissues²⁶¹. In addition, the floxed *stop* codon is deleted which leads to the expression of yellow fluorescent protein (YFP), driven by the endogenous mouse *Gt(ROSA)26Sor* promoter. The YFP can be used

for fate mapping and recombination reporter studies. Adult mice (6-weeks old) received 3 mg/day subcutaneous doses of tamoxifen (two doses in total over three days) to induce *Cre* recombination (**Section 2.4.2**). Animals were then analysed 4 days after the final dose. Initial investigation of PB revealed significant reductions to the majority of the analysed blood parameters, including the Hgb level and RBC counts, although platelets were significantly elevated (**Table 3.3**).

Table 3.3 *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice display abnormalities in their peripheral blood counts

Analysis of peripheral blood counts of 6-week old *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* and WT littermate controls (*RosaCre^{ERT2-};Kitl^{Δex7/Δex7}*). Key as for **Table 3.2**. (n=3 mice per genotype, values shown are the mean±SEM p<0.05*, <0.01** with p values determined using the unpaired Student t-test).

	<i>RosaCre^{ERT2-};Kitl^{Δex7/Δex7}</i>	<i>RosaCre^{ERT2+};Kitl^{Δex7/Δex7}</i>
WBC (μl)	10267±147	3200±10**
RBC (x10 ⁴ /μl)	739±41	613±12*
Hgb (g/dl)	12.8 ± 0.6	10.2±0.4*
Hct (%)	40.70±0.02	30.90±0.02**
MCV (fl)	55.1±1.7	50.4±0.1*
MCH (pg)	17.3±0.2	16.6±0.2*
Plt (x10 ⁴ / μl)	1136±91	1888±26**

The BM was analysed to investigate haematopoiesis in greater detail. The *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice displayed a significant reduction in total BM cellularity (75 % less than WT littermate controls, *RosaCre^{ERT2-};Kitl^{Δex7/Δex7}* ; **Figure 3.18a**). MNCs fell from 91.0 ± 4.8 x 10⁶ in WT to 23.0 ± 3.3 x 10⁶ in *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* (p<0.0005***). Flow cytometric analysis revealed that these mice also had very few cells to analyse, almost entirely lacking c-Kit⁺ cells, which included a significant reduction in both LSK cells (16.9 ± 16.5 x 10³ vs. 83.3 ± 5.8 x 10³ in WT, p<0.05*)

and SLAM-defined HSCs (LSK CD48⁻CD150⁺, 273 ± 243 vs. 7616 ± 471 in WT, $p < 0.001^{***}$, Figures 3.18b-d).

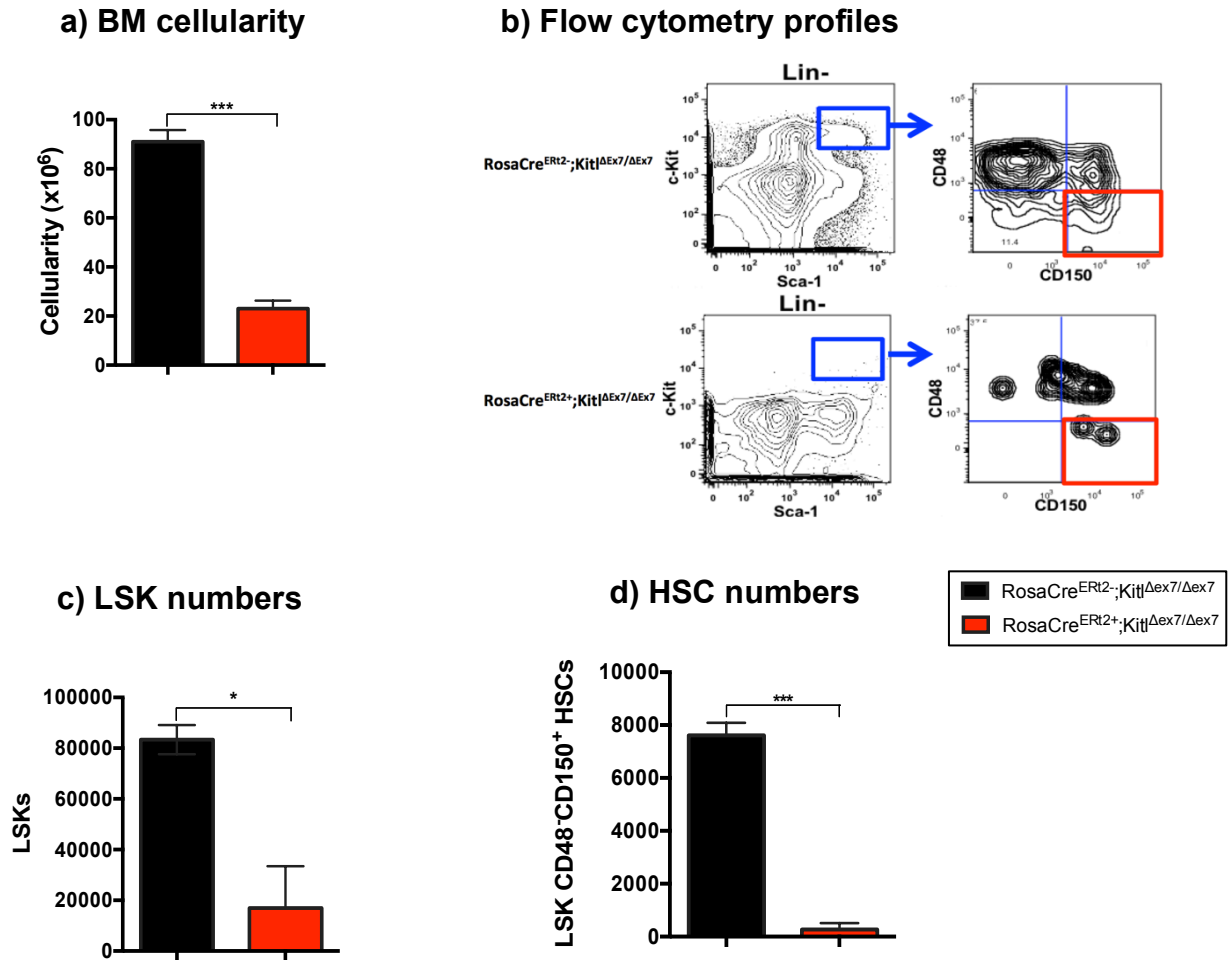


Figure 3.18 *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice exhibit defective haematopoiesis (I)

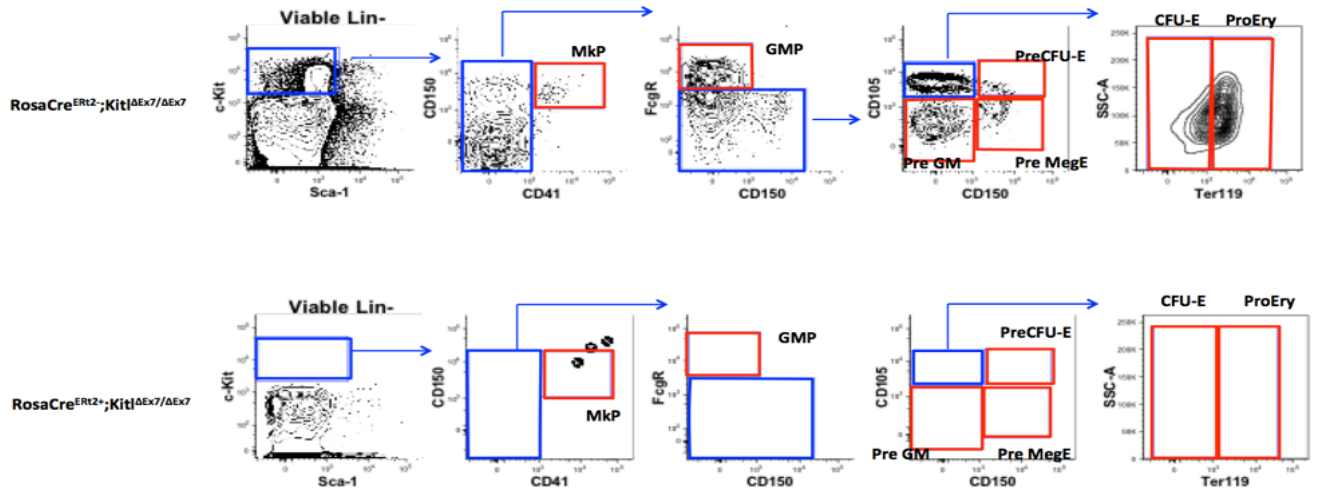
Analysis of 6-week old *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* and WT *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* littermate control BM (from 2 femurs and 2 tibiae per mouse, n=3 mice per genotype, values quoted are the mean±SEM, with p values determined using the unpaired Student t-test):

- Total BM MNC numbers ($p < 0.0005^{***}$).
- Representative flow cytometry profiles for LSKs (blue gates) and LSK CD48⁻CD150⁺ HSCs from the BM (red gates).
- Absolute number of LSKs ($p < 0.05^*$).
- Absolute number of phenotypic LSK CD48⁻CD150⁺ HSCs ($p < 0.001^{***}$).

A significant loss across all MPC populations was seen in the

RosaCre^{ERT2+};Kitl^{Δex7/Δex7} mice, with the erythroid lineage cells (Pre CFU-Es, CFU-Es and Pro Ery) most affected and were almost completely absent (**Figure 3.19**).

a) Myeloid progenitor staging



b) Myeloid progenitor numbers

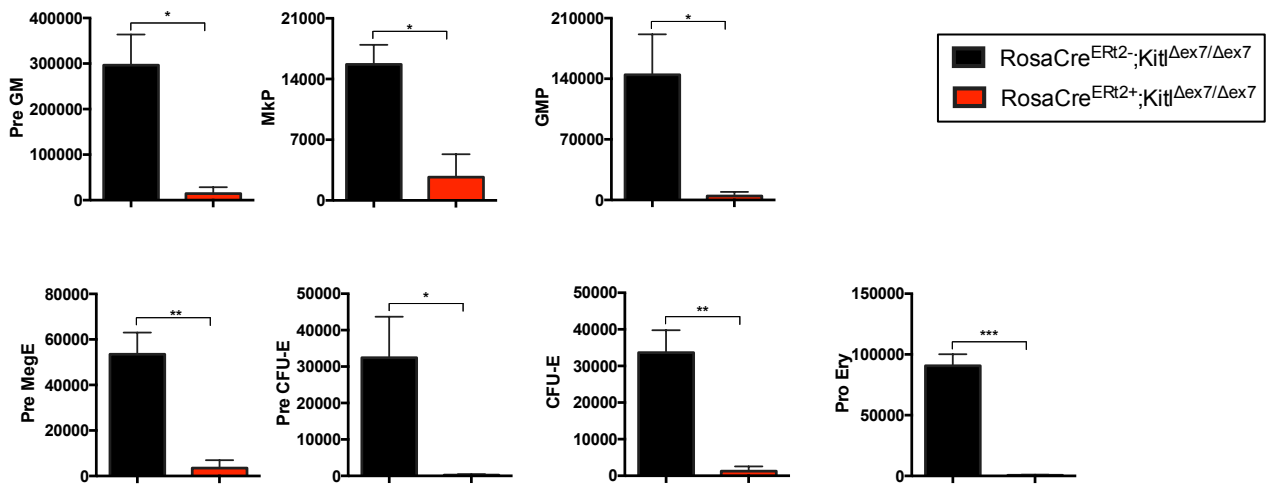


Figure 3.19 *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice exhibit defective haematopoiesis (II)

Analysis of 6-week old *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* and WT *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* littermate control BM (from 2 femurs and 2 tibiae per mouse, n=3 mice per genotype, values shown are the mean±SEM, with p values determined using the unpaired Student t-test):

- Representative myeloid progenitor staging from the BM.
- Absolute numbers of myeloid progenitors. Cell populations are as defined in **Figure 3.11** (mean±SEM, p<0.05*, p<0.005**, p<0.0005***).

Circulating sKitl levels were again determined using the ELISA kit.

RosaCre^{ERT2+};Kitl^{Δex7/Δex7} mice demonstrated a significant reduction in the level of serum sKitl compared to WT controls (*RosaCre^{ERT2+};Kitl^{Δex7/Δex7}*; **Figure 3.20**). The average concentration of systemic sKitl in *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice was 55.7 ± 13.3 pg/ml compared to their WT littermates with 145.7 ± 15.0 pg/ml (62.0 % reduction, $p < 0.001^{**}$).

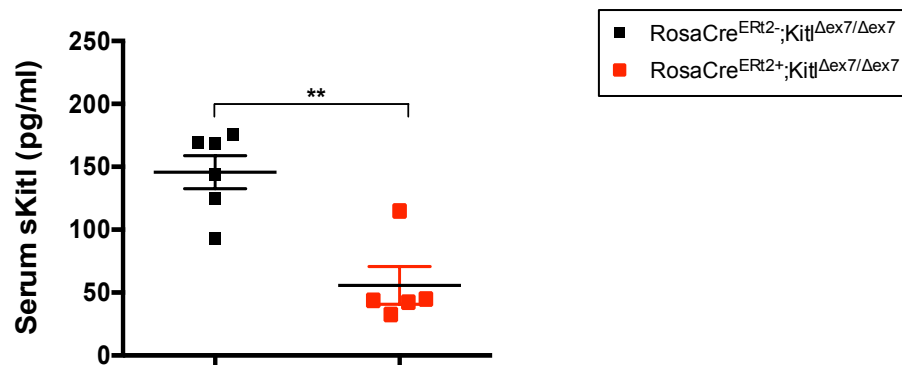


Figure 3.20 *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice display reduced levels of circulating sKitl

Analysis of serum sKitl levels determined by ELISA from 6-week old *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice and WT *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* littermate controls (n=5-6 mice per genotype, values shown are the mean $\pm$ SEM, $p < 0.001^{**}$ with p values determined using the unpaired Student t-test).

The use of the *RosaCre^{ERT2}* tamoxifen inducible model can be associated with potential haematopoietic toxicity²⁷², which could in part account for the more severe phenotype seen in this model. Accordingly, an appropriate control model (*RosaCre^{ERT2+};Kitl^{+/+}*) was developed and revealed gene-related toxicity (reduced growth, thymus size and testicular aberrations) following tamoxifen administration (Dr. Mario Buono, personal communication). Thus, the likely effects seen in *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice may be in part intensified by tamoxifen-mediated *Cre* toxicity, which makes interpretation of the above data difficult.

3.3 Discussion

Cytokines have been demonstrated to be important within haematopoiesis for HSC maintenance, proliferation and differentiation^{26,80,273}. It is also known that several of these ligands are expressed as membrane-bound and soluble proteins, both of which possess biological activity¹⁶⁰. However, it has yet to be fully elucidated if and how these isoforms of the same cytokine can differentially influence HSCs within the haematopoietic niche.

In the studies described here, the specific role of the membrane bound form of Kitl was initially explored using the naturally occurring compound heterozygote *Sl/Sl^d* mouse model. The studies were then expanded, using a targeting construct to generate new mouse models to investigate the role of mKitl more specifically.

Sl/Sl^d mice have previously been shown to lose expression of *mKitl* due to an intragenic deletion but were believed to retain the potential to encode a truncated sKitl^{217,259}. *Sl/Sl^d* mice were confirmed to display defective haematopoiesis, namely significantly reduced BM MNCs and HSCs. The current studies have also confirmed that *Sl/Sl^d* mice possess the same *Kitl* sequence as previously published²⁵⁹, which when cloned was shown to be expressible *in vitro* in 293T cells, with the truncated protein found to be active in a MC/9 cell proliferation assay. These results are comparable to those obtained by Brannan *et al.* and Flanagan *et al.*^{217,259}.

However, *in vivo*, this truncated sKitl was only detected at negligible levels in *Sl/Sl^d* mice, despite the use of 2 sets of Abs and an ELISA method (systemic levels were close to zero, with mean levels of 1.6 pg/ml compared to WT animals, which had a mean level of 154.6 pg/ml), shown to recognise an antigenic portion of this protein.

Furthermore, by staining the same BM sections with VE-Cadherin, a marker known to be expressed on ECs (a cell population which also expresses Kitl⁸⁹), it was confirmed that the lack of staining was not due to a lack of Kitl⁺ cells but a lack of Kitl itself. Given the confirmed adequate detection of the S^dKitl protein by IHC and notably by ELISA, the studies reported here have for the first time demonstrated that S/S^d mice do not in fact reveal sKitl protein *in vivo* in anything other than negligible or non-detectable levels.

It can be postulated that the lack of sKitl protein in S/S^d mice may arise as a result of the deletion in the S^d allele preventing RNA translation, alternatively there may be only low level production of the S^dKitl protein and if produced, is not secreted or may be unstable and is rapidly degraded, and as a result fails to be detected. Therefore these mice were considered unsuitable for further use in the investigation of the specific role of mKitl.

Accordingly, a specific *mKitl* KO mouse (*Kitl*^{ex7fl/ex7fl}) was used to develop two models, *Kitl*^{Δex7/Δex7} (germline deleted) and *RosaCre*^{ERT2+};*Kitl*^{Δex7/Δex7} (inducible, conditional KO). The *RosaCre*^{ERT2+};*Kitl*^{Δex7/Δex7} model was investigated in addition to the *Kitl*^{Δex7/Δex7} model to overcome the issue of the embryonic lethality and reduced birth rates of *Kitl*^{Δex7/Δex7} mice. Furthermore, as Kitl/c-Kit signalling is known to be important during embryogenesis^{231,232,274}, *Kitl*^{Δex7/Δex7} mice may undergo defective development, which contributes to the phenotypic defects revealed by the analysis of 3-week old mice. By allowing *RosaCre*^{ERT2+};*Kitl*^{Δex7/Δex7} to develop with functional mKitl to 6 weeks of age before inducing its deletion, it was postulated that this would permit investigation of the specific loss of *mKitl* on adult steady-state haematopoiesis.

These two models were confirmed to lack mKitl by combinations of sequencing, flow cytometry, genotyping and IHC. Sequence analysis of *Kitl* in *Kitl*^{Δex7} mice revealed an altered nucleotide sequence with a full-length ExC domain, a truncated TM domain and no cytoplasmic domain. An Ab specific for the IC domain of mKitl readily detected the protein in the BM of WT mice using IHC, but not unexpectedly given the lack of a predicted cytoplasmic domain, was unable to detect mKitl in the BM of *Kitl*^{Δex7/Δex7} mice.

A second experiment failed to detect the cell-surface expression of Kitl on 293T cells transfected with a vector containing the *Kitl* sequence encoded in *Kitl*^{Δex7} mice, however cells transfected with a WT *Kitl* sequence encoding the full-length protein expressed Kitl on their cell surface. If the *Kitl* sequence encoded in *Kitl*^{Δex7} mice had led to the expression of mKitl on these 293T cells, given its gene sequence contains an ExC domain, cell-surface expression of Kitl would have been detected by flow cytometry following staining with an Ab which recognises an ExC portion of the protein. Similarly, genotyping of *RosaCre*^{ERT2+};*Kitl*^{Δex7/Δex7} mice, which have the same *Kitl* sequence as *Kitl*^{Δex7/Δex7} mice, showed that they lacked the sequence encoding exon 7. In contrast, gene sequencing confirmed that *sKitl* was encoded and ELISA data, both *in vitro* and *in vivo*, indicated the presence of sKitl protein. Therefore, unlike the *Sl/Sl*^d mouse, both the *Kitl*^{Δex7/Δex7} and *RosaCre*^{ERT2+};*Kitl*^{Δex7/Δex7} models express a biologically active, sKitl *in vivo*, albeit at significantly reduced levels systemically (>60 %) compared to WT.

Germline deletion of *Kitl* exon 7 in *Kitl*^{Δex7/Δex7} mice *in vivo* leads to a multi-faceted phenotype, which includes reduced viability, altered fur colour and impaired blood development. These animals, born at reduced Mendelian ratios, exhibited

significantly reduced BM cellularity and significantly decreased numbers of HSCs and erythroid progenitors (CFU-E and Pro Ery) at 3 weeks of age. Overall the *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice showed more pronounced haematopoietic effects. However, given the gene toxicity following tamoxifen administration to the control *RosaCre^{ERT2+};Kitl^{+/+}*, other than confirming through genotyping the loss of exon 7 of *Kitl*, the data from *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice cannot be utilised to evaluate the roles of mKitl and sKitl.

If systemic levels of sKitl are a measure of its overall expression, then a consequence of deleting *mKitl* would appear to be a marked reduction in the level of sKitl expression. These data, in particular from the germline *Kitl^{Δex7/Δex7}* mice demonstrate that deletion of *mKitl* produces marked deficiencies in haematopoiesis, which may result in part from the absence of the membrane-bound protein itself, but also from a reduction in the level of the circulating soluble ligand. Thus the phenotypes seen (**Section 3.2.2 – 4**) cannot be attributed solely to loss of the membrane-bound form, given the impact on the circulating sKitl levels. Soluble Kitl is predominantly thought to be produced by splicing to give rise to a TM precursor (KL1) which is then proteolytically cleaved. However, it can also arise by proteolytic cleavage of mKitl (KL2)^{172,275}. As the latter isoform of the protein with the capacity to give rise to sKitl via proteolytic cleavage cannot be produced following exon 7 deletion, it is perhaps unsurprising that the level of circulating sKitl is reduced in these mouse models.

In conclusion, deletion of *mKitl* from all tissues results in a marked impact upon haematopoiesis and is associated with significant reductions in the level of sKitl *in vivo*. Having established a requirement for mKitl in haematopoiesis, the next step

was to determine if expression of *mKitl* by a specific cell type(s) is essential for HSC maintenance *in vivo* (**Chapter 4**).

4 Investigating the Impact of Cell-specific Loss of *Kitl* on Haematopoiesis

4.1 Introduction

As indicated previously, the HSC niche is comprised of a variety of cell types, which are known to express and secrete different cytokines important for HSC maintenance³. Preliminary assessments for the involvement of cytokines in the regulation of HSCs were undertaken *in vitro* and confirmed through the analysis of genetically modified animal models exhibiting selective deletion of individual cytokines^{4,41,276,277}.

Furthermore, by deleting cytokines from specific cell populations, groups have investigated if cell-specific expression is important for HSC regulation. As indicated in the **Introduction**, two groups independently demonstrated that deletion of CXCL12 from diverse cell types had different impacts on haematopoiesis. Deletion of CXCL12 from osterix⁺ cells led to a reduction in lymphoid progenitors but did not affect the HSCs, whereas EC or PVC-specific loss of CXCL12 caused impaired HSC maintenance^{99,100}. These data indicated that HSCs are supported by CXCL12-expressing vascular and perivascular cells, whereas B lymphoid progenitors are maintained by CXCL12-expressing osterix⁺ cells⁹⁹. These findings appeared to indicate that there are functional niches for different stem and progenitor populations and that the same cytokine can influence different haematopoietic cell types, depending on the stromal niche cell type it is expressed on or secreted from. Although, it should be remembered that certain cytokines implicated in haematopoiesis can be produced, in part or in certain cases largely outside of the BM niche^{175,178}.

The most definitive study to date to investigate the cell-specific role of *Kitl*, was conducted in the Morrison lab (with *Kitl* designated as SCF in their paper⁸⁹). They developed two models, one which revealed the distribution of *Kitl* within the BM (*Scf^{gfp}* mice) and the second (*Scf^{fl}* mice), which was used to conditionally KO *Kitl* from a range of stromal cells and haematopoietic cells by crossing with several *Cre* mouse lines. These models revealed that *Kitl* was primarily expressed by sinusoidal ECs and LepR⁺ PVCs and its deletion from these cells, but not from any other stromal or haematopoietic cell populations (*Vav1*⁺ HSCs, CD45⁺ and Ter119⁺ cells) led to impaired HSC maintenance. Resulting mice from these crosses (*Tie2Cre*⁺; *Scf^{fl/-}* and *LepRCre*⁺; *Scf^{fl/-}*) were found to show ~80 % and 85 % reductions in HSC numbers, respectively. Furthermore, simultaneous deletion of total *Kitl* from both cell populations in *Tie2Cre*⁺; *LepRCre*⁺; *Scf^{fl/-}* mice induced the most dramatic phenotype, reducing HSCs to 5 % of WT levels⁸⁹. The authors claimed that ECs and LepR⁺ PVCs were two important components of the vascular niche and both cell types produce *Kitl* for HSC maintenance/expansion.

Their findings demonstrated that ECs and LepR⁺ PVCs express the highest levels of *Kitl* expression in BM. It was also shown that the loss of *Kitl* from OBs, Nes⁺ cells and haematopoietic cells had no impact upon haematopoiesis. Despite these observations, the study might be considered to have certain limitations. All *Cre*⁺ mice investigated in this study were hemizygous and had one complete allele missing for *Kitl*. It is assumed that there would therefore be a 50 % reduction in the expression of total *Kitl* protein in all tissues and consequently, any effects on haematopoiesis seen might result from a combination of both decreased overall expression of *Kitl* and its specific KO in the specified perivascular cell populations. Therefore homozygous models, which targeted KO of *Kitl* in ECs and LepR⁺ PVCs, were considered worth

generating and investigating. In addition, the Morrison study did not determine the individual protein expression of mKitl and sKitl *in vivo* or attempt to distinguish between the roles of mKitl and sKitl and accordingly, it was considered necessary to develop models with the specific KO of *mKitl* from these cell populations.

In summary, having established that the complete loss of mKitl *in vivo* has a major impact upon murine haematopoiesis (**Chapter 3**), the next step was to confirm which cell(s) expressed *mKitl* at the highest levels within the adult BM niche. Once determined, the aim was to generate murine models to conditionally delete *mKitl* from the identified cells, ECs but also include LepR⁺ PVCs (given their emerging importance within the BM niche⁸⁹) in order to assess haematopoietic impacts following *mKitl* deletion in individual cell populations or simultaneously in these cell populations.

Given the HSC intrinsic and microenvironmental changes which occur with age, the haematopoietic impact of deletion of *mKitl* in the above models at different stages of ontogeny (young vs. aged mice) was also assessed.

To address some of the limitations of the Morrison group study (outlined above) and to be able to more definitively determine the specific roles for mKitl and sKitl, mouse models, which had total loss of *Kitl* from the aforementioned cell types were also investigated and compared to models with cell-specific loss of *mKitl*.

4.2 Results

4.2.1 Endothelial cells highly express *mKitl*

To determine the gene expression levels of *mKitl* and total *Kitl* by different stromal cell populations, MSCs, OBs and ECs cells were prepared and isolated from BLCs of WT (C57BL/6) BM (**Section 2.3**). They were then FACS sorted according to the gating strategy indicated in **Figure 4.1a**, including Abs against markers which would ensure exclusion of haematopoietic cells (CD45 and Ter119). Cells were sorted into a lysis/pre-amplification mix and then underwent reverse transcription of target transcripts and pre-amplification of corresponding cDNA. These cDNA sample mixes and Taqman assays were prepared, loaded onto a chip and run using the Fluidigm nanofluidic gene expression platform (for quantitative gene expression, conducted jointly by *R.F.* and *S.M.*, **Section 2.7.1**). This enabled the quantitative determination of the expression of a large number of genes including several cytokines implicated in haematopoiesis (*Cxcl12*, *Flt3l*, *Csf1* and *Ang1*).

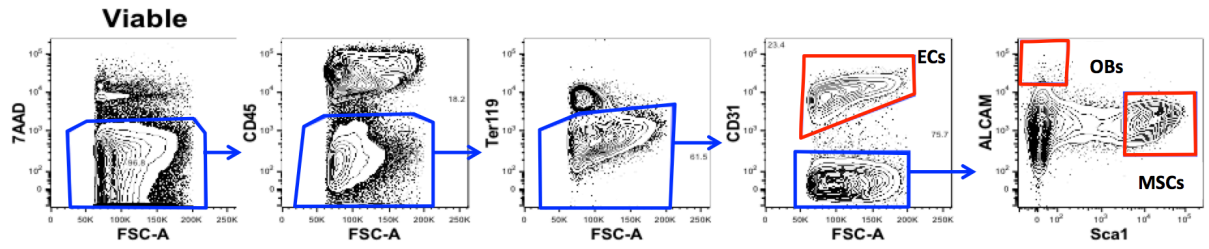
The genes investigated also included those known to be expressed by specific stromal cell populations (such as *Thy1* (MSCs), *Osx* (OBs) and *Tie2* (ECs)). The expression of haematopoietic cell markers such as *Cd45* was also investigated and confirmed not to be present in the stromal cell populations investigated (data not shown). Expression levels of ‘housekeeping genes’ such as *Hprt* and *Gapdh* were also determined with the former being used to calculate relative expression levels of the genes of interest (**Figure 4.1b**).

These analyses, which were conducted before the Morrison group paper⁸⁹ was published, confirmed that expression of both total *Kitl* and *mKitl* were respectively an average of 11.4 and 12.4 fold higher in ECs than in the other two stromal cell

populations. The use of isoform-specific Taqman[®] probes made possible the determination of the expression level of total *Kitl* and *mKitl*. These results were in broad agreement with the findings of the Morrison group, which revealed that VE-Cad⁺ ECs expressed almost 10 fold more of the long form of *Scf* (corresponding to *mKitl*) than whole BM. In addition, the newly-identified LepR⁺ PVC population was shown to express even higher levels of the long form in whole BM by the Morrison group⁸⁹.

Cell-specific genes (*Thy1*, *Osx* and *Tie2*) were confirmed to be predominantly expressed in their corresponding stromal cell types (MSCs, OBs and ECs, respectively; **Figure 4.1b**). Of the other factors investigated, *Flt3l* appeared to be expressed in all three cell populations, with the highest expression seen in MSCs. *Csf1* was more highly expressed on MSCs. OBs had markedly lower expression of *Cxcl12* than MSCs and ECs, whilst ECs had significantly lower expression of *Ang1* than MSCs or OBs. The low level of *Cxcl12* in OBs might be considered unexpected, although the expression in these cells has been reported to be mainly in immature OBs and highly expressed in ECs⁴⁶. Additionally, the low OB-expression of *Cxcl12* correlates with a recent study, which demonstrated that mineralising OB-specific loss of CXCL12 did not impact HSCs or early lymphoid progenitors⁹⁹.

a) BLC gating strategy



b) Quantitative gene expression

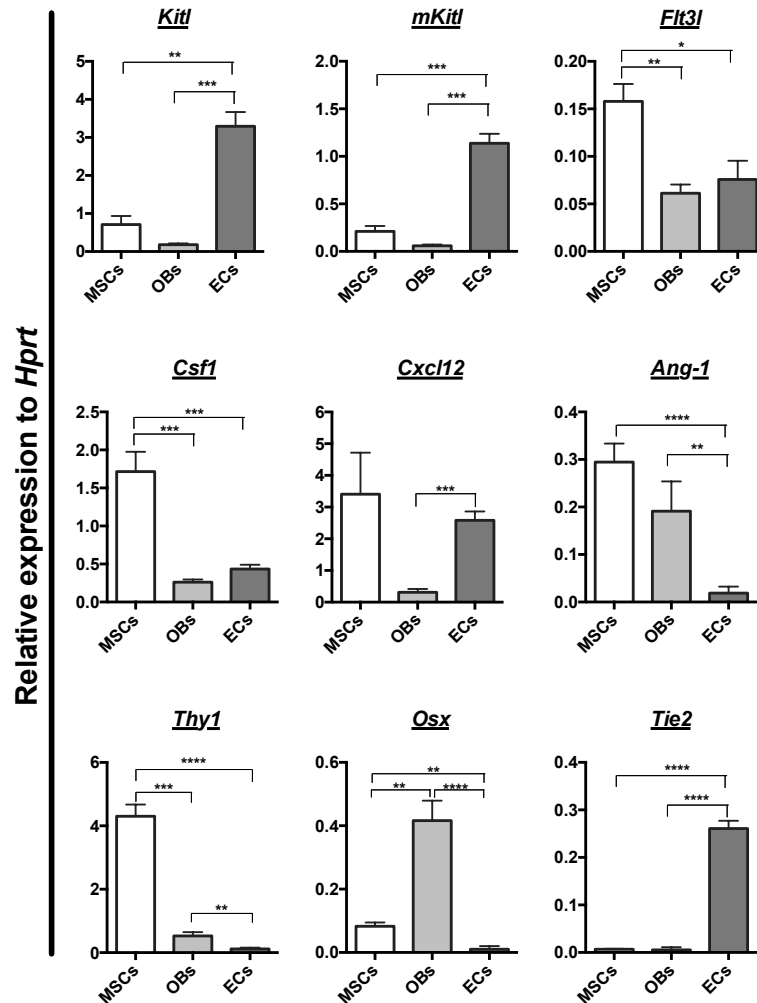


Figure 4.1 BLC endothelial cells express high levels of *mKitl* and total *Kitl*

- Representative FACS profiles and gating strategies used to sort ECs, OBs and MSCs from 3-week old WT C57BL/6 mouse BLC stromal populations stained with antibodies against CD45, Ter119, CD31, ALCAM and Sca1 and by cell viability staining (7AAD).
- Quantitative gene expression analysis of FACS purified mesenchymal stem cells (MSCs; CD45⁻ Ter119⁻CD31⁻Sca1⁺), osteoblasts (OBs; CD45⁻Ter119⁻CD31⁻ALCAM⁺) and endothelial cells (ECs; CD45⁻Ter119⁻CD31⁺) from 3-week old WT C57BL/6 mice (100 cells per replicate). Average expression levels are relative to *Hprt* (n=6, values shown are the mean±SEM, p < 0.05*, p < 0.01**, p < 0.005*** p < 0.001**** with p values determined using the unpaired Student t-test).

4.2.2 Endothelial and perivascular cell loss of *mKitl* results in mild haematopoietic defects in young mice

Gene expression analysis from this work and from the Morrison group indicated that ECs and LepR⁺ PVCs express the highest levels of *mKitl* and total *Kitl* within the stromal cell populations. These findings led me to investigate the *in vivo* impact of the loss of *mKitl* from ECs and LepR⁺ PVCs. To do so, *Kitl* ^{Δ ex7fl/ Δ ex7fl} mice were crossed with *Tie2Cre*⁺ and *LepRCre*⁺ mice to generate mice lacking *mKitl* from ECs (*Tie2Cre*⁺;*Kitl* ^{Δ ex7/ Δ ex7}), LepR⁺ PVCs (*LepRCre*⁺;*Kitl* ^{Δ ex7/ Δ ex7}) and from both populations simultaneously (*Tie2Cre*⁺;*LepRCre*⁺;*Kitl* ^{Δ ex7/ Δ ex7}). The genotypes of these mice were confirmed by PCR (**Section 2.2**).

Both single KO (sKO) and the double KO (dKO) models retained their black coat colour (data not shown) unlike the *Kitl* ^{Δ ex7/ Δ ex7} mice, which are white (**Section 3.2.2**). PB parameters were investigated at 4 weeks and the results obtained are shown in **Figure 4.2**. Neither of the sKO (*Tie2Cre*⁺;*Kitl* ^{Δ ex7/ Δ ex7} or *LepRCre*⁺;*Kitl* ^{Δ ex7/ Δ ex7}) mice displayed any abnormalities in their blood profile, however, the dKO *Tie2Cre*⁺;*LepRCre*⁺;*Kitl* ^{Δ ex7/ Δ ex7} mice exhibited small yet significant reductions to both the number of RBCs by 13.3 % ($6.78 \pm 0.27 \times 10^6$ vs. $7.82 \pm 0.28 \times 10^6$ / μ L) and the level of Hgb by 9.0 % (12.2 ± 0.3 vs. 13.4 ± 0.3 g/dL) compared to WT controls (*Cre*⁻;*Kitl* ^{Δ ex7/ Δ ex7}) indicating a mild anaemic phenotype (both $p < 0.05^*$).

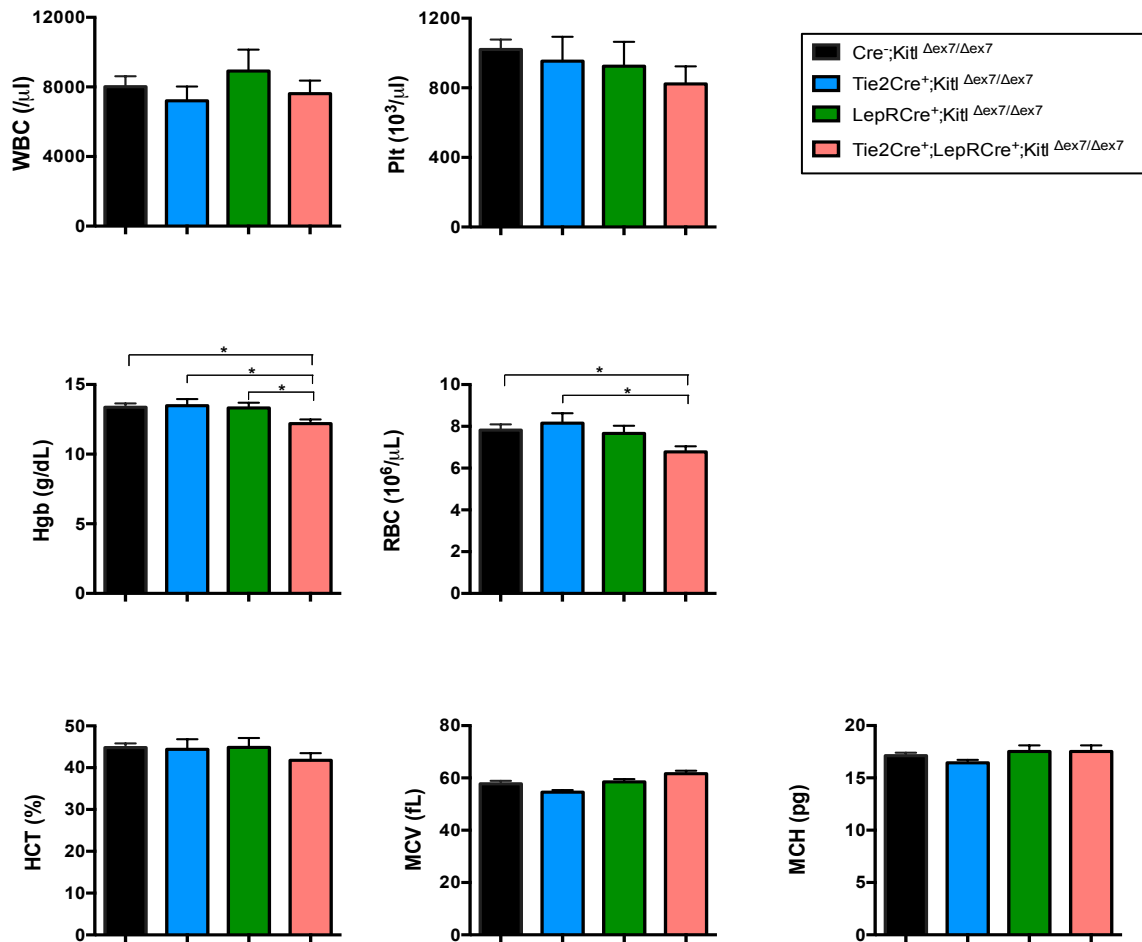


Figure 4.2 *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* mice exhibit mild anaemia

Analysis of peripheral blood counts of 4-week old *Tie2Cre⁺;Kitl^{Δex7/Δex7}*, *LepRCre⁺;Kitl^{Δex7/Δex7}*, *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* and WT (*Cre⁻;Kitl^{Δex7/Δex7}*) littermate controls (n=6-11 mice per genotype, values shown are the mean $\pm$ SEM, $p < 0.05^*$ with p values determined using the unpaired Student t-test). Key: WBC (white blood cells), Plt (platelets), Hgb (haemoglobin), RBCs (red blood cells), HCT (haematocrit), MCV (mean cell volume), MCH (mean cell haemoglobin).

Further analyses of these sKO (*Tie2Cre⁺;Kitl^{Δex7/Δex7}* and *LepRCre⁺;Kitl^{Δex7/Δex7}*) and dKO (*Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}*) mice showed that compared to WT, they maintained normal BM cellularity (WT: $46.8 \pm 1.8 \times 10^6$ vs. respectively $41.4 \pm 3.0 \times 10^6$ vs. $43.0 \pm 2.4 \times 10^6$ vs. $43.7 \pm 2.2 \times 10^6$ / femur and tibia) and spleen cellularity (WT: $148.0 \pm 8.1 \times 10^6$ vs. respectively $158.9 \pm 12.9 \times 10^6$ vs. $145.9 \pm 9.3 \times 10^6$ vs. $135.1 \pm 7.9 \times 10^6$, **Figure 4.3a and b**).

Flow cytometric analysis revealed that compared to WT controls, the KO models (*Tie2Cre⁺;Kitl^{Δex7/Δex7}*, *LepRCre⁺;Kitl^{Δex7/Δex7}* and *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}*) had lower overall numbers of phenotypic LSK CD48⁺CD150⁺ HSCs within the BM (WT: 3588 ± 461 vs. respectively 2897 ± 837 vs. 2851 ± 325 vs. 2967 ± 326 / femur and tibia, **Figure 4.3c**), although these reductions were not statistically significant.

Similarly, the sKO mice had no statistically significant changes to their MPC profiles (**Figure 4.3d**). However, compared to WT, the loss of *mKitl* in the dKO

Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7} mice led to a significant reduction in the number of CFU-Es (74.6 % reduction, WT: $58.2 \pm 12.8 \times 10^3$ vs. $14.8 \pm 3.8 \times 10^3$, $p < 0.05^*$).

Furthermore, these mice also had significantly fewer erythroid progenitors (Pro Ery, 66.0 % reduction, WT: $87.2 \pm 19.8 \times 10^3$ vs. $29.6 \pm 4.9 \times 10^3$, $p < 0.05^*$), granulocyte-macrophage progenitors (Pre GM 39.5 % reduction, WT: $184.4 \pm 12.8 \times 10^3$ vs.

$111.5 \pm 12.4 \times 10^3$, $p < 0.01^{**}$ and GMP 69.1 % reduction, WT: $259.9 \pm 49.1 \times 10^3$ vs.

$80.3 \pm 19.8 \times 10^3$, $p < 0.05^*$) and megakaryocytic progenitors (MkP 37.0 % reduction, WT: $12.8 \pm 1.6 \times 10^3$ vs. $8.1 \pm 0.4 \times 10^3$, $p < 0.05^*$), indicating that the loss of *mKitl*

from multiple stromal cell types leads to an impairment of haematopoiesis (**Figure 4.3d**).

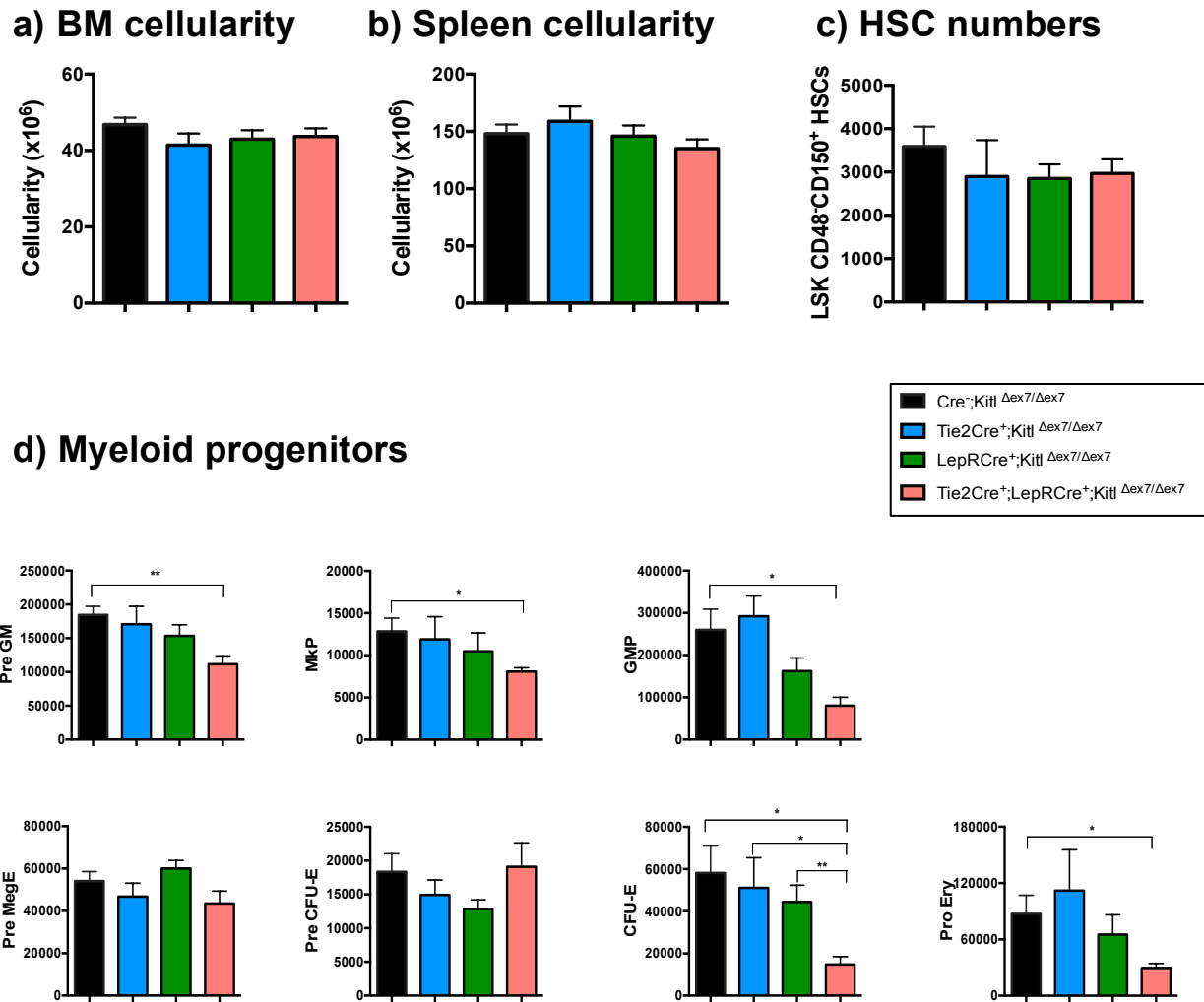


Figure 4.3 EC and PVC loss of *mKitl* results in haematopoietic defects in young mice

Analysis of 4-week old Tie2Cre⁺;Kitl ^{Δ ex7/ Δ ex7}, LepRCre⁺;Kitl ^{Δ ex7/ Δ ex7}, Tie2Cre⁺;LepRCre⁺;Kitl ^{Δ ex7/ Δ ex7} and WT (Cre⁻;Kitl ^{Δ ex7/ Δ ex7}) littermate controls (values shown are the mean $\pm$ SEM, with p values determined using the unpaired Student t-test):

- BM cellularity from 1 femur and 1 tibia (n=6-8 mice per genotype).
- Spleen cellularity (n=6-8 mice per genotype).
- Absolute number of phenotypic LSK CD48⁺CD150⁺ HSCs from 1 femur and 1 tibia (n=5-10 mice per genotype).
- Absolute number of myeloid progenitors from 1 femur and 1 tibia (n=5-10 mice per genotype, p<0.05*, p<0.01**). Cell populations are as defined as in **Figure 3.11**.

I then wanted to establish if loss of *mKitl* in the specific stromal cell populations would cause a reduction in the level of circulating systemic sKitl. Analysis by ELISA (Section 2.6.1) of the serum from the different cell-specific KO models demonstrated that loss of *mKitl* from ECs resulted in a statistically significant 26.6 % reduction in systemic sKitl compared to WT controls (152.7 ± 10.4 vs. WT 210.7 ± 14.8 pg/ml, $p < 0.05^*$). This reduction was marginally greater in the dKO model (30.8 % reduction to 145.7 ± 26.6 pg/ml, $p < 0.05^*$). Although lower by 14.5 % compared to WT, the level of systemic sKitl (180.8 ± 25.6 pg/ml) was not statistically reduced by the LepR⁺ PVC-specific loss of *mKitl* (Figure 4.4).

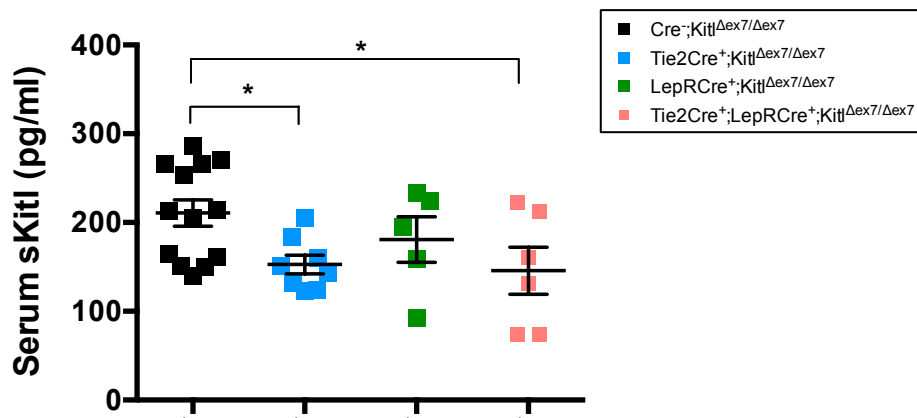


Figure 4.4 Endothelial-specific loss of *mKitl* causes a reduction in the level of systemic sKitl

Analysis of serum sKitl levels determined by ELISA from 4-week old Tie2Cre⁺;Kitl^{Δex7/Δex7}, LepRCre⁺;Kitl^{Δex7/Δex7}, Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7} and WT (Cre⁻;Kitl^{Δex7/Δex7}) littermate controls (n=5-13 mice per genotype, values shown are the mean±SEM; $p < 0.05^*$ with p values determined using the unpaired Student t-test)

The deletion efficiency of the Cre models was then assessed, especially given the limited phenotypes in the sKO models. I sorted ECs from the BLCs of Tie2Cre⁺;Kitl^{Δex7/Δex7} mice and LepR⁺ PVCs from the CBM of LepRCre⁺;Kitl^{Δex7/Δex7} mice and WT controls (Section 2.3.1.3-4 and Section 2.3.2). RNA was isolated from each cell type and converted to cDNA, which was then used for qPCR analysis (Section 2.7.2.2). The expression levels were determined relative to the

housekeeping gene, *Hprt* and calculated on the basis of $\Delta\Delta C_t$ values. The results revealed that the *Tie2Cre* model gave extremely high deletion of *mKitl* (~ 99 %, $p < 0.0001$ ****) compared to WT (**Figure 4.5a**), with a 33.6 % reduction in the expression of total *Kitl* (**Figure 4.5b**). The *LepRCre* model was not as efficient, demonstrating a deletion efficiency of only ~ 20 % for *mKitl* (**Figure 4.5c**) and no reduction of total *Kitl* compared to WT (**Figure 4.5d**) (qPCR performed by Dr. Tiago Luis, T.L.).

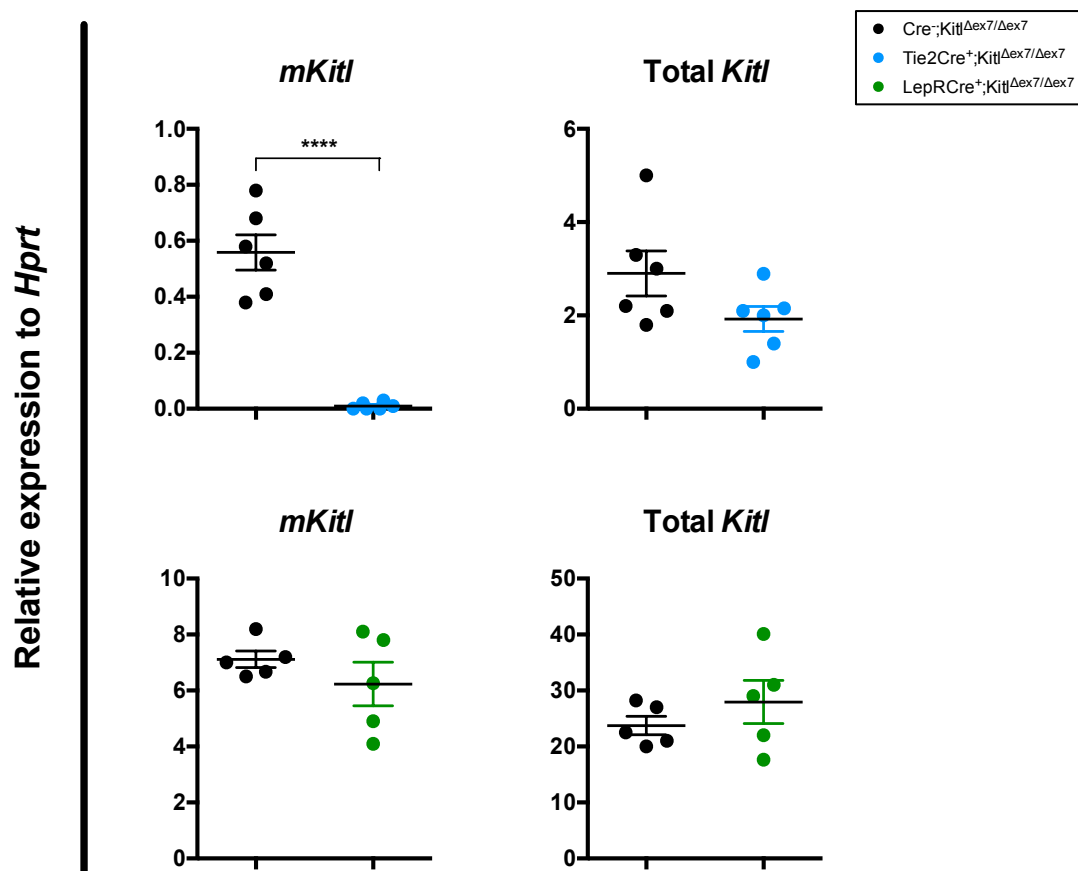


Figure 4.5 *mKitl* and total *Kitl* deletion efficiency in *Tie2Cre*⁺;*Kitl*^{Δex7/Δex7} and *LepRCre*⁺;*Kitl*^{Δex7/Δex7} mice

Gene expression analysis of FACS purified BLC ECs (CD45⁺Ter119⁻CD31⁺) and CBM LepR⁺ PVCs (CD45⁺Ter119⁻LepR⁺CD31⁺) from 4-6 week old *Tie2Cre*⁺;*Kitl*^{Δex7/Δex7}, *LepRCre*⁺;*Kitl*^{Δex7/Δex7} and WT littermate controls (*Cre*⁻;*Kitl*^{Δex7/Δex7}), 2000 cells per replicate. Average expression levels are relative to *Hprt*. (n=5-6, values shown are the mean±SEM, $p < 0.0001$ **** with p values determined using the unpaired Student t-test).

A second approach was used to confirm the low deletion efficiency of the *LepRCre* line, to rule out the possibility of insensitivity of the assay, which had used RNA isolated from the *LepR*⁺ PVCs. I therefore crossed *LepRCre*⁺ mice with *Rosa26YFP*⁺ mice to generate a *LepRCre*⁺;*Rosa26YFP*⁺ model and confirmed the genotype by PCR (Section 2.2). CBM cells were prepared and analysed by flow cytometry using the principle that all *LepR*⁺ cells should be marked with YFP. It was found that 53 % of *LepRCre*⁺;*Rosa26YFP*⁺ CBM stromal cells were YFP⁺, compared to the *LepRCre*⁻;*Rosa26YFP*⁺ controls which contained 0 % YFP⁺ cells (T.L., Figure 4.6). This level of recombination efficiency in combination with the qPCR data further suggested that this model does induce some but not complete deletion of *mKitl* from *LepR*⁺ cells.

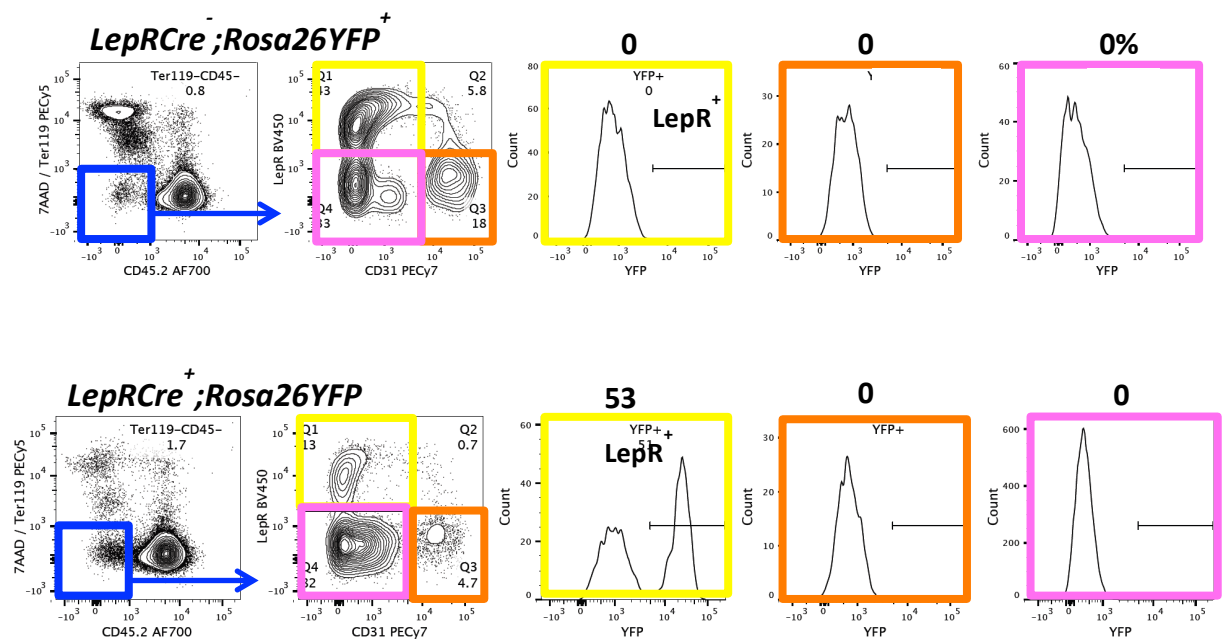


Figure 4.6 *LepRCre* line displays >50% recombination efficiency

Representative FACS profiles from 6-week old *LepRCre*⁺;*Rosa26YFP*⁺ (bottom panel) and WT littermate control (*LepRCre*⁻;*Rosa26YFP*⁺, top panel) CBM, demonstrating the proportion of YFP⁺ cells within different stromal cell population (n=3 mice per genotype, mean values).

This low deletion efficiency was unexpected since the Morrison paper had used the identical *LepRCre* model⁸⁹ (estimated to be >70 % deletion when the *Cre* was used hemizygously) but potentially incomplete using homozygous *Cre* (flox/flox) mice,

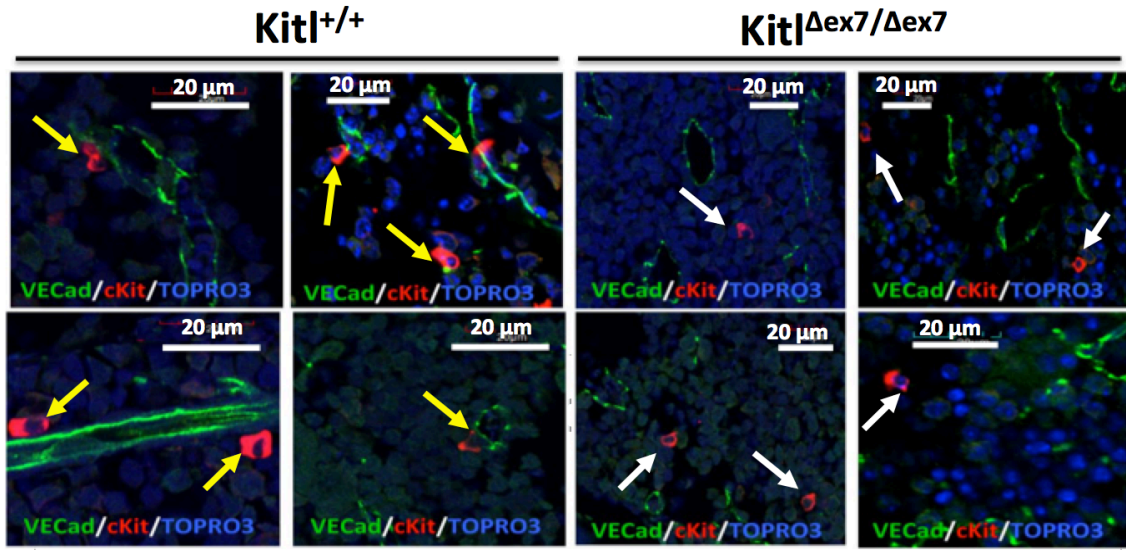
which in part may be the reason why hemizygous (flox/null) mice were investigated and reported in their paper.

4.2.3 Impact of cell-specific loss of *mKitl* on BM HSPC localisation and cell cycle status

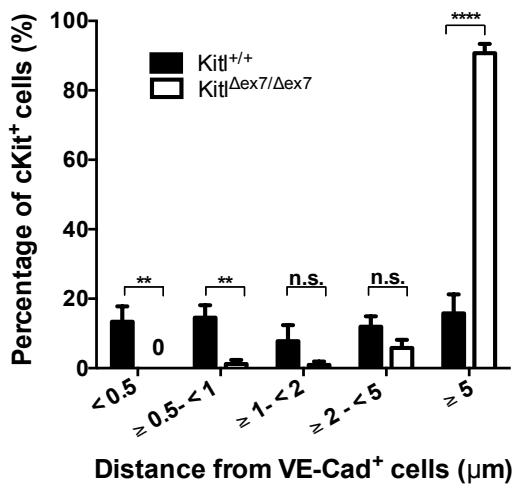
Under steady-state conditions, most stem cells within the BM niche are found in direct contact with stromal cells and are quiescent, with only a small proportion actively cycling^{26,278-280}. Kitl/c-Kit signalling has previously been shown to be essential not only for HSC maintenance but also in the anchoring of these cells and therefore important for maintaining HSCs in quiescence^{250,278}. Therefore I wanted to establish if the loss of *mKitl* in ECs and PVCs would affect the localisation of HSPCs within the niche or alter their cell cycle status.

Firstly, using IHC, I investigated the spatial relationship between ECs and HSPCs in the BM. To validate this method, BM sections from germline *mKitl* deleted *Kitl* ^{Δ ex7/ Δ ex7} mice (**Chapter 3**) were stained with VE-Cad Ab to mark the endothelium, c-Kit Ab to identify HSPCs and TO-PRO[®]-3 (**Figure 4.7a**). In WT control mice (*Kitl*^{+/+}), 84.2 % of c-Kit⁺ HSPCs were located less than 5 μ m from VE-Cad⁺ endothelium (**Figure 4.7b**). Furthermore, 13.4 % of HSPCs were in direct contact with or <0.5 μ m away from ECs. This relationship was lost in *Kitl* ^{Δ ex7/ Δ ex7} BM sections, with 90.7 % of c-Kit⁺ HSPCs residing >5 μ m ($p < 0.0001^{****}$) from the endothelium and none in direct contact ($p < 0.01^{**}$). This altered positioning of c-Kit⁺ progenitor cells was not attributable to a loss of ECs, which was determined by measuring VE-Cad⁺ ECs in both *Kitl* ^{Δ ex7/ Δ ex7} and WT sections (**Figure 4.7c**). The above data demonstrate that *mKitl* has a specific role in anchoring HSPCs via cell-cell contact within the BM HSC niche.

a) Representative BM sections



b) c-Kit⁺ to VE-Cad⁺ distances



c) VE-Cad⁺ ECs

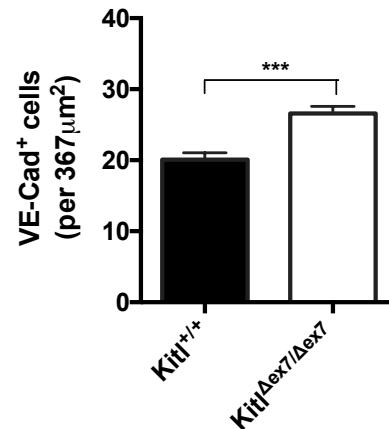


Figure 4.7 Loss of *mKitl* in *Kitl*^{Δex7/Δex7} mice leads to loss of proximity between niche cells and haematopoietic stem and progenitor cells

- Interaction of c-Kit⁺ progenitors (red, primary + donkey anti-rat IgG Cy5) and VE-Cadherin⁺ vascular endothelial cells (green, primary + donkey anti-goat IgG 488) in the BM of 3-week old *Kitl*^{Δex7/Δex7} and WT *Kitl*^{+/+} mice. Progenitors attached (yellow arrows) and not attached (white arrows) to VE-Cad⁺ ECs. Results are representative of 8 mice analysed per genotype.
- Percentage of c-Kit⁺ cells at indicated distances from VE-Cad⁺ ECs. 93 and 77 c-Kit⁺ cells from *Kitl*^{Δex7/Δex7} and WT *Kitl*^{+/+} respectively (n=4 mice per genotype, p<0.01**, p<0.0001****).
- Number of VE-Cad⁺ ECs in *Kitl*^{Δex7/Δex7} and WT *Kitl*^{+/+} BM sections (n=4 mice per genotype, p<0.005***).

Values shown are the mean±SEM, with p values determined using the unpaired Student t-test.

To determine if this phenotype was also seen in KO mice with the EC and LepR⁺ PVC-specific loss of *mKitl*, a similar approach was employed. I prepared BM samples from 4-week old *Tie2Cre⁺;Kitl^{Δex7/Δex7}*, *LepRCre⁺;Kitl^{Δex7/Δex7}*, *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* and WT (*Cre⁻;Kitl^{Δex7/Δex7}*) controls. Tissue sections were stained in this experiment with an Ab against the endothelial marker MECA-32, in combination with Abs against c-Kit and with lineage and SLAM markers to more definitively identify HSCs (IHC on these samples was conducted by S.M).

My analysis of the resulting slides (**Figure 4.8a**) revealed that 82.3 % of Lin⁻cKit⁺CD48⁻CD150⁺ HSCs were associated with MECA-32⁺ endothelium in WT BM (**Figure 4.8b**) whereas in the cell-specific KO mice, these values were 40.0 % (p<0.01**), 59.0 % (p<0.05*) and 42.7 % (p<0.01**) in respectively *Tie2Cre⁺;Kitl^{Δex7/Δex7}*, *LepRCre⁺;Kitl^{Δex7/Δex7}* and *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* BM sections. The number of MECA-32⁺ ECs was maintained in each of the models (**Figure 4.8c**), which ruled out that the loss of attachment of HSCs was due to a decline in ECs.

The least effect was seen in the *LepRCre⁺;Kitl^{Δex7/Δex7}* sections, but in theory none should have occurred. Indeed, how can reducing *mKitl* in LepR⁺ cells in *LepRCre⁺;Kitl^{Δex7/Δex7}* appear to cause a reduction in HSC association with ECs? One can speculate but cannot confirm at this stage, that this may reflect a close association between LepR⁺ PVCs and ECs. This needs to be confirmed by co-staining BM sections with Abs against LepR, and EC and HSC markers. These findings confirm that the expression of *mKitl* specifically by endothelial and potentially also perivascular cells maintain HSPCs at defined locations within the niche, close to ECs.

a) Representative BM sections

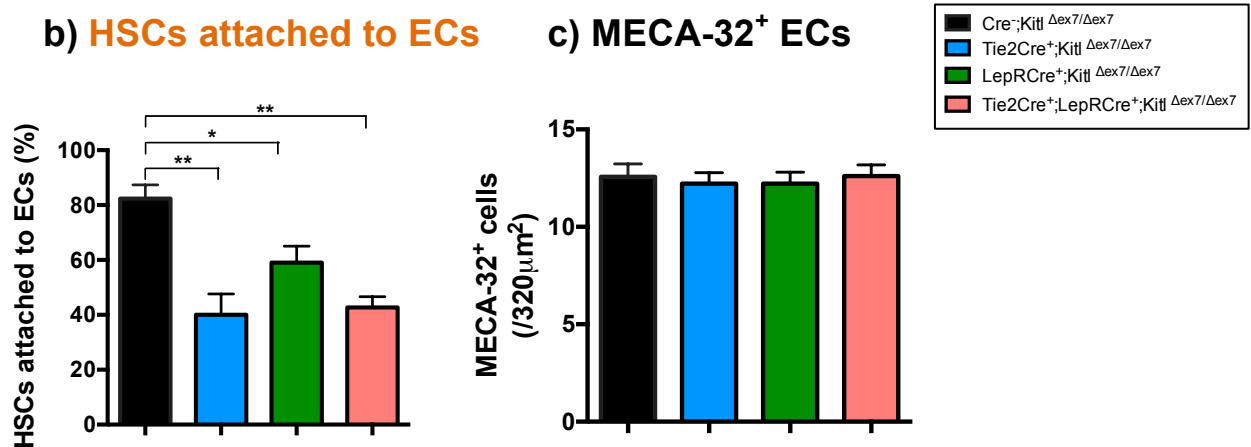
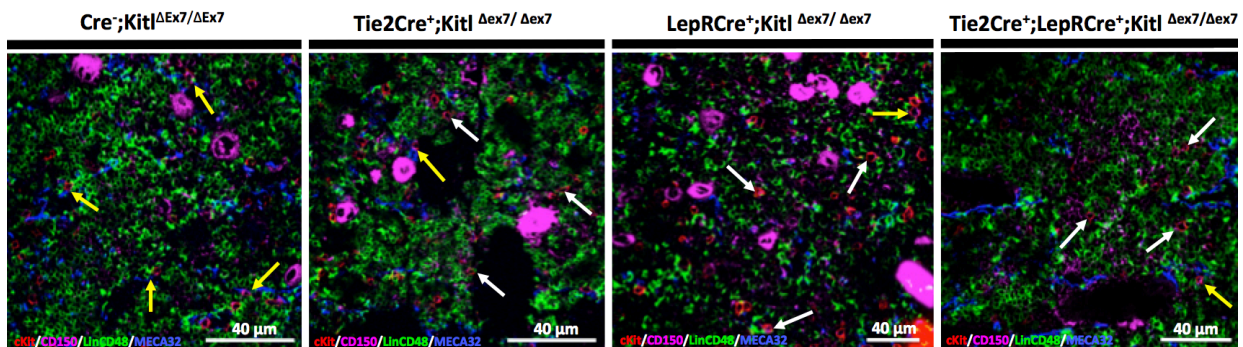


Figure 4.8 Endothelial and perivascular cell loss of *mKitl* is specifically required for anchoring HSCs to the endothelium in adult BM

Analysis of 4-week old *Tie2Cre⁺;Kitl^{Δex7/Δex7}*, *LepRCre⁺;Kitl^{Δex7/Δex7}*, *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* and WT (*Cre⁻;Kitl^{Δex7/Δex7}*) littermate controls (values shown are the mean±SEM with p values determined using the unpaired Student t-test):

- Interaction of Lin⁻CD48⁻ (green), CD150⁺ (pink), cKit⁺ (red) HSCs and MECA-32⁺ vascular endothelium (blue) in the BM. HSCs attached (yellow) and detached (white) to MECA-32⁺ ECs.
- Percentage Lin⁻c-Kit⁺ CD48⁻CD150⁺ HSCs attached to MECA-32⁺ ECs. Mean results from 15 sections and 80 Lin⁻c-Kit⁺CD48⁻CD150⁺ cells (n= 4 mice per genotype, p<0.05*, p<0.01**).
- Number of MECA-32⁺ ECs in BM sections (n=4 mice per genotype).

Loss of anchoring can lead to increased cell cycling as cells come out of quiescence²⁷⁹. The loss of interaction between HSCs and stroma in our KO mice led me to investigate if the loss of *mKitl* from ECs or LepR⁺ PVCs altered the cell cycle status of HSCs within the BM. Using Ki-67 staining and the nuclear stain DAPI, in

combination with antibody markers for HSCs (Lin, Sca1, c-Kit, CD48 and CD150), it was possible to determine which phase of the cell cycle HSCs occupied. Ki-67 is a protein that acts as a cellular marker for proliferation, as it is present during the active phases of the cell cycle (G_1 , S, G_2 and M)²⁸¹. Using this method (**Section 2.3.2.1**), cells were defined as being in G_0 (quiescence) or in the different stages of cycling (G_1 or S/ G_2 /M).

Cell cycle analysis of HSCs from cell-specific $Cre^+Kitl^{\Delta ex7/\Delta ex7}$ models revealed that 4-week old $LepRCre^+;Kitl^{\Delta ex7/\Delta ex7}$ and $Tie2Cre^+;LepRCre^+;Kitl^{\Delta ex7/\Delta ex7}$ mice had a significantly lower proportion of LSK CD48⁻ CD150⁺ HSCs in G_0 (52 % and 54 % respectively vs. 72 % in WT ($Cre^-;Kitl^{\Delta ex7/\Delta ex7}$) controls (both $p < 0.05^*$, **Figure 4.9**). They had a concomitant significant increase in the proportion of cells in G_1 compared to WT littermate controls (44 % and 38 % respectively vs. WT 22 %, both $p < 0.05^*$). The frequency of cells in both G_0 and G_1 from the $LepRCre^+;Kitl^{\Delta ex7/\Delta ex7}$ and the dKO was very similar indicating that loss of *mKitl* from ECs does not appear to contribute to increased cycling. In fact, $Tie2Cre^+;Kitl^{\Delta ex7/\Delta ex7}$ mice actually showed a slight increase in the proportion of cells in G_0 (83 % vs. 72 % in WT) and a decrease in G_1 (10 % vs. 22 % in WT), but these differences were not statistically significant.

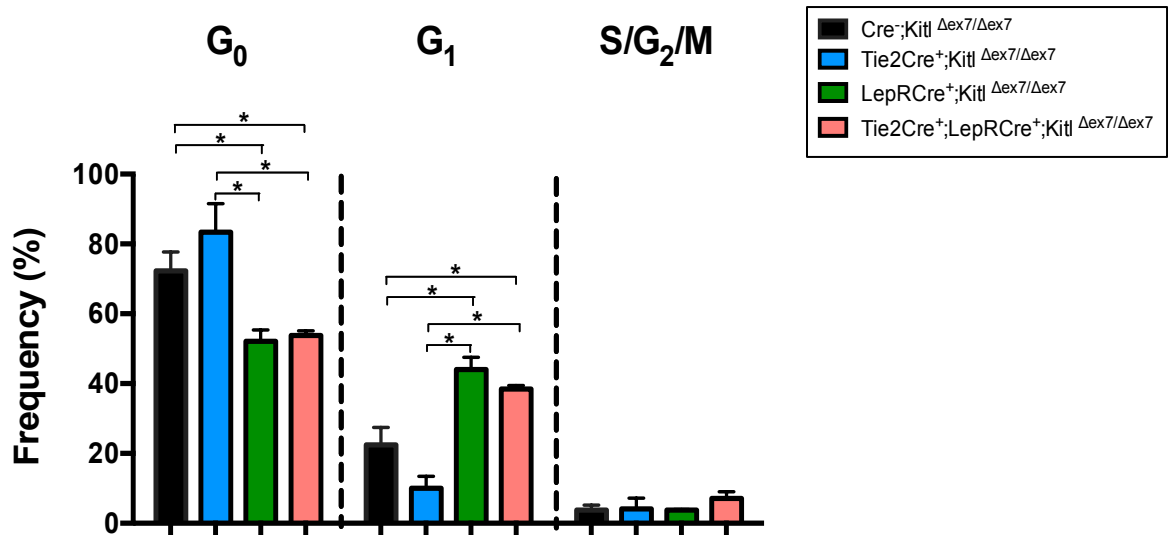


Figure 4.9 LepR⁺ PVC-loss of *mKitl* leads to increased HSC cycling *in vivo*

Frequencies of quiescent and cycling phenotypic LSK CD48⁺CD150⁺ HSCs in 4-week old *Tie2Cre⁺;Kitl^{Δex7/Δex7}*, *LepRCre⁺;Kitl^{Δex7/Δex7}*, *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* and WT (*Cre⁺;Kitl^{Δex7/Δex7}*) littermate controls. Bar chart shows frequency of HSCs in G₀ (Ki67⁺DAPI⁻), G₁ (Ki67⁺DAPI⁻) and S/G₂/M (Ki67⁺DAPI⁺). (n=4 mice per genotype, values shown are the mean±SEM, p<0.05* with p values determined using the unpaired Student t-test).

4.2.4 The impact of endothelial and perivascular cell loss of *mKitl* increases with age

To determine whether or not the role of *mKitl* in the regulation of haematopoiesis changes during the ageing process, *Cre⁺;Kitl^{Δex7/Δex7}* mice aged between 12-13 months were investigated. It was not possible to analyse germline deleted *Kitl^{Δex7/Δex7}* mice, as these never survive past 6 weeks.

Initial investigation of PB indicated that overall, aged mice exhibited more defects in their blood profiles vs. WT controls than observed in corresponding comparisons in younger mice. However, no statistically significant changes in the blood profile of aged *Tie2Cre⁺;Kitl^{Δex7/Δex7}* compared to WT control mice were seen (**Figure 4.10**). This was comparable to the situation seen in their 4-week old counterparts (**Figure 4.2**).

Compared to WT littermate controls, in the case of aged *LepRCre⁺;Kitl^{Δex7/Δex7}* mice, there were small but significant reductions in the Hgb level (WT: 13.9 ± 0.2 vs. 13.1 ± 0.1 g/dL, $p < 0.05^*$) and the HCT (WT: 47.9 ± 0.7 vs. 44.0 ± 0.3 %, $p < 0.05^*$, **Figure 4.10**); not evident in their 4-week old counterparts (**Figure 4.2**). It must be emphasised, however, that the reductions seen in these aged *LepRCre⁺;Kitl^{Δex7/Δex7}* mice represent only small decreases in these parameters, respectively 6.4 % and 8.2 %.

In older dKO mice, there appeared to be more anaemia-related changes with time than seen in 4-week old mice. Compared to WT littermate controls, there were statistically significant decreases in HCT (47.9 ± 0.7 vs. 42.9 ± 1.4 %, $p < 0.05^*$), Hgb levels (13.9 ± 0.2 vs. 12.9 ± 0.4 g/dL, $p < 0.05^*$) and RBCs (8.8 ± 0.2 vs. $7.2 \pm 0.2 \times 10^6 / \mu\text{L}$, $p < 0.005^{***}$) and statistically significant increases in MCV (54.4 ± 0.9 vs. 59.8 ± 0.7 fL, $p < 0.01^{**}$) and MCH (15.8 ± 0.3 vs. 18.0 ± 0.2 pg, $p < 0.005^{***}$) values (**Figure 4.10**), whereas in young mice, only RBCs and Hgb levels showed significant changes (**Figure 4.2**). In the 12-13 month old dKO mice, RBCs fell by 18.6 % compared to WT littermate controls, whereas this decline was 13.3 % in 4-week old dKO mice compared to their WT littermates.

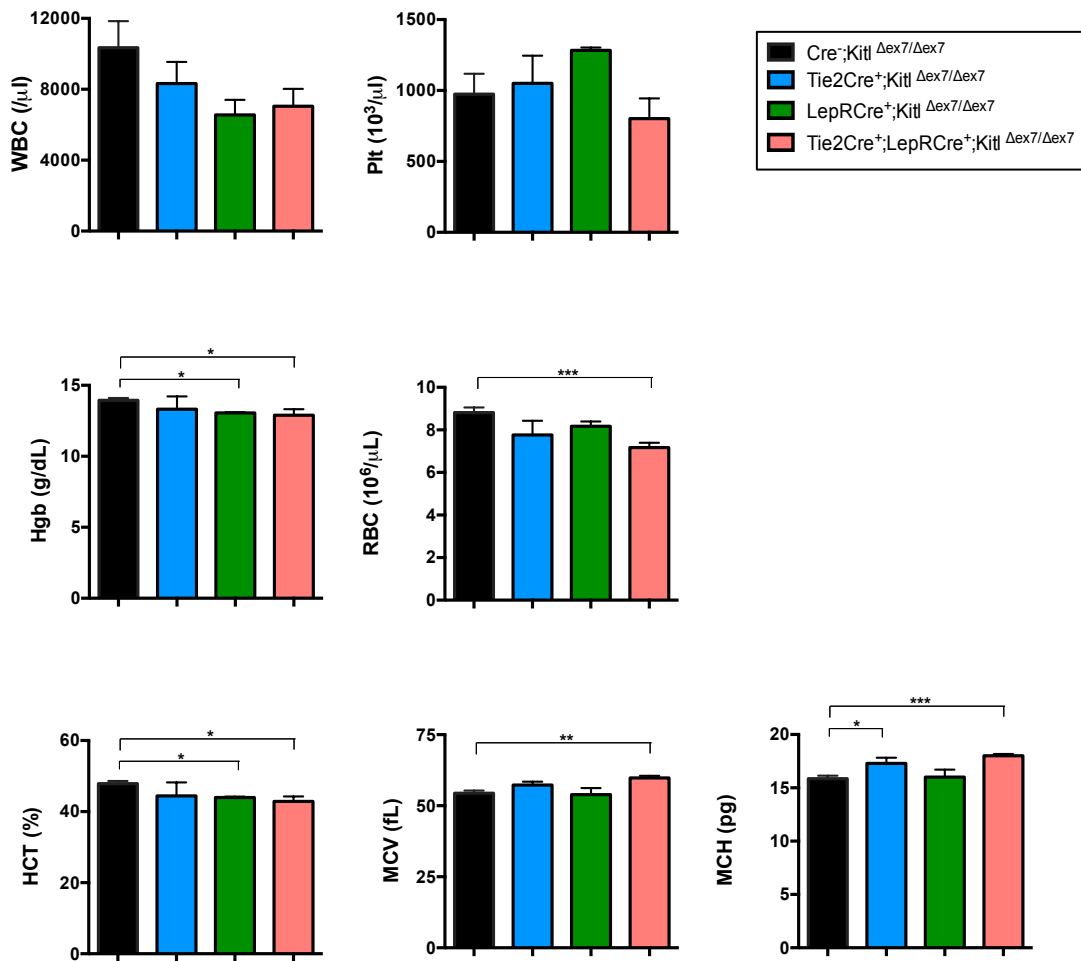


Figure 4.10 Aged *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* mice exhibit more anaemic features than young mice

Analysis of peripheral blood counts of 12-13 month old *Tie2Cre⁺;Kitl^{Δex7/Δex7}*, *LepRCre⁺;Kitl^{Δex7/Δex7}*, *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* and WT littermate controls (*Cre-;Kitl^{Δex7/Δex7}*, n=3-5 mice per genotype, values shown are the mean $\pm$ SEM, p<0.05*, p<0.01**, p<0.005*** with p values determined using the unpaired Student t-test). Key as indicated in **Figure 4.2**.

The analysis of the BM from these aged mice revealed that loss of *mKitl* from ECs and LepR⁺ PVCs resulted in an affected haematopoietic cell phenotype.

Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7} showed a reduction to BM cellularity ($86.4 \pm 1.31 \times 10^6$ vs. WT $120.0 \pm 10.0 \times 10^6$ cells/ 2 femurs and tibiae), however this change was not found to be statistically significant (**Figure 4.11a**). Investigation of haematopoietic cells by flow cytometry revealed that unlike in young mice, loss of *mKitl* in the dKO mice led to a statistically significant 58.5 % reduction in the absolute number of LSK CD48⁻CD150⁺ HSCs ($2.2 \pm 0.4 \times 10^4$ vs. WT $5.3 \pm 1.2 \times 10^4$ cells/ 2 femurs and tibiae, $p < 0.05^*$, **Figure 4.11b**).

In addition, compared to WT controls there were also statistically significant reductions in *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* mice in the number of GMPs (31.0 % reduction: $380.0 \pm 35.4 \times 10^3$ vs. $262.0 \pm 36.5 \times 10^3$, $p < 0.05^*$) and erythroid progenitor subsets including Pre MegE (51.0 % reduction, WT: $153.9 \pm 17.0 \times 10^3$ vs. $75.4 \pm 5.4 \times 10^3$, $p < 0.01^{**}$), Pre CFU-E (56.2 % reduction, WT: $31.8 \pm 5.7 \times 10^3$ vs. $17.9 \pm 0.7 \times 10^3$, $p < 0.01^{**}$) and Pro Ery (46.8 % reduction, WT: $35.3 \pm 6.9 \times 10^3$ vs. $18.8 \pm 3.6 \times 10^3$, $p < 0.05^*$) cells (**Figure 4.11c**).

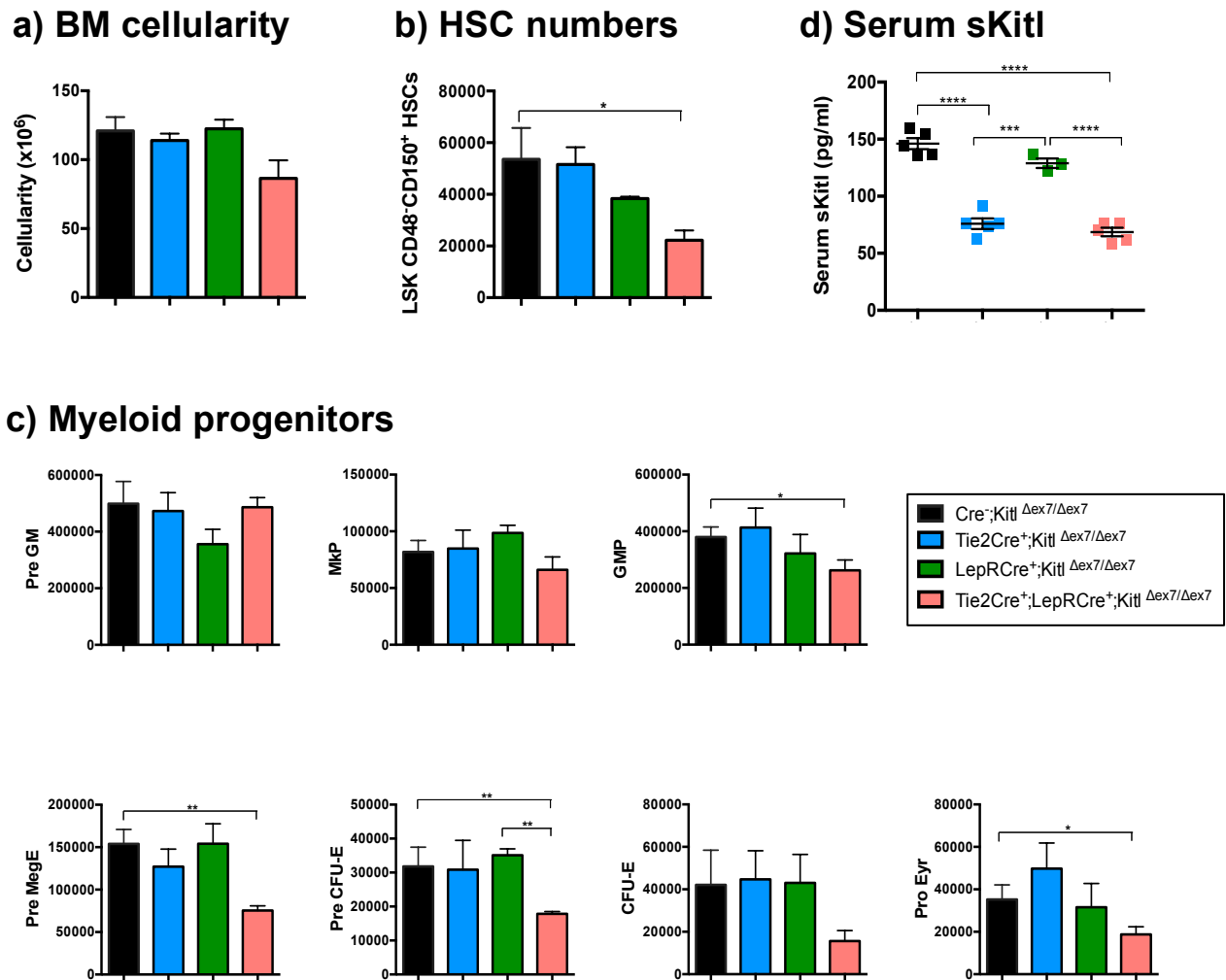


Figure 4.11 EC and PVC loss of *mKitl* results in haematopoietic defects in older mice

Analysis of 12-13 month old *Tie2Cre⁺;Kitl^{Δex7/Δex7}*, *LepRCre⁺;Kitl^{Δex7/Δex7}*, *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* and WT littermate controls (*Cre⁻;Kitl^{Δex7/Δex7}*, n=3-5 mice per genotype, values shown are the mean±SEM with p values determined using the unpaired Student t-test):

- BM cellularity from 2 femurs and 2 tibiae.
 - Absolute number of phenotypic LSK CD48⁺CD150⁺ HSCs from 2 femurs and 2 tibiae (p<0.05*).
 - Absolute numbers of myeloid progenitors per 2 femurs and 2 tibiae (p<0.05*, p<0.01**).
- Cell populations are as defined as in **Figure 3.11**.
- Serum sKitl levels determined by ELISA (p<0.0005***, p<0.0001****).

Analysis of systemic sKitl from the above aged mice revealed appreciably lower levels across the four genotypes including WT compared to the 4-week old mice (**Figure 4.11d** and **Figure 4.4**). There were again significant reductions in the level of ligand in *Tie2Cre⁺;Kitl^{Δex7/Δex7}* and *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* aged mice but not in *LepRCre⁺;Kitl^{Δex7/Δex7}* compared to WT littermate controls (respectively 75.9 ± 4.6 ($p < 0.0001^{****}$), 68.6 ± 3.8 ($p < 0.0001^{****}$) and 128.9 ± 4.2 vs. WT: 146.1 ± 4.8 pg/ml; **Figure 4.11d**). These reductions for *Tie2Cre⁺;Kitl^{Δex7/Δex7}* and *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* mice were greater than those determined in their 4-week old counterparts. The aged mice showed 48.0 % and 51.0 % reductions respectively (**Figure 4.11d**), compared to 26.6 % and 30.8 % in the young mice (**Figure 4.4**).

Based on the analysis of PB, these data appear to indicate that the combined loss of *mKitl* from both EC and LepR⁺ PVCs results in a slight increase in the anaemic profile in aged mice. Furthermore, aged mice showed a significant reduction of LSK CD48⁻CD150⁺ HSCs in the dKO model compared to WT, but this was not the case in 4-week old mice. There was also a trend in the sKO aged mice to show a decline in these cells, although not significant, notably in *LepRCre⁺;Kitl^{Δex7/Δex7}* mice. However, both the 4-week old and aged dKO mice appear to show marked effects on myeloid progenitor populations, although some differences were observed. For example, compared to their WT controls Pre CFU-Es were significantly reduced in aged mice but were unaffected in young mice. In the latter, pre GMs were significantly reduced whereas the same cells were not affected in aged mice.

4.2.5 Endothelial and perivascular cell loss of total *Kitl* (*mKitl* + *sKitl*) results in more pronounced haematopoietic defects than loss of *mKitl* alone

To more definitely investigate the differing roles of *Kitl* within haematopoiesis, *Scf*^{f1/f1} mice obtained from the Morrison lab were crossed with *Tie2Cre*⁺ and *LepRCre*⁺ mice, to generate *TieCre*⁺;*Scf*^{f1/f1}, *LepRCre*⁺;*Scf*^{f1/f1} and *TieCre*⁺;*LepRCre*⁺;*Scf*^{f1/f1} mice. As indicated above, their previously published study had used hemizygous mice, which were therefore null for 1 allele of *Scf*. Mice generated for the studies reported here were analysed at 8 weeks to be age matched to those used in the Morrison group study. The PB and the BM profiles of these crosses were determined and also compared to those obtained from the 4-week old *Cre*⁺;*Kitl*^{Δex7/Δex7} mice.

Analysis of PB demonstrated that the total loss of *Kitl* in these crosses caused similar trends across analysed parameters compared to the *Cre*⁺;*Kitl*^{Δex7/Δex7} mouse models, which had deletion only of *mKitl* in specific cell populations. In the sKO *Cre*⁺;*Scf*^{f1/f1} mice, there were no changes in PB profiles except for increased MCV in *LepRCre*⁺;*Scf*^{f1/f1} mice (66.0 ± 2.2 vs. WT 57.3 ± 1.5 fL, p<0.05*).

Compared to WT littermate controls, dKO *Tie2Cre*⁺;*LepRCre*⁺;*Scf*^{f1/f1} mice showed significant decreases in both the number of RBCs (7.7 ± 0.2 vs. 5.3 ± 0.0 10⁶/μL, p<0.005***) and the Hgb levels (12.8 ± 0.7 vs. 9.0 ± 0.1 g/dL, p<0.01**) both reduced by ~30 %, **Figure 4.12**). These reductions are greater compared to 4-week old mice with the same cell-specific losses but of *mKitl* only (**Figure 4.2**). In addition, these mice and the *LepRCre*⁺;*Scf*^{f1/f1} mice showed a significant increase in MCV. There is indication therefore of anaemic features in the dKO *Tie2Cre*⁺;*LepRCre*⁺;*Scf*^{f1/f1} mouse.

The results seen here with the *Tie2Cre⁺;Scf^{fl/fl}* mice replicate those seen with *Tie2Cre⁺;Scf^{fl/-}* mice, which are reported to have normal blood counts⁸⁹. It should be noted that the blood counts for both *LepRCre⁺;Scf^{fl/-}* and *Tie2Cre⁺;LepRCre⁺;Scf^{fl/-}* are not stated in the paper and therefore no comparison can be made to the data generated here.

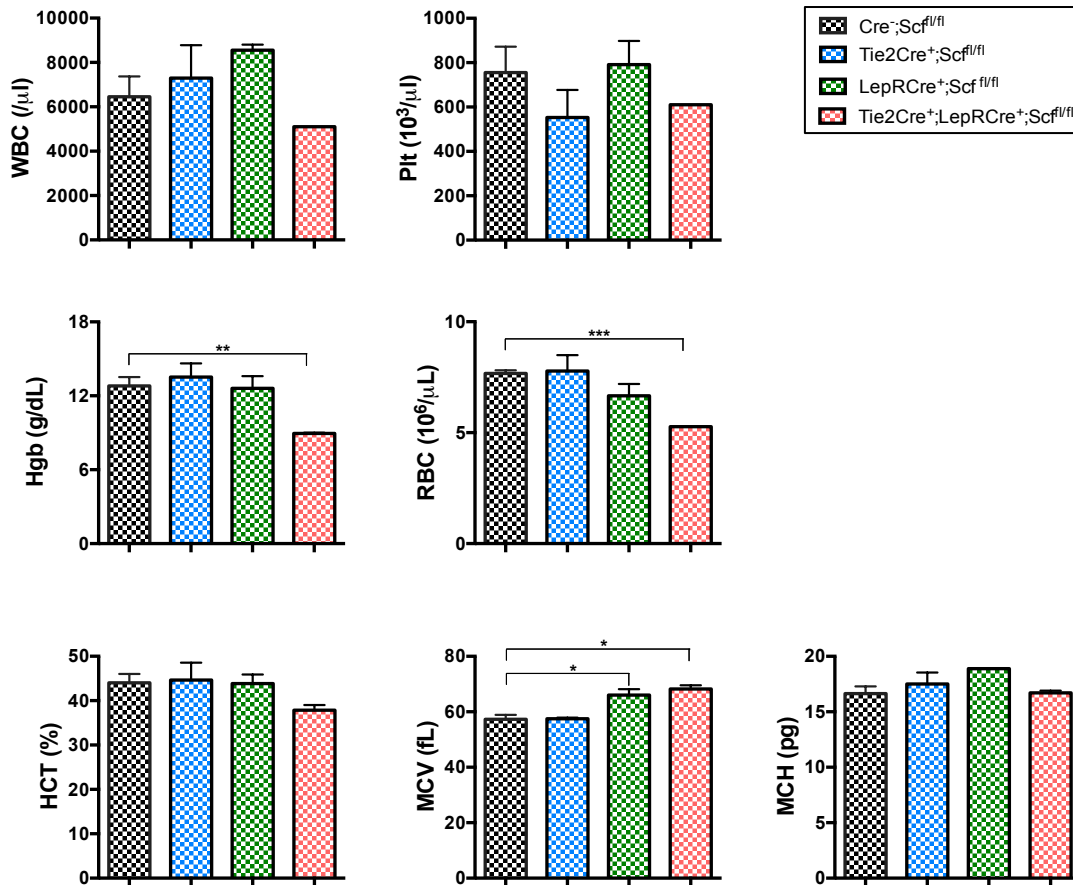


Figure 4.12 *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* mice display anaemic features

Analysis of peripheral blood counts of 8-week old *Tie2Cre⁺;Scf^{fl/fl}* (n=6) *LepRCre⁺;Scf^{fl/fl}* (n=2), *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* (n=2) and WT littermate controls (*Cre⁻;Scf^{fl/fl}*, n=6). Values shown are the mean±SEM, p<0.05*, p<0.01**, p<0.005*** with p values determined using the unpaired Student t-test). Key as for **Figure 4.2**.

To investigate the impact of total *Kitl* loss within the BM of these mice, the cellularity was determined. In the different *Cre*⁺;*Scf*^{*fl/fl*} models there was a trend for a decrease in BM cellularity (*Tie2Cre*⁺;*LepRCre*⁺;*Scf*^{*fl/fl*} MNCs were $84.0 \pm 5.0 \times 10^6$ cells compared to WT littermate controls *Cre*⁺;*Scf*^{*fl/fl*} with $114.5 \pm 7.8 \times 10^6$ cells / 2 femurs and 2 tibiae), however this decline was not significant (**Figure 4.13a**). No changes were also seen in *Cre*⁺;*Kitl* ^{Δ ex7/ Δ ex7} mice (**Figure 4.3a**). These data in *Cre*⁺;*Scf*^{*fl/fl*} KO mice are consistent with the published findings using *Cre*⁺;*Scf*^{*fl/-*} mice⁸⁹.

Flow cytometric analysis was undertaken to further determine the resultant effect of total *Kitl* loss on haematopoiesis. Contrary to what was observed in the *Cre*⁺;*Kitl* ^{Δ ex7/ Δ ex7} mice (**Figure 4.3c**), the *Tie2Cre*⁺;*Scf*^{*fl/fl*} and the *Tie2Cre*⁺;*LepRCre*⁺;*Scf*^{*fl/fl*} mice showed significant reductions in the numbers of phenotypic LSK CD48⁻CD150⁺ HSCs (respectively 5032 ± 1486 and 2697 ± 176) compared to WT (9452 ± 1061 , both $p < 0.05^*$, **Figure 4.13b**). Loss of total *Kitl* from ECs or from LepR⁺ PVCs alone was sufficient to cause a decrease in the total number of HSCs by 50 % and 30 %, respectively. This was significant for the *Tie2Cre*⁺;*Scf*^{*fl/fl*} mice as indicated, but not for the *LepRCre*⁺;*Scf*^{*fl/fl*} mice. The concomitant loss of total *Kitl* in *Tie2Cre*⁺;*LepRCre*⁺;*Scf*^{*fl/fl*} mice resulted in a greater reduction in total HSC number by 70 % compared to WT. These reductions were higher in the corresponding *Cre*⁺;*Scf*^{*fl/-*} mice reported by Morrison⁸⁹.

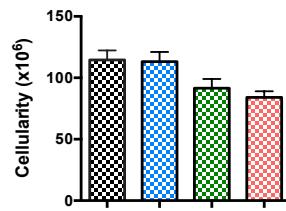
To determine if the reduction in stem cells had impacted upon progenitors further down the cell hierarchy, additional flow cytometric analysis was undertaken.

Tie2Cre⁺;*Scf*^{*fl/fl*} mice maintained normal MPC numbers. *LepRCre*⁺;*Scf*^{*fl/fl*} mice exhibited marked reductions in the numbers of MkPs ($18.1 \pm 2.7 \times 10^3$ vs. WT $30.6 \pm 4.4 \times 10^3$), Pre CFU-Es ($24.3 \pm 1.5 \times 10^3$ vs. WT $44.6 \pm 6.8 \times 10^3$) and Pro Erys (45.6

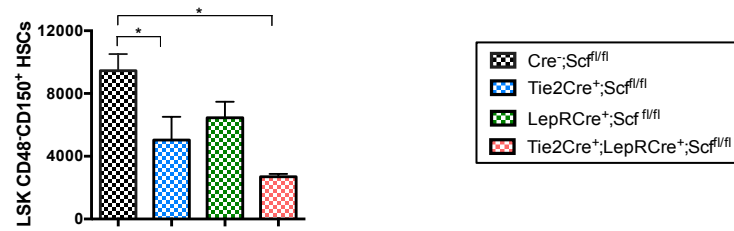
$\pm 18.7 \times 10^3$ vs. WT $126.5 \pm 16.8 \times 10^3$, only the last of which is statistically significant (decreases of 41.1 %, 45.6 % and 63.9 % ($p < 0.05^*$), respectively). It was found that most MPC populations were markedly reduced in the dKO *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* mice, with the exception of Pre GM (**Figure 4.13c**), which is in contrast to results in *Tie2Cre⁺;LepRCre⁺;Kitl ^{$\Delta_{ex7/\Delta_{ex7}}$}* mice, in which Pre GM were significantly reduced and there was no significant change in Pre MegE or Pre CFU-Es (**Figure 4.3d**). The most marked reduction in the *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* mice was in their numbers of erythroid progenitors subsets (Pre CFU-Es, $6.9 \pm 1.2 \times 10^3$ vs. WT $44.6 \pm 6.8 \times 10^3$, $p < 0.01^{**}$; CFU-Es, $7.7 \pm 2.9 \times 10^3$ vs. WT $32.0 \pm 4.9 \times 10^3$, $p < 0.05^*$; Ery Pros, $6.9 \pm 0.2 \times 10^3$ vs. WT $126.5 \pm 16.8 \times 10^3$, $p < 0.01^{**}$). These populations were respectively 24 %, 15 % and 5 % of WT numbers. These data further confirm that the expression of *Kitl* by both ECs and LepR⁺ PVCs together is important for MPC proliferation.

Systemic levels of sKitl in *Tie2Cre⁺;Scf^{fl/fl}* and *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* mice were significantly lower, 87.2 ± 15.3 and 86.6 ± 9.4 pg/ml compared to WT 140.1 ± 19.2 pg/ml, with reductions of 41.0 % and 38 % respectively (both $p < 0.05^*$, **Figure 4.13d**). However, *LepRCre⁺;Scf^{fl/fl}* mice maintained similar sKitl levels to WT mice (123.7 ± 7.2 vs. 140.1 ± 19.2 pg/ml, respectively). These percentage reductions in sKitl levels between WT and both *Tie2Cre⁺;Scf^{fl/fl}* and *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* mice were greater than those seen in the corresponding *Cre⁺;Kitl ^{$\Delta_{ex7/\Delta_{ex7}}$}* mice (**Figure 4.4**), which may reflect the additional deletion of sKitl from the targeted stromal cells.

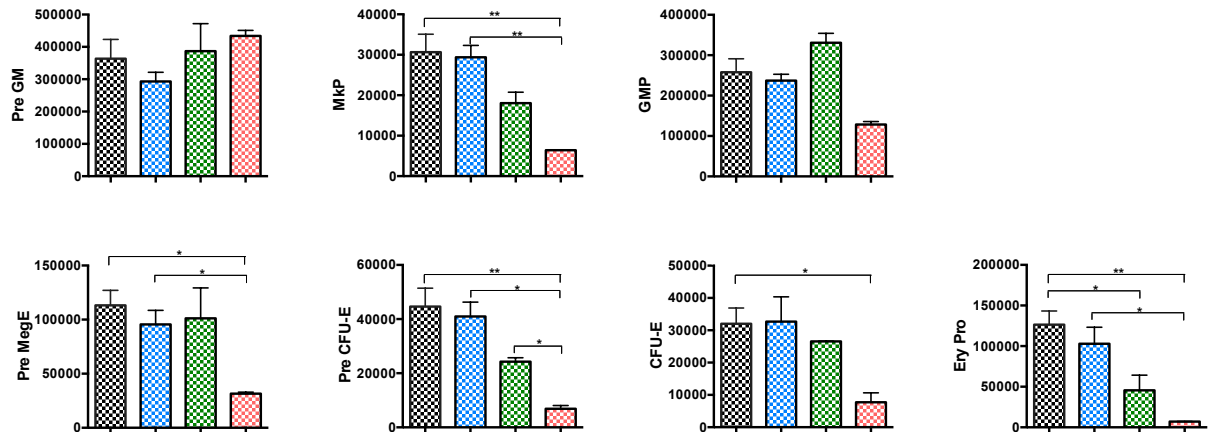
a) BM cellularity



b) HSC numbers



c) Myeloid progenitors



d) Serum sKitl

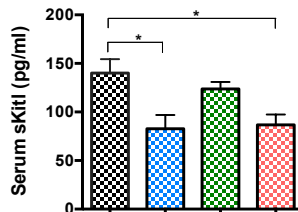


Figure 4.13 Endothelial and LepR⁺ PVC loss of total *Kitl* results in impaired haematopoiesis and reduced systemic levels of *Kitl*

Analysis of 8-week old *Tie2Cre⁺;Scf^{fl/fl}* (n=6) *LepRCre⁺;Scf^{fl/fl}* (n=2), *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* (n=2) and WT littermate controls (*Cre⁻;Scf^{fl/fl}*, n=6). Values shown are the mean±SEM with p values determined using the unpaired Student t-test):

- BM cellularity from 2 femurs and 2 tibiae.
- Absolute number of phenotypic LSK CD48⁺CD150⁺ HSCs from 2 femur and 2 tibiae (p<0.05*).
- Absolute numbers of MPCs per 2 femurs and 2 tibiae (p<0.05*, p<0.01**). Cell populations are as defined as in **Figure 3.11**.
- Serum sKitl levels detected by ELISA (p<0.05*).

4.3 Discussion

The primary aim of the work reported in this chapter was to further discern the roles of mKitl and sKitl by investigating the loss of *mKitl* from specific stromal cell populations in homozygous models as opposed to the cell-specific deletion of total *Kitl* (*mKitl* + *sKitl*) in hemizygous models previously reported⁸⁹. Murine models were developed and used to assess the impact of cell-specific loss of *mKitl* on i) systemic sKitl levels, ii) the spatial relationship between HSPCs and Kitl⁺ stromal cells in the BM, iii) overall MNC, HSC and MPC numbers and iv) PB parameters. Additionally, to better understand the roles of mKitl and sKitl, the data obtained from these models was then compared to those obtained in corresponding cell-specific homozygous models, in which total *Kitl* was deleted.

Initially, the expression levels of total *Kitl* and *mKitl* in different stromal cells were evaluated and ECs were confirmed to highly express both total *Kitl* and the membrane-bound isoform *mKitl*; data which correlate with previous findings, showing that *Kitl* was highly expressed in ECs^{89,172}. This led me to investigate the impact of *mKitl* loss and total *Kitl* loss from ECs and also from the recently identified PVCs expressing the leptin receptor; studies on the latter cells following on from the work of the Morrison group⁸⁹.

Individual KO of *mKitl* in one or other cell population had little impact on HSC proliferation or maintenance. It is clear, that despite the minimal HSC phenotype seen in the *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice, there is efficient deletion (~99 %) of *mKitl* in addition to a reduction in the level of circulating systemic sKitl. The incomplete deletion of *mKitl* from the *LepRCre⁺* model (~20-50 %) makes it difficult to draw firm conclusions about the full extent of mKitl's role when expressed by LepR⁺ PVCs. As

indicated above, this low deletion efficiency was unexpected since the Morrison paper had used the identical *LepRCre* model. However, it seems that in these homozygous models, deletion is distinctly less than complete and this may in part explain their use of a hemizygous model to demonstrate sufficiently marked haematopoietic effects.

In the sKO models of total *Kitl* deletion, there were some effects on HSCs, which were significantly reduced in the case of *Tie2Cre⁺;Scf^{fl/fl}* mice. However, dKO of either *mKitl* or total *Kitl* in the two stromal cell populations (ECs and LepR⁺ PVCs) led to more marked and consistent haematopoietic defects. These included a combination of anaemic features in the blood profile and impact on myeloid progenitors. In the case of the *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* mice, with deletion of total *Kitl* from both cell types, there was a significant reduction in the absolute number of HSCs, which is not seen in the corresponding *mKitl* dKO *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* mouse. The fall seen in HSCs in the *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* mice is markedly greater than in the sKO *Tie2Cre⁺;Scf^{fl/fl}* mice.

Thus, similarly to the work of the Morrison group, loss of total *Kitl* from both cell types together caused the greatest reduction in HSCs, however the losses seen in their *Cre⁺;Scf^{fl/-}* mice were greater than evident in the corresponding *Cre⁺;Scf^{fl/fl}* mice investigated here. One possible explanation might be the use of hemizygous models in their study, which are null for 1 allele of *Scf* and therefore have a 50 % tissue-wide reduction in *Kitl* expression, in addition to the stromal cell-specific losses of total *Kitl*. The greater reported deletion in the Morrison model may also be a contributing factor, however deletion efficiency is only shown for one of their models, the

LepRCre model and is calculated based on the percentage of CD45⁺/Ter119⁻ *LepRCre*;YFP⁺ cells which express PDGFR α ⁸⁹.

The loss of expression of *mKitl* by both ECs and LepR⁺ PVCs together in *Tie2Cre⁺;LepRCre⁺;Kitl ^{Δ ex7/ Δ ex7}* mice has an impact on haematopoiesis, resulting in reductions in RBCs, Hgb and certain myeloid progenitors. However, KO of total *Kitl* in both cell populations in *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* mice has more pronounced haematopoietic effects, notably the decline in HSCs, which may indicate not only a role for localised effects of the membrane-bound but also the soluble ligand, which in these KO mice may only be present at reduced levels in the niche.

In both dKO models and the sKO *Tie2Cre⁺* models, there is also a reduction in systemic sKitl, which appears to arise from the deletion of total *Kitl* or *mKitl* in ECs, but not from PVCs in the *LepRCre⁺* models (possibly resulting from incomplete deletion of *mKitl* or total *Kitl* in these models). The *Tie2Cre⁺;Kitl ^{Δ ex7/ Δ ex7}* mouse, which has loss of *mKitl* from ECs, demonstrates no effects on haematopoiesis, whereas the *Tie2Cre⁺;Scf^{fl/fl}* mouse, which has loss of total *Kitl* from ECs, appears to have significantly reduced HSCs. This might be taken as a further indication in the latter of the additional importance of sKitl, given the added deletion of *sKitl* from ECs and the further reduced systemic levels of sKitl observed in *Tie2Cre⁺;Scf^{fl/fl}* mice compared to the *Tie2Cre⁺;Kitl ^{Δ ex7/ Δ ex7}* model. Therefore, in addition to localised levels of sKitl and mKitl in the BM niche, there is the possibility that changes in the circulating levels of sKitl (which can decrease following the loss of *mKitl* or total *Kitl*) also have an impact upon haematopoiesis. This could indicate that whilst EC-expression of *mKitl* is important to help maintain the systemic level of sKitl, EC-expression of total *Kitl* (both *mKitl* and *sKitl*) may have a combined role in HSC maintenance.

In the $Cre^+;Kitl^{\Delta ex7/\Delta ex7}$ models, which have deletion of *mKitl*, there was a significant disruption of the association between HSCs and ECs upon investigation of the localisation of these cells within the BM using IHC. This correlates with previous findings, which have highlighted the role of Kitl in anchoring via cell-cell contact during haematopoiesis^{172,278}. However the apparent reduction in the number of HSCs associated with ECs in BM sections from $LepRCre^+;Kitl^{\Delta ex7/\Delta ex7}$ mice was unexpected. This would not have been anticipated given that *mKitl* expression in ECs in these mice should be unaffected. I hypothesise that this is the result of delocalisation of HSCs from LepR⁺ PVCs, which may be in very close association with ECs and therefore give the appearance of altered positioning relative to ECs. Work is ongoing to develop a method to simultaneously stain BM sections with Abs against LepR, and EC and HSC markers. Once these data are obtained, it may be possible to confirm whether HSCs are also found anchored or in close proximity to LepR⁺ PVCs.

In the $Tie2Cre^+;Kitl^{\Delta ex7/\Delta ex7}$ mice there is no change in the HSC cell cycle status, whilst in $LepRCre^+;Kitl^{\Delta ex7/\Delta ex7}$ mice there is a significant reduction in the frequency of HSCs in G₀ and a resultant increase in G₁, which is comparable to that seen in the dKO mice. However, unlike in the dKO, this appears not to translate into any haematopoietic effects either in blood parameters or MPCs. This effect in $LepRCre^+;Kitl^{\Delta ex7/\Delta ex7}$ mice is marked given that *mKitl* has only been deleted ~20-50 % from LepR⁺ cells. This may suggest that in these mice, the *mKitl* in the ECs and other stromal cells and the systemic levels of sKitl, which are maintained at WT levels, compensate and help to maintain normal HSC numbers.

Analysis of young (4-week old) and aged (12-13 months) mice simultaneously lacking *mKitl* from both ECs and LepR⁺ PVCs revealed haematopoietic defects. The

mice had significant reductions in their RBCs and Hgb levels, indicative of anaemia, which was slightly more pronounced in aged mice, in addition to changes in HCT, MCV and MCH. These older dKO mice also showed a significant reduction of LSK CD48⁻ CD150⁺ HSCs compared to WT, but this was not the case in young mice. There was also a trend in the sKO aged mice to show a decline in HSCs, although not significant, notably in *LepRCre⁺;Kitl^{Δex7/Δex7}* mice. However, both the young and aged dKO mice appear to show marked effects on myeloid progenitors, although some differences in the cell populations affected were evident (Pre GMs and Pre CFU-Es).

As indicated, the phenotype seen in older mice was more prominent for certain parameters, notably significantly fewer HSCs in the dKO, which may be indicative of a changing requirement over time for Kitl by HSCs. It has been shown previously that in viable *c-Kit* null 'Vickid' mutants, there is a progressive loss of B and T cells with age²⁸², indicating Kitl/c-Kit signalling may be differentially important for haematopoiesis over time. However, the normal changes in haematopoiesis that occur during ageing, such as the increase in the number of HSCs, which are preferentially biased towards myeloid differentiation^{139,142} needs to be taken into consideration when evaluating the differences between WT and dKO aged mice. It should be noted therefore that dKO mice with reduced *mKitl* expression in ECs & LepR⁺ PVCs, exhibited reduced systemic levels of sKitl, 69 pg/ml at 12-13 months vs. 146 pg/ml in their 4-week old counterparts, but still possessed the capacity to increase HSC numbers from ~3,000 (young) to ~11,000 cells (aged) / femur and tibia.

Overall these findings indicate that the combined presence of mKitl on ECs and LepR⁺ PVCs, localised sKitl and potentially systemic sKitl are important for maintaining haematopoiesis within the adult BM HSC niche. However, it remains to be determined if and how Kitl and specifically mKitl act locally within the BM niche. A limitation of the mouse models investigated here is that they delete *mKitl* or total *Kitl* from ECs and LepR⁺ PVCs not only in the BM, but also potentially in other tissues. One would assume that the majority of the effects seen arise from loss of these cytokine isoforms from BM stromal cells, but a contribution to the phenotypes seen by deletion from these cells in other tissues cannot be completely ruled out. Accordingly, the localised role of mKitl and total Kitl were further assessed through explant studies and by investigating the spermatogonial niche (**Chapter 5**).

5 Investigating the Localised Role of Kitl Within the Niche

5.1 Introduction

As indicated above, in the murine models developed and evaluated (**Chapters 3 and 4**), a range of impacts on haematopoiesis were seen following deletion of either *mKitl* or total *Kitl*, which was also associated in certain models, with a fall in the levels of systemic sKitl. However, a potential limitation of these models is that the cells targeted for deletion of *mKitl* or total *Kitl*, are not only present in the BM but also in a range of other tissues. These factors make interpretation of the respective roles of mKitl and sKitl difficult to discern and therefore do not permit determination of the specific localised role of this cytokine within the BM niche. To try and address these factors, kidney subcapsular explantation (KSE) with embryonic femurs from murine KO models, which had deletion of *mKitl* or total *Kitl*, were conducted in WT C57BL/6 mice. Additionally, to further dissect out the localised role of mKitl and sKitl, the spermatogonial niche was investigated in *AmhCre⁺;Kitl^{Δex7/Δex7}* mice.

To understand niche biology, ectopic transplantation to the subcapsular region of the kidney has been used to investigate the differentiation, development and function of a variety of tissues and has successfully been used to explant pancreatic cells²⁸³, thymii²⁸⁴, gonads²⁸⁵ and bone²⁸⁶. The kidney is used as it is highly vascularised and its capsule can physically accommodate different tissues with the capability of expanding as the tissue grows²⁸⁷.

In a haematological context, KSE can be used to investigate the BM niche in a localised setting *in vivo*. By explanting femurs which are deleted of *mKitl* or total *Kitl*

into a WT system, it was deemed possible to assess the impact of their local loss solely on the BM. Transplantation of BM cells or whole bones under the kidney capsule provides a method to determine if multipotent HSCs can engraft and be maintained by this tissue²⁸⁸. Murine E15.5 bones are small enough to fit under the kidney capsule, permitting the intact BM microenvironment to be explanted. In addition, at E15.5 of gestation, bones contain stromal cells but lack HSCs, as stem cells are known to migrate to the BM around E17¹⁶. Consequently any HSCs detected inside these explanted foetal bones will arise from those which initially migrated there from the systemic circulation of the WT recipient.

Understanding of the BM niche has also been informed by investigations of other niches such as those in the gut and gonads^{10,289}. The spermatogonial niche has been previously studied and has provided additional insight into the processes and mechanisms occurring in niches. Stem cells within the spermatogonial niche are known as spermatogonial stem cells (SSCs) and their downstream progenitors are called spermatogonial progenitor cells (SPCs). Sustained spermatogenesis in adult males relies on the activity of SSCs and SPCs. The number of SSCs and SPCs is strictly controlled and their functions regulated by niche cells within the testis such as Leydig cells and Sertoli cells²⁹⁰.

The importance of Kitl/c-Kit signalling has been demonstrated within the spermatogonial niche²⁹¹. Sertoli cells located in the seminiferous tubules of the testes express Kitl²⁹² and are found in close contact with c-Kit⁺ SPCs²⁹³. This interaction is known to be critical for germ cell survival and proliferation, with homozygote *W* and *Sl* mice being infertile^{241,248}. The requirement of the interaction between the ligand expressed on Sertoli cells and the receptor on germ cells is compatible with a

potential specific requirement for mKitl, but this has not been previously investigated. Given the defined cellular composition and expression of Kitl and c-Kit within the spermatogonial niche, studies to specifically delete *mKitl* in Sertoli cells may provide further 'proof of principle' of the importance of the membrane-bound isoform in niche biology and potentially offer additional insights into its localised role within the BM niche.

5.2 Results

5.2.1 Localised BM loss of EC-*mKitl* expression in *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice does not impede HSC homing or maintenance in femur explants *in vivo*

In an attempt to better define the role of mKitl local to the BM niche, relative to the potential impact of circulating sKitl levels on HSC maintenance, two of the previously investigated mouse models, (*Tie2Cre⁺;Kitl^{Δex7/Δex7}* and *Tie2Cre⁺;Scf^{fl/fl}* mice; **Chapter 4**) with differing impacts upon haematopoiesis, were investigated using KSE. Both models selected had demonstrated significant reductions in the level of systemic sKitl in previous studies (**Chapter 4**), which had made it difficult to clearly determine the extent to which *mKitl* was responsible for the phenotypes seen.

Accordingly, through experiments explanting embryonic bones from the above mouse models with a defective BM microenvironment into a WT system (**Figure 5.1**), I sought to investigate how this would impact haematopoiesis, by determining if HSCs were able to home to these explanted BM niches, self-renew and differentiate.

For each of the two *Cre⁺* models indicated above, timed matings were set up between *Cre⁺* stud males and *Cre⁻* females. Following successful plugging, pregnant

females were sacrificed and the embryos taken at E15.5. From each embryo, both femurs were dissected and then explanted under the kidney capsule of 8-12 week old WT C57BL/6 recipients (**Section 2.4.1**). The genotypes of the embryos used for explantation were confirmed by PCR. By transplanting the embryonic bones under the kidney of WT mice, the bones should have received normal levels of circulating sKitl. After 3 weeks, the recipient mice were sacrificed and the explanted bones removed and analysed. The experimental design was similar to that reported by Chan *et al.*²⁸⁸.

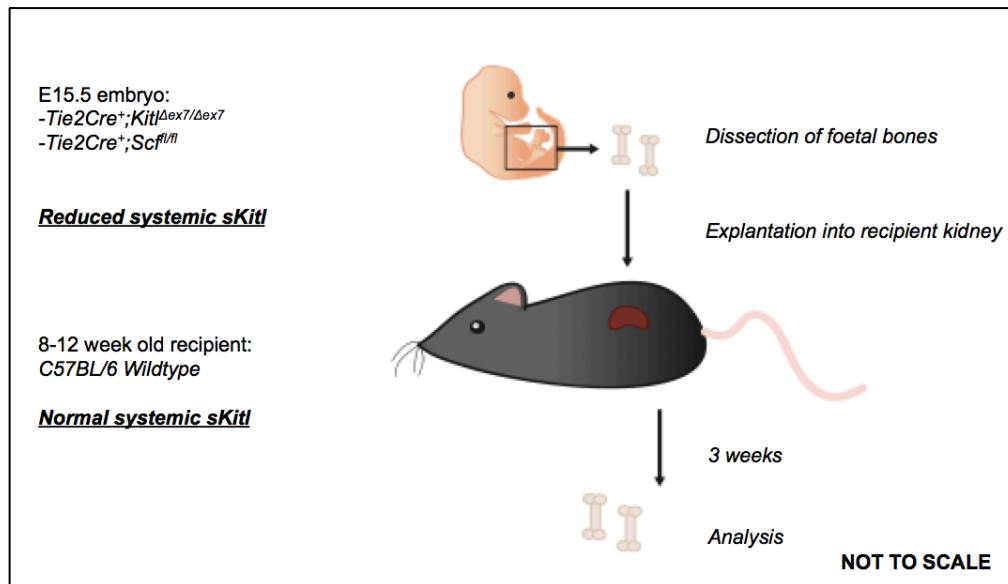


Figure 5.1 Experimental design of kidney subcapsular explantation of foetal bones

E15.5 femurs from *Cre⁺* or *Cre⁻* littermate controls were dissected and explanted under the left kidney capsule of WT C57BL/6 recipient mice for 3-4 weeks before analysis.

To validate this technique as a method to investigate the localised role of mKitl, *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice were chosen as the initial model to be evaluated. Femurs from E15.5 *Tie2Cre⁺;Kitl^{Δex7/Δex7}* and *Tie2Cre⁻;Kitl^{Δex7/Δex7}* littermate control embryos were explanted under the kidney capsule of WT C57BL/6 recipients. These donor *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice have a high deletion efficiency of *mKitl* from ECs but are capable of maintaining HSC numbers despite a reduction in systemic sKitl *in vivo*

(Chapter 4). Therefore the explanted BM from these *Tie2Cre⁺;Kitl^{Δex7/Δex7}* embryos should exhibit unaffected cellularity or impact upon HSC or MPC numbers in the presence of circulating sKitl from the recipient WT C57BL/6 mice.

Explanted embryonic bones were dissected out from the WT recipient kidneys for analysis 3 weeks later. Investigation of the explanted *Tie2Cre⁺;Kitl^{Δex7/Δex7}* bones revealed that consistent with previous *in vivo* experiments in the whole animal (4-week old *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice, **Section 4.2.2**), the loss of *mKitl* from ECs did not impact haematopoiesis (**Figure 5.2**). Total BM cellularity in the explanted femurs was almost identical between *Tie2Cre⁻;Kitl^{Δex7/Δex7}* (WT littermate controls) and *Tie2Cre⁺;Kitl^{Δex7/Δex7}* femurs ($14.2 \pm 1.4 \times 10^6$ vs. $14.4 \pm 2.4 \times 10^6$ cells/ 2 femurs respectively, **Figure 5.2a**). Furthermore, flow cytometric analysis showed that HSC numbers were maintained in *Tie2Cre⁺;Kitl^{Δex7/Δex7}* explanted bones (360 ± 64 vs. 361 ± 60 cells in WT littermate controls, **Figure 5.2b**). Similarly, there were no significant changes in the MPC numbers (**Figure 5.3c**).

To confirm that the explanted bones were receiving normal levels of systemic sKitl, PB was taken from the WT recipients to isolate the serum, which was then analysed by ELISA (**Figure 5.2d**). Recipients who had received either *Tie2Cre⁻;Kitl^{Δex7/Δex7}* or *Tie2Cre⁺;Kitl^{Δex7/Δex7}* femurs had very similar levels of systemic sKitl (117.7 ± 25.2 vs. 122.6 ± 26.4 pg/ml, respectively).

Despite the high deletion efficiency of *mKitl* in the ECs of *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice (**Chapter 4**), there was no difference seen in the analysis of *Tie2Cre⁺;Kitl^{Δex7/Δex7}* and *Tie2Cre⁻;Kitl^{Δex7/Δex7}* foetal bones. In the absence of *mKitl* expression in BM ECs of *Tie2Cre⁺;Kitl^{Δex7/Δex7}* explanted femurs, homing to the BM by HSCs readily appears to

still take place. In addition, this BM microenvironment is able to maintain comparable HSC numbers to WT littermate controls, in the presence of essentially equivalent systemic sKitl levels.

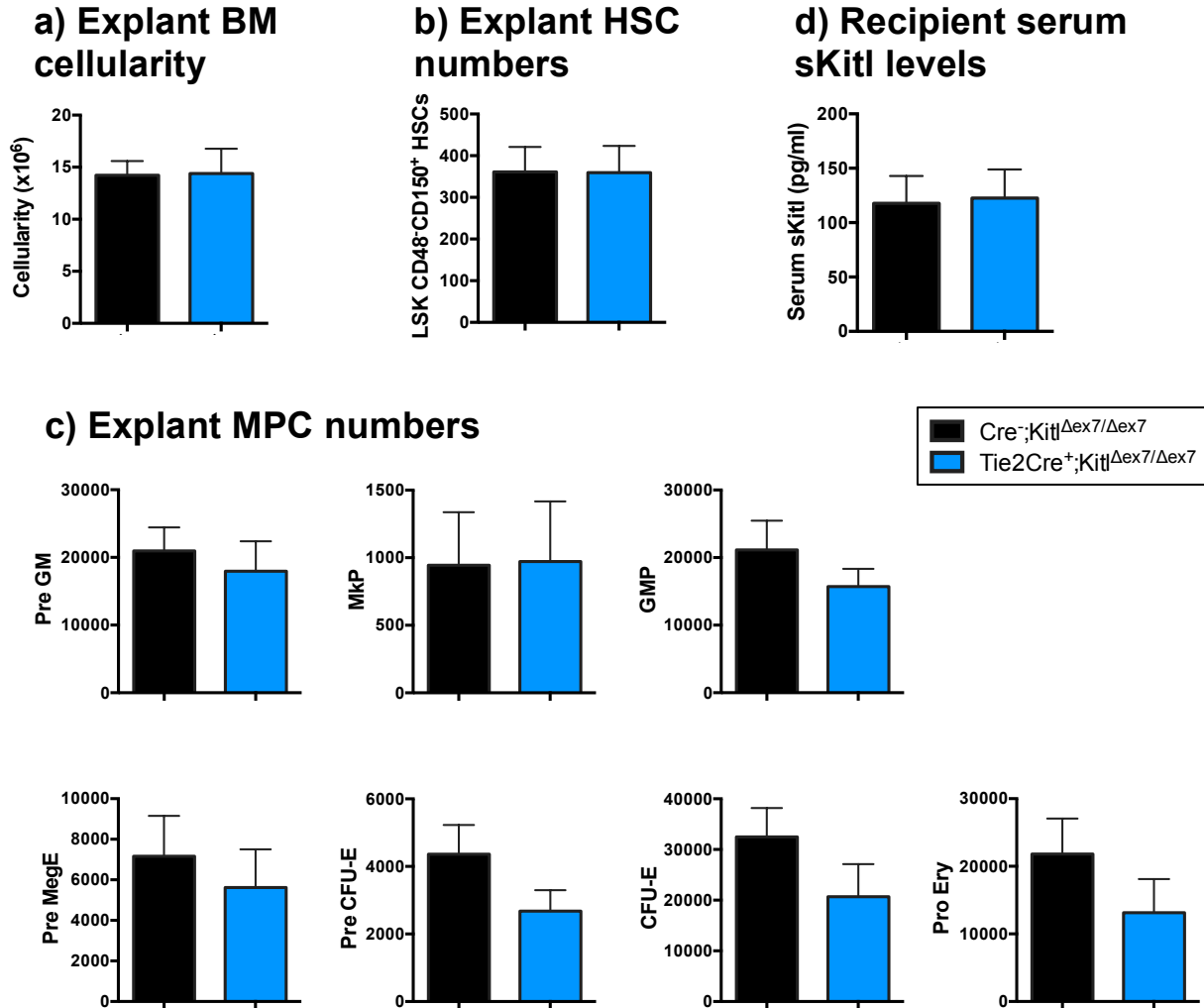


Figure 5.2 Localised loss of *mKitl* from BM ECs of *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice does not alter haematopoiesis in femur explants

Analysis of *Tie2Cre⁺;Kitl^{Δex7/Δex7}* or *Tie2Cre⁻;Kitl^{Δex7/Δex7}* littermate control femurs 3 weeks post-KSE into WT C57BL/6 recipients (n=5-8 mice per genotype, values shown are the mean±SEM, statistical analysis undertaken using the unpaired Student t-test):

- Total MNCs per two transplanted femurs per recipient.
- Total number of phenotypic LSK CD48⁻CD150⁺ HSCs per two transplanted femurs per recipient.
- Total number of MPCs per two transplanted femurs per recipient. Cell populations are as defined as in **Figure 3.11**.
- Serum sKitl levels determined by ELISA from recipient WT C57BL/6 mice transplanted with E15.5 *Tie2Cre⁺;Kitl^{Δex7/Δex7}* or *Tie2Cre⁻;Kitl^{Δex7/Δex7}* littermate control femurs.

5.2.2 Systemic sKitl compensates for the localised loss of total *Kitl* (*mKitl* + *sKitl*) from ECs in *Tie2Cre⁺;Scf^{fl/fl}* femur explants *in vivo*

I next wanted to investigate if loss of total *Kitl* (*mKitl* + *sKitl*) from ECs would affect haematopoiesis in the presence of maintained systemic sKitl levels. *In vivo*, adult *Tie2Cre⁺;Scf^{fl/fl}* mice had previously been shown to have significantly fewer HSCs and also a reduction in levels of the soluble, circulating ligand (**Section 4.2.5**). Timed matings were set up using *Tie2Cre⁺;Scf^{fl/fl}* males and *Tie2Cre⁻;Scf^{fl/fl}* females. Embryos were taken at E15.5, femurs dissected and then used for KSE to explant into C57BL/6 WT recipients. The genotypes of the embryos used for explantation were confirmed by PCR.

Three weeks after KSE surgery, *Tie2Cre⁺;Scf^{fl/fl}* and WT littermate *Tie2Cre⁻;Scf^{fl/fl}* foetal bones were dissected from the kidneys of the WT recipients. The BM of these femurs was determined for total cellularity. Consistent with the findings seen in adult *Tie2Cre⁺;Scf^{fl/fl}* mice *in vivo*, the cellularity of explanted embryonic femurs was similar between *Tie2Cre⁺;Scf^{fl/fl}* and *Tie2Cre⁻;Scf^{fl/fl}*. In the explanted femurs, the total MNCs averaged $20.5 \pm 4.4 \times 10^6$ and $22.4 \pm 2.9 \times 10^6$ cells, respectively (**Figure 5.3a**). Furthermore, flow cytometric analysis indicated that HSC numbers were comparable in *Tie2Cre⁺;Scf^{fl/fl}* and *Tie2Cre⁻;Scf^{fl/fl}* (416 ± 98 vs. 420 ± 119 cells, respectively; **Figure 5.3b**).

Serum isolated from the WT C57BL/6 recipient mice, which had received explanted *Tie2Cre⁺;Scf^{fl/fl}* and *Tie2Cre⁻;Scf^{fl/fl}* femurs, revealed the presence of comparable levels of sKitl in their circulation (**Figure 5.3c**). This confirmed that the recipients of both *Tie2Cre⁻;Scf^{fl/fl}* and *Tie2Cre⁺;Scf^{fl/fl}* KSE femurs had comparable levels of sKitl in their circulation (173.4 ± 9.9 vs. 166.5 ± 10.3 pg/ml, respectively).

These data would appear to suggest that in the absence of mKitl and sKitl (owing to loss of total *Kitl*) from ECs in the BM of *Tie2Cre⁺;Scf^{fl/fl}* explanted femurs, normal haematopoiesis can be achieved; with HSCs homing to and being maintained in this niche, potentially by circulating sKitl levels provided by the WT recipient. It should be emphasised that in adult *Tie2Cre⁺;Scf^{fl/fl}* mice (**Section 4.2.5**), despite the expression of *Kitl* in other BM niche stromal cells, this does not appear to compensate for the reduced levels of circulating sKitl as HSC defects are not prevented in this model.

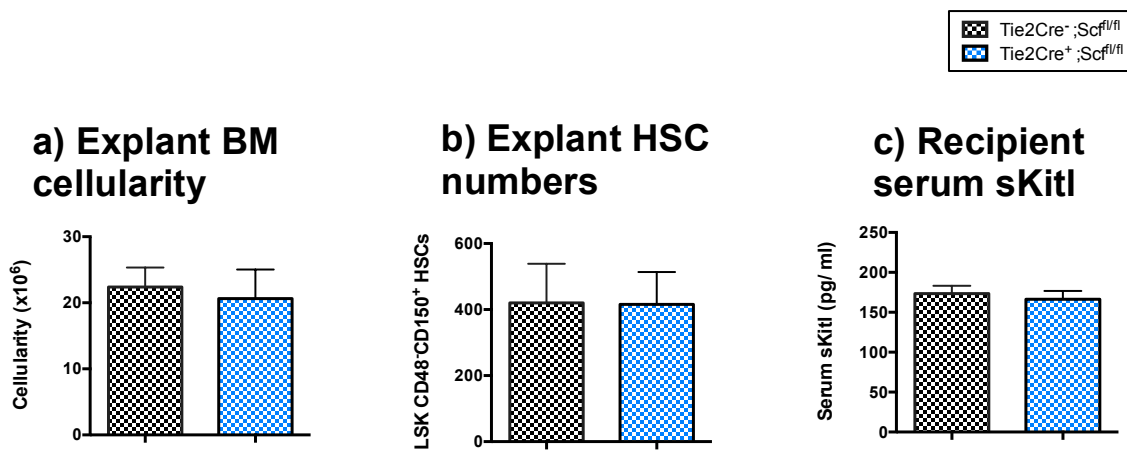


Figure 5.3 Localised loss of total *Kitl* from BM ECs of *Tie2Cre⁺;Scf^{fl/fl}* explanted femurs does not impact haematopoiesis in the presence of systemic sKitl

Analysis of *Tie2Cre⁺;Scf^{fl/fl}* or *Tie2Cre⁻;Scf^{fl/fl}* littermate control femurs 4 weeks post-KSE into WT C57BL/6 recipients (n=4-5 mice per genotype, values shown are the mean±SEM, statistical analysis undertaken using the unpaired Student t-test):

- Total MNCs per two transplanted femurs per recipient.
- Total number of phenotypic LSK CD48⁻CD150⁺ HSCs per two transplanted femurs per recipient.
- Serum sKitl levels determined by ELISA from recipient WT C57BL/6 mice transplanted with E15.5 *Tie2Cre⁺;Scf^{fl/fl}* or *Tie2Cre⁻;Scf^{fl/fl}* littermate control femurs.

5.2.3 Loss of *mKitl* in *AmhCre⁺;Kitl^{Δex7/Δex7}* mice leads to impaired spermatogenesis within the spermatogonial niche and is a localised effect

To further understand the role *Kitl*/c-Kit signalling in the broader context of niche biology in order to gain potential additional insight into what occurs within the BM, the spermatogonial niche was also investigated. This niche has to a degree been characterised and *Kitl*/c-Kit signalling is known to be important²⁹¹. Sertoli cells, which express *Kitl*, co-express and secrete anti-Müllerian hormone (AMH) and therefore a Sertoli-cell specific *Cre* mouse model was required.

AmhCre⁺ mice were crossed with *Kitl^{Δex7fl/Δex7fl}* mice over 2 generations to give *AmhCre⁺;Kitl^{Δex7/Δex7}* mice, which lacked expression of *mKitl* in Sertoli cells. Genotypes were confirmed by PCR. Male mice were taken at 4 weeks of age and the testes removed for analysis. Initial observations indicated that the testes of *AmhCre⁺;Kitl^{Δex7/Δex7}* mice were much smaller than their male *AmhCre⁻;Kitl^{Δex7/Δex7}* WT littermate controls (**Figure 5.4a**) and weighed significantly less (34.7 ± 4.1 vs. 78.5 ± 6.6 mg, respectively, $p < 0.0001^{****}$; **Figure 5.4b**).

To investigate the impact of Sertoli cell-specific loss of *mKitl* on SSCs and SPCs, both IHC and flow cytometric analysis was undertaken. I prepared testes for sectioning (**Section 2.5.1.2**), which were then stained (S.M.) with antibodies against GDNF family receptor alpha-1 (*GFRα1*) expressed on SSCs and c-Kit expressed on SPCs (**Figure 5.4c**). Sections from *AmhCre⁺;Kitl^{Δex7/Δex7}* mice revealed an almost complete loss of c-Kit⁺ SPCs in seminiferous tubules, with a 96 % reduction compared to *AmhCre⁻;Kitl^{Δex7/Δex7}* WT littermate controls (1.0 ± 0.6 vs. WT: 53.0 ± 21.0 cells, $p < 0.001^{***}$; **Figure 5.4e, right**). This loss of SPCs was concurrent with an

increase in the number of GFR α 1⁺ SSCs (5.0 ± 2.4 vs. WT: 2.2 ± 1.0 , $p < 0.01^{**}$; **Figure 5.4e, left**), which were on average 56 % more abundant in these mice.

Investigation of SSCs and SPCs by flow cytometry demonstrated similar trends (S.M., **Figure 5.4d and f**). SPCs, (defined as CD45⁻ CD51⁻ CD9⁺ Thy-1^{low} c-Kit⁺)²⁹⁴ were significantly reduced in KO testes (8900 ± 3676 vs. WT: 26000 ± 2646 cells, $p < 0.05^*$; **Figure 5.4f, right**), with the total number per testis only 34 % of *AmhCre*⁻; *Kitl* ^{$\Delta_{ex7}/\Delta_{ex7}$} littermate controls. These mice also had an average increase of 24 % in the number of CD45⁻ CD51⁻ CD9⁺ Thy-1^{low} GFR α 1⁺ SSCs²⁹⁴ (25000 ± 12662 vs. WT: 17333 ± 6936 cells, **Figure 5.4f, left**), although this change was not statistically significant. These findings indicate that the loss of *mKitl* from Sertoli cells appears to cause a block in the differentiation to SPCs, resulting in an expansion of SSCs. This first step in the differentiation of SSCs into SPCs is however claimed to be a Kitl/c-Kit independent process²⁹⁵. Overall these data suggest that expression of *mKitl* by Sertoli cells is required for spermatogenesis *in vivo*.

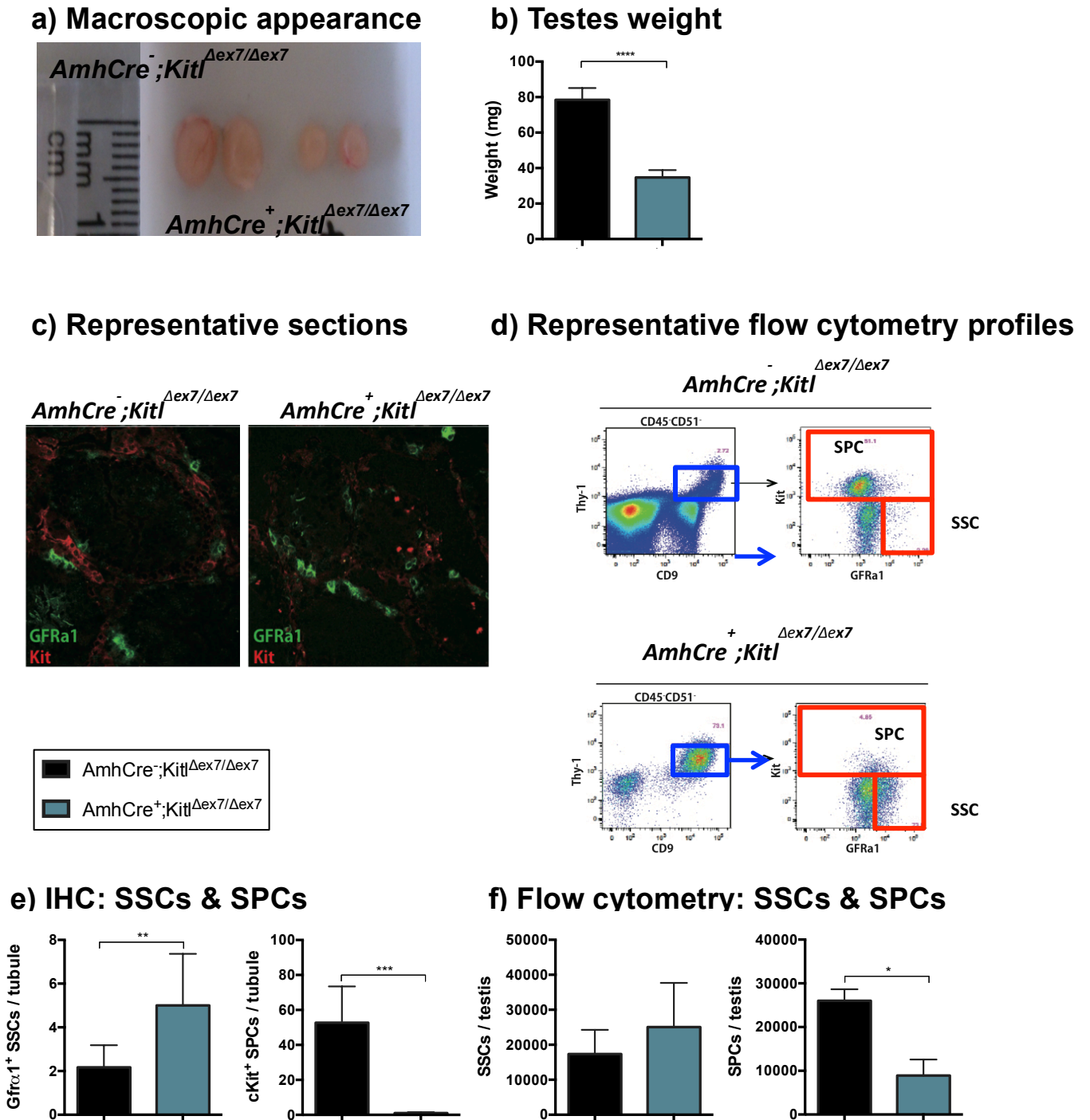


Figure 5.4 Sertoli cell expression of *mKitl* in *AmhCre*^{+/+};*Kitl*^{Δex7/Δex7} mice is critical for maintenance of c-Kit⁺ germ cell precursors (SPCs) in testes

Analysis of 4-week old *AmhCre*^{+/+};*Kitl*^{Δex7/Δex7} and *AmhCre*^{-/-};*Kitl*^{Δex7/Δex7} littermate control testes (values shown are the mean±SEM, p values determined using the unpaired Student t-test):

- The macroscopic appearance of testes. Representative of each genotype.
- Weight of testis (n=8 and 12 mice per genotype, p<0.0001***).
- Immunostaining with Abs against GFRα1 (green) and c-Kit (red). Representative results of 12-15 sections from 3 mice of each genotype.
- Representative flow cytometry profiles for SSCs and SPCs.
- c-Kit⁺ SPC (left) and GFRα1⁺ SSC (right) numbers per seminiferous tubule. At least 50 round cross

sections of seminiferous tubules were counted per genotype ($p < 0.01^{**}$, $p < 0.001^{***}$).

- f. Absolute numbers of $CD45^{-}CD51^{-}CD9^{+}Thy-1^{low}GFR\alpha1^{+}$ SSCs and $CD45^{-}CD51^{-}CD9^{+}Thy-1^{low}c-Kit^{+}$ SPCs ($n = 4-6$ mice per genotype, $p < 0.05^{*}$).

Systemic levels of sKitl were determined in the serum of *AmhCre⁺;Kitl^{Δex7/Δex7}* and *AmhCre⁻;Kitl^{Δex7/Δex7}* mice by ELISA. Levels of circulating sKitl were very similar between *AmhCre⁻;Kitl^{Δex7/Δex7}* and *AmhCre⁺;Kitl^{Δex7/Δex7}* mice (154.9 ± 23.7 and 158.8 ± 26.1 pg/ml respectively, **Figure 5.5**). This confirms that the loss of *mKitl* alone from Sertoli cells, was sufficient to cause the disruption to spermatogenesis and provides evidence for the specific role of mKitl within this niche.

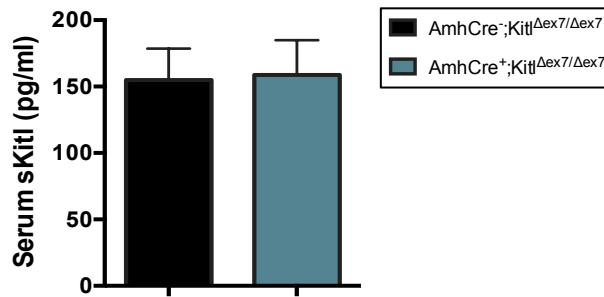


Figure 5.5 Sertoli cell loss of *mKitl* does not affect the levels of systemic sKitl in

***AmhCre⁺;Kitl^{Δex7/Δex7}* mice**

Analysis of serum Kitl levels determined by ELISA from the serum of 4-week old *AmhCre⁺;Kitl^{Δex7/Δex7}* and *AmhCre⁻;Kitl^{Δex7/Δex7}* littermate controls ($n = 6$ mice per genotype, values shown are the mean $\pm$ SEM, statistical analysis undertaken using the unpaired Student t-test).

To ensure that the loss of *mKitl* from Sertoli cells was localised to the testes and had not affected other tissues where Kitl/c-Kit signalling is important, the BM and thymii from *AmhCre⁺;Kitl^{Δex7/Δex7}* mice were isolated and analysed (**Figure 5.6**). The cellularity from both tissues was found to be similar between the KO mice and their *AmhCre⁻;Kitl^{Δex7/Δex7}* littermate controls (**Figure 5.6a**). The average total MNC numbers were $115.1 \pm 8.8 \times 10^6$ and $138.3 \pm 6.7 \times 10^6$ respectively in BM, whilst in the thymus, the average total thymocyte numbers were $299.8 \pm 39.4 \times 10^6$ and 284.9

$\pm 52.8 \times 10^6$, respectively.

Haematopoiesis was investigated by flow cytometry to determine if the stem and progenitors cells were maintained in these mice. Flow cytometry indicated that HSPCs in these mice were unaffected as both LSK and LSK CD48⁻CD150⁺ HSC numbers were similar between *AmhCre⁺;Kitl^{Δex7/Δex7}* and *AmhCre⁻;Kitl^{Δex7/Δex7}* mice (3.4 ± 0.4 vs. $3.4 \pm 0.2 \times 10^4$ LSKs and 12.6 ± 1.3 vs. $12.2 \pm 0.7 \times 10^3$ HSCs, respectively).

Investigation of myeloid progenitors also confirmed these cells were unaffected (data not shown). Thymopoiesis was analysed by staining cells with Abs against c-Kit, CD25 and Flt3 to identify the early thymic progenitors (ETPs) and the subsets of double negative thymocyte (DN) progenitors (**Figure 5.6c**). None of the DN populations or ETP cells were reduced in number in the absence of the expression of *mKitl* from Sertoli cells (**Figure 5.6d**).

Within the spermatogonial niche, loss of *mKitl* expression in Sertoli cells seems to have a specific role in spermatogenesis, specifically in SPC maintenance.

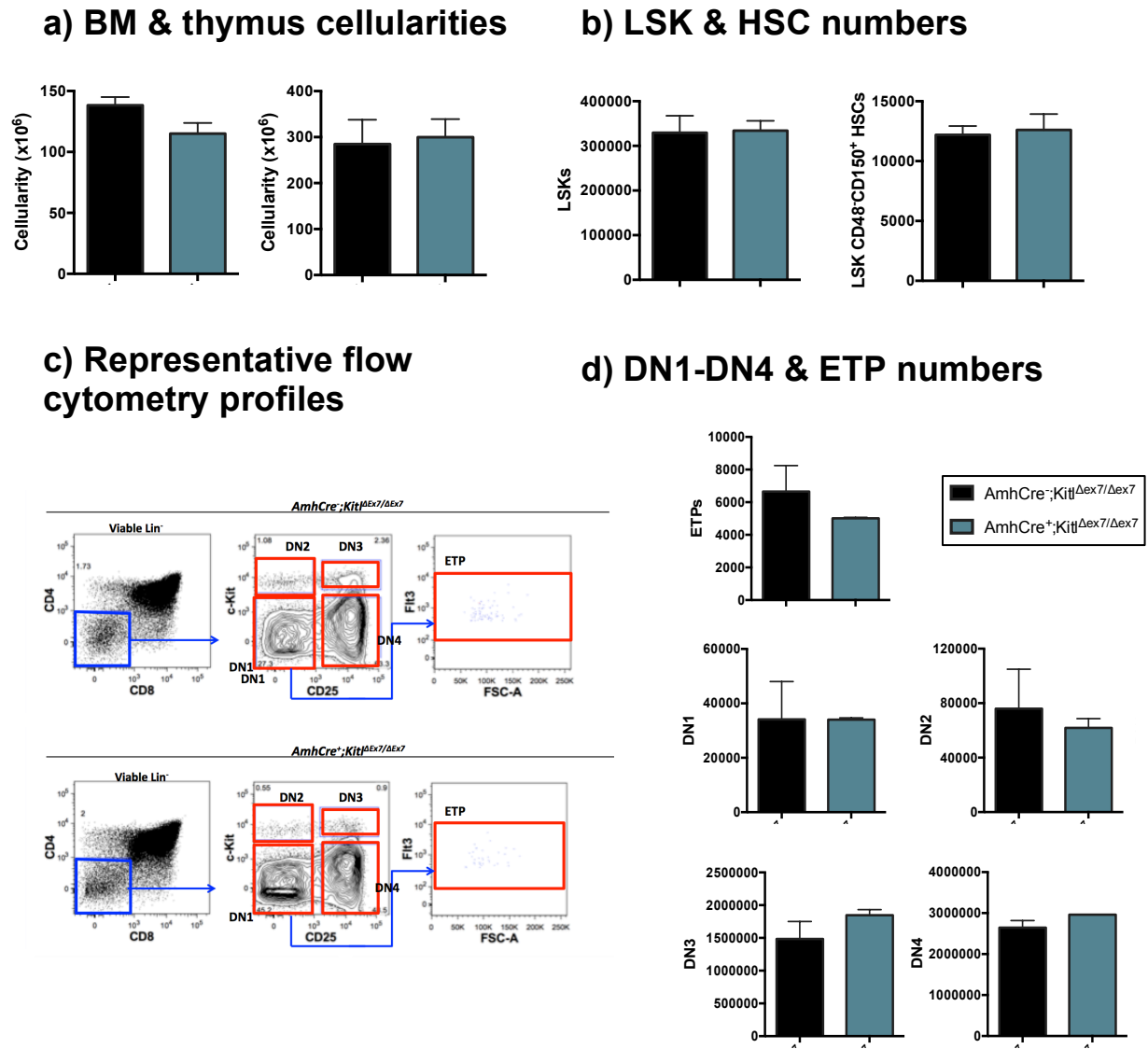


Figure 5.6 *AmhCre⁺;Kitl^{Δex7/Δex7}* mice maintain haematopoiesis and thymopoiesis

Analysis of 4-week old *AmhCre⁺;Kitl^{Δex7/Δex7}* and *AmhCre⁻;Kitl^{Δex7/Δex7}* littermate controls (n=6 mice per genotype, values shown are the mean±SEM, statistical analysis undertaken using the unpaired Student t-test):

- Total cellularity in 2 femurs and 2 tibiae (left) and thymii (right).
- Absolute number of BM LSKs (left) and phenotypic LSK CD48⁺CD150⁺ HSCs from 2 femurs and 2 tibiae (right).
- Representative flow cytometry profile for thymic DN1-DN4 and ETP cells. Blue indicates gating and red boxes indicate populations of interest.
- Absolute number of DN1-DN4 and ETP cells per thymus.

5.3 Discussion

Cytokines act by both paracrine and endocrine signalling to exert their effects as both membrane-bound and soluble proteins. Experimental techniques have been developed to establish if these different modes of action of a ligand can have specific roles. The aim of the studies reported in this chapter was to dissect out the role of localised expression *mKitl* following deletion of its expression or that of total *Kitl* from the BM ECs whilst maintaining systemic levels of sKitl,

mKitl is highly deleted from the ECs of *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice, yet there was no difference seen in the BM cellularity or HSC numbers of *Tie2Cre⁺;Kitl^{Δex7/Δex7}* compared to *Tie2Cre⁻;Kitl^{Δex7/Δex7}* explanted femurs. In the absence of *mKitl* expression in BM ECs of *Tie2Cre⁺;Kitl^{Δex7/Δex7}* explanted femurs, homing to the BM by HSCs readily appears to still take place. In addition, this BM microenvironment is able to maintain comparable HSC numbers to *Tie2Cre⁻;Kitl^{Δex7/Δex7}* littermate controls, in the presence of essentially equivalent systemic sKitl levels.

It should be remembered that these *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice still possess a full complement of mKitl, associated with other stromal cell populations and certainly the Morrison group has indicated that *mKitl* (*Scf long form*) is expressed at ~1000 fold higher levels in LepR⁺ PVCs compared to whole BM and ~100 fold higher than in VE-Cad⁺ ECs⁸⁹. This and the fact that all BM stromal cells in this model will produce local concentrations of sKitl, may explain why knocking out *mKitl* expression in ECs may cause a change in the localisation of haematopoietic progenitors relative to the ECs themselves (**Section 4.2.3**), yet is insufficient to result in altered numbers of MNCs, HSCs and MPCs.

In vivo, deletion of total *Kitl* (*mKitl* and *sKitl*) from ECs in adult *Tie2Cre⁺;Scf^{f/f}* mice, resulted in a significant reduction in the number of HSCs and in the level of circulating sKitl. Despite this phenotype, there was no difference seen in the BM cellularity or HSC numbers between *Tie2Cre⁺;Scf^{f/f}* and *Tie2Cre⁻;Scf^{f/f}* explanted femurs into WT C57BL/6 recipients in which systemic sKitl levels were maintained. The only difference between these two experiments is the level of systemic sKitl, which suggests that in KSE studies, the expression of *Kitl* by other cell types within the BM niche and the contribution of circulating levels of the soluble ligand provided by the WT recipient are capable of maintaining HSCs in explanted femurs.

Within the spermatogonial niche, the expression and role of total *Kitl* has been documented^{291,296}. However until this work, it had yet to be determined whether the cell-specific loss of the membrane-bound isoform played a non-redundant role in spermatogenesis. Analysis of *AmhCre⁺;Kitl^{Δex7/Δex7}* mice revealed several tissue-specific defects including smaller sized testes and increased numbers of SSCs and significant decreased numbers of their downstream SPCs. Investigation of other niches in the BM and thymus, demonstrated that *Kitl*/c-Kit signalling remains functional within these niches in this mouse model. These *AmhCre⁺;Kitl^{Δex7/Δex7}* mice were also shown to maintain levels of circulating sKitl, indicating that the phenotype seen in the testes was a localised niche effect. Within the spermatogonial niche, loss of *mKitl* expression in Sertoli cells therefore seems to have a key role in spermatogenesis, specifically in SPC maintenance, which correlates with *Kitl*'s known role as a survival/anti-apoptotic factor^{234,297}.

Overall the results from the KSE indicate that deletion of *mKitl* or total *Kitl* expression in only ECs has no impact upon haematopoiesis, as their loss appears to be

compensated by *Kitl* expression in other stromal cells and maintained systemic sKitl levels. The studies in testes indicate that Sertoli cell-specific expression of *mKitl* is required for spermatogenesis. The phenotype of *AmhCre⁺;Kitl^{Δex7/Δex7}* mice demonstrates that systemic sKitl cannot compensate for the loss of *mKitl* from Sertoli cells and therefore the latter is required in the spermatogonial niche.

6 Overall Discussion

The haematopoietic stem cell niche was first suggested in the late 1970s¹³ and its existence has been confirmed by multiple studies over the last four decades. This work has shown that the haematopoietic niche within the BM is a complex system, which plays host to the interplay between various cells, factors and localised conditions^{10,26}. The complexity of this microenvironment has made the task of definitively characterising the niche more difficult and a number of questions remain unresolved.

Cytokines such as CXCL12, Flt3l and Kitl have been shown by multiple groups to be important within the BM niche, as loss of ligand/receptor signalling results in impaired haematopoiesis^{41,89,163}. These factors are expressed and secreted by multiple cell types within the niche and are capable of acting on haematopoietic cells of distinct lineages, making them extremely important for the regulation of haematopoiesis¹⁵⁵. Furthermore, certain of these cytokines such as Flt3l and Kitl exist in multiple forms (membrane and soluble)^{163,298}. The potential biological differences in the roles of each isoform of these proteins have only been inferred from mutant mouse models^{217,259}.

Despite several investigations of the cell-specific expression of cytokines, no study to date has investigated the role of cytokine isoforms in a cell-specific manner. The studies in this thesis aimed to determine the expression of *mKitl* and total *Kitl* by stromal cells within the BM niche and using this information, evaluate the impact upon haematopoiesis when *mKitl* or total *Kitl* are absent from these cell types. By comparing complete loss of *mKitl* from all tissues, loss of *mKitl* from different stromal cell populations and total loss of *Kitl* (*mKitl* + *sKitl*) from stromal cell populations, the

aim was to determine the specific roles of the membrane-bound isoform, mKitl and the soluble isoform, sKitl. To gain greater insight into the biological role of this cytokine, its localised role was additionally investigated in the spermatogonial niche.

The initial investigation into the role of mKitl focused on the *Sl/Sl^d* mouse model, which was chosen as it has been considered the 'proof of principle' for the specific requirement of mKitl. Compound heterozygote *Sl/Sl^d* mice have one *Sl* allele (complete loss of the *Kitl* gene) and one hypomorphic *Sl^d* allele. The *Sl^d* allele contains a deletion, which has previously been reported to only encode and express a truncated, soluble form of the protein and to not express mKitl^{217,259}. These *Sl/Sl^d* mice are known to possess many phenotypic similarities to mice lacking all *Kitl*, such as infertility and impaired haematopoiesis, resulting in anaemia and reduced numbers of HSPCs^{213,214,217}.

Sl/Sl^d mice were confirmed to possess significantly fewer BM MNCs and HSCs compared to their WT littermates. The current studies also confirmed that *Sl/Sl^d* mice possess the same *Kitl* sequence as previously published²⁵⁹, which when cloned was shown to be expressible *in vitro* in 293T cells, with the truncated protein found to be active in a MC/9 cell proliferation assay. These results were comparable to those obtained by Brannan *et al.* and Flanagan *et al.*^{217,259}. However, contrary to what may have been inferred from their *in vitro* studies, which looked at the expression of the truncated protein and its activity^{217,259}, further analysis reported here confirmed that sKitl protein expression is essentially absent in the *Sl/Sl^d* mouse *in vivo* with no detectable staining of BM sections and only negligible systemic levels, quantifiable in 1 of 4 mice. Thus, the studies reported here have for the first time demonstrated that

Sl/Sl^d mice do not in fact produce sKitl protein *in vivo* in anything other than negligible or non-detectable levels.

The experiments undertaken do not provide an explanation for the lack of sKitl protein in *Sl/Sl^d* mice but postulates can be made. Given the alteration to the encoded sequence, there may be several consequences. First, the mRNA may be unable to be successfully translated *in vivo*. Although this may be negated by the fact that the gene sequence can readily produce an active protein *in vitro*, as indicated in the studies here and also by the work of Brannan *et al.*²⁵⁹. Second, even if the truncated sKitl protein is produced, it may not be secreted or it may exhibit an altered tertiary structure, which is unstable *in vivo*. As WT Kitl is known to function as a dimer²²⁵⁻²²⁷, dimerisation of *Sl^d*Kitl may not be possible and once recognised as 'foreign', could be targeted for rapid degradation. This is seen in *Sl^{17H}* mice, which produce a truncated Kitl protein with reduced dimerisation capability and decreased stability²²⁷. Any of these could result in the inability of the protein to be detected. Brannan *et al.* themselves speculated that lack of *mRNA* translation, failure to produce sKitl in sufficient quantities and defective protein secretion could all contribute to the phenotype seen in *Sl/Sl^d* mice²⁵⁹. Further work would need to be conducted to determine the molecular basis for this finding.

Overall, the *in vivo* absence of detectable sKitl from *Sl/Sl^d* mice reported in these studies here, made them an inappropriate model to further investigate the specific role of mKitl. Accordingly, a specific *mKitl* KO mouse (*Kitl^{ex7fl/ex7fl}*) was used to develop two models, *Kitl^{Δex7/Δex7}* (germline deletion of *Kitl* exon 7) and *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* (conditional deletion of *Kitl* exon 7 following tamoxifen induction). These two models reflect constitutive absence from conception and

induced ubiquitous deletion in adults of *Kitl* exon 7, respectively. The *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* model was investigated in addition to the *Kitl^{Δex7/Δex7}* model in an attempt to overcome the issue of the embryonic lethality and reduced birth rates evident in *Kitl^{Δex7/Δex7}* mice. Additionally, given the importance of Kitl/c-Kit signalling during embryogenesis^{231,232,274}, *Kitl^{Δex7/Δex7}* mice might undergo defective development, which may be reflected in the phenotypic defects seen on analysis of 3-week old mice. By allowing *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* to develop with functional mKitl to 6 weeks of age before inducing its deletion, it was postulated that this would permit investigation of the specific loss of *mKitl* on adult steady-state haematopoiesis.

These models were confirmed to lack mKitl by combinations of sequencing, flow cytometry, genotyping and IHC. Sequence analysis of *Kitl* in these *Kitl^{Δex7}* mice models revealed an altered nucleotide sequence containing a full-length ExC domain, a truncated TM domain but no cytoplasmic domain. An Ab specific for the IC domain of mKitl readily detected the protein in the BM of WT mice using IHC, but as expected given the lack of a predicted cytoplasmic domain, was unable to detect mKitl in the BM of *Kitl^{Δex7/Δex7}* mice.

Furthermore, Kitl expression could not be detected on the surface of 293T cells transfected with a vector containing the *Kitl* sequence encoded in *Kitl^{Δex7}* mice, while cells transfected with WT *Kitl* sequence encoding the full-length protein were found to express Kitl on their cell surface. If the *Kitl* sequence encoded in *Kitl^{Δex7}* mice had resulted in expression of mKitl on these 293T cells, given its gene sequence encodes an ExC domain, cell-surface expression of mKitl would have been detected by flow cytometry following staining with an Ab, which recognises an ExC portion of the protein. Similarly, genotyping of *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice, which have the same

Kitl sequence as *Kitl*^{Δex7/Δex7} mice, showed that they lacked the sequence encoding exon 7. Gene sequencing however, confirmed that *sKitl* was encoded and both *in vitro* and *in vivo* ELISA data confirmed the presence of sKitl protein. In contrast therefore to the *Sl/Sl*^d mouse, both the *Kitl*^{Δex7/Δex7} and *RosaCre*^{ERT2+};*Kitl*^{Δex7/Δex7} models expressed a biologically active, sKitl *in vivo*, albeit at significantly reduced levels systemically (>60 %) compared to WT.

Kitl^{Δex7/Δex7} mice with the germline deletion of *Kitl* exon 7, which do not express *mKitl*, exhibited characteristic *Sl* defects such as impaired pigmentation resulting in white fur and abnormalities in their PB counts. These mice were born at frequencies significantly less than predicted by Mendelian genetics. At 3 weeks of age, the animals that did survive were significantly smaller than their WT littermates and had significantly fewer BM MNCs, in addition to significantly decreased numbers of HSCs and certain erythroid progenitors (CFU-E and Pro Ery). The significant reductions in erythroid parameters including RBC numbers and Hgb levels are consistent with anaemia and defective erythropoiesis, which are associated with both *W* and *Sl* defects and are some of the defining phenotypes of *W* and *Sl* mutants^{238,241,299}.

Total *mKitl* deletion from all tissues also resulted in a significant reduction in the level of systemic sKitl compared to WT. Therefore a consequence of deleting *mKitl* would appear to be a marked reduction in the level of sKitl expression. Thus, deletion of *mKitl*, which produces marked deficiencies in haematopoiesis, may result in part from the absence of the membrane-bound protein itself, but also from a reduction in the level of the circulating soluble ligand. The phenotype seen cannot therefore, be attributed solely to loss of the membrane-bound form, in light of the impact on the circulating sKitl levels. This is further emphasised by the more severe phenotype

seen in *Sl/Sl^d* mice compared to *Kitl^{Δex7/Δex7}* mice, given that the former in contrast to the latter, express essentially no sKitl protein *in vivo*.

Soluble Kitl can be produced by splicing (KL1 and proteolytic cleavage of this TM precursor) or can alternatively arise by proteolytic cleavage of mKitl (KL2)^{172,275}. As mKitl protein (KL2) is not produced in *Kitl^{Δex7/Δex7}* mice, it cannot give rise to sKitl by proteolytic cleavage and it is therefore not surprising that systemic levels of sKitl are reduced. Although the relative contribution of each of the above mechanisms to overall systemic sKitl levels remains to be fully elucidated, it can be postulated that proteolytic cleavage of mKitl (KL2) may contribute ~60 % to total sKitl given the homozygous loss of *mKitl* from all tissues in these mice.

Overall, the loss of *mKitl* from all tissues *in vivo* resulted in phenotypic defects concurrent with previously published *Sl* mutants^{241,248,299}. In addition, the loss of *mKitl* was associated with a marked reduction in the level of circulating soluble ligand. The phenotypic features seen were not solely and directly attributable to the loss of the membrane-bound form from all tissues, given the impact on systemic sKitl levels in these mice, which may also contribute to the phenotype seen. However, as sKitl levels appear to fall in the absence of *mKitl*, it would appear that indirectly or directly, the loss of *mKitl* results in the phenotype.

RosaCre^{ERT2+};Kitl^{Δex7/Δex7} adult mice which lack expression of *mKitl* in all tissues displayed more pronounced haematopoietic effects than *Kitl^{Δex7/Δex7}* mice. However, given the preliminary indication of gene related toxicity in the *RosaCre^{ERT2+};Kitl^{+/+}* controls, the haematopoietic effects observed in *RosaCre^{ERT2+};Kitl^{Δex7/Δex7}* mice are likely to be increased by tamoxifen-mediated *Cre* toxicity, which therefore limits the

interpretation of the data from this model. It may be possible to evaluate the impact of complete loss of *mKitl* within the BM by using germline *Kitl*^{Δex7/Δex7} foetal bones (*mKitl* deletion from all tissues) for KSE. These experiments may be challenging, given the embryonic lethality of this model at approximately E15. However, these experiments would permit evaluation of whether systemic *sKitl* can fully compensate for the loss of *mKitl* from all cells within the BM niche.

Having evaluated the loss of *mKitl* from all tissues, which had established a requirement for *mKitl* in haematopoiesis, I proceeded to investigate the impact of loss of *mKitl* and total *Kitl* (*mKitl* + *sKitl*) from specific stromal cell populations to determine if expression of *mKitl* by a specific cell type(s) was essential for HSC maintenance *in vivo*. These studies were undertaken in the knowledge that different stromal cell populations within the BM niche had previously been demonstrated to be important for haematopoiesis^{76,83} and that the expression of specific cytokines by these defined cell populations is required for HSPC maintenance^{89,99,100}.

The expression levels of total *Kitl* and *mKitl* in MSCs, OBs and ECs was assessed in WT C57BL/6 mice and ECs were confirmed to highly express both total *Kitl* and *mKitl*; data which correlate with the findings of other groups showing that *Kitl* was highly expressed in ECs^{89,172}. This led me to investigate the impact in turn of *mKitl* loss and total *Kitl* loss from ECs and also from the more recently identified PVCs expressing the leptin receptor⁸⁹.

As a result, I generated *Tie2Cre*⁺;*Kitl*^{Δex7/Δex7}, *LepRCre*⁺;*Kitl*^{Δex7/Δex7} and *Tie2Cre*⁺;*LepRCre*⁺;*Kitl*^{Δex7/Δex7} mice, with the deletion of *Kitl* exon 7 from respectively ECs, LepR⁺ PVCs and simultaneous deletion in ECs & LepR⁺ PVCs resulting in loss

of *mKitl* from these cell types *in vivo*. I also generated *Tie2Cre⁺;Scf^{fl/fl}*, *LepRCre⁺;Scf^{fl/fl}* and *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* mice, which had all *Kitl* deleted from respectively, ECs, LepR⁺ PVCs and both cell populations simultaneously to permit the comparison of loss of total *Kitl* versus *mKitl* alone.

Individual KO of *mKitl* from ECs or LepR⁺ PVCs had no significant impact on HSC proliferation or maintenance. Despite the minimal HSC phenotype seen in the *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice, there was efficient deletion (~99 %) of *mKitl* in addition to an ~27 % reduction in the level of systemic sKitl. The incomplete deletion of *mKitl* from the *LepRCre⁺* model (≤53 %) prevents firm conclusions from being drawn about the full extent of mKitl's role when expressed by LepR⁺ PVCs. The low deletion efficiency was however unexpected, since the Morrison paper had used the identical *LepRCre* model. The difference between the studies reported here and those of Morrison, is that we used homozygous models and they used a hemizygous model, in order to possibly obtain sufficiently marked haematopoietic effects.

In the case of the sKO models of total *Kitl* deletion (*Tie2Cre⁺;Scf^{fl/fl}* and *LepRCre⁺;Scf^{fl/fl}*), there was some indication of effects on HSCs which were significantly reduced only in the case of the former. However, dKO of either *mKitl* or total *Kitl* in the two stromal cell populations (ECs and LepR⁺ PVCs) simultaneously, led to more marked and consistent haematopoietic defects. These included a combination of anaemic features in the blood profile and impact on myeloid progenitors. In the case of the *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* mice, which have deletion of total *Kitl* from both cell types, there was a significant reduction in the absolute number of HSCs which was not seen in the corresponding *mKitl* dKO *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* mouse. The fall seen in HSCs in the

Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl} mice was markedly greater than in the sKO *Tie2Cre⁺;Scf^{fl/fl}* mice.

Thus, similarly to the work of the Morrison group, loss of total *Kitl* from both cell types together caused the greatest reduction in HSCs, however the losses seen in the Morrison *Cre⁺;Scf^{fl/-}* mice were greater than evident in the corresponding *Cre⁺;Scf^{fl/fl}* mice investigated here. One possible explanation might be the use of hemizygous models in their study, which are null for one allele of *Scf* and therefore have a 50 % tissue-wide reduction in *Kitl* expression, in addition to the stromal cell-specific losses of total *Kitl*. The greater reported deletion in the Morrison model may also be a contributing factor, however deletion efficiency is only assessed for one of their models, the *LepRCre* model and is calculated on the basis of CD45⁺/Ter119⁻ *LepRCre;YFP⁺* cells which express PDGFR α ⁸⁹. There is no confirmation that the *LepRCre;YFP⁺* cells express LepR on their cell surface by Ab staining and are only confirmed to be LepR⁺ cells by their co-expression of PDGFR α . Furthermore, the expression of PDGFR α is not restricted to LepR⁺ cells, but is also expressed on other cell types including ECs and mesenchymal cells from a range of tissues^{300,301}. A more precise method might have been to first define the LepR⁺ cells and from these, determine the percentage of YFP⁺ cells.

In contrast to the sKO models, the loss of *mKitl* expression in both ECs and LepR⁺ PVCs together in the dKO *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* mice, impacted haematopoiesis with reductions in RBCs, Hgb and subsets of myeloid progenitors. Additionally, KO of total *Kitl* in both cell populations in *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* mice had more pronounced haematopoietic effects, notably a significant decline in HSCs, which may indicate not only a role for localised effects of the membrane-bound but

also the soluble ligand, which in these KO mice may only be present at reduced levels in the niche.

Reduction in systemic sKitl is evident in both sKO *Tie2Cre*⁺ models and both dKO models and appears to occur following the deletion of total *Kitl* or *mKitl* in the *Tie2Cre*⁺ ECs, as the level of circulating sKitl is unaffected in the two *LepRCre*⁺ mice models (*LepRCre*⁺;*Kitl* ^{Δ ex7/ Δ ex7} and *LepRCre*⁺;*Scf*^{fl/fl}; possibly resulting from incomplete deletion of *mKitl* or total *Kitl* in these models). Furthermore, this is emphasised by the fact that the level of reduction is similar between the *Tie2Cre*⁺;*Kitl* ^{Δ ex7/ Δ ex7} versus *Tie2Cre*⁺;*LepRCre*⁺;*Kitl* ^{Δ ex7/ Δ ex7} mice and *Tie2Cre*⁺;*Scf*^{fl/fl} versus *Tie2Cre*⁺;*LepRCre*⁺;*Scf*^{fl/fl} mice. The *Tie2Cre*⁺;*Kitl* ^{Δ ex7/ Δ ex7} mouse, which has loss of *mKitl* from ECs, exhibited unaffected haematopoiesis, whereas the *Tie2Cre*⁺;*Scf*^{fl/fl} mouse, which has loss of total *Kitl* from ECs, appeared to have significantly fewer HSCs. This HSC phenotype in *Tie2Cre*⁺;*Scf*^{fl/fl} mice could be taken as a further indication of the additional importance of sKitl, given the added deletion of *sKitl* from ECs and the further reduced systemic levels of sKitl observed in these mice compared to the *Tie2Cre*⁺;*Kitl* ^{Δ ex7/ Δ ex7} model. In addition to localised levels of sKitl and mKitl in the BM niche, there is also therefore the possibility that changes in the circulating levels of sKitl (which can decrease following the loss of *mKitl*) also have an impact upon haematopoiesis. This could indicate that whilst EC-expression of *mKitl* is important to help maintain the systemic level of sKitl, EC-expression of total *Kitl* (both *mKitl* and *sKitl*) may have a combined role in HSC maintenance.

In the different stromal *Cre*⁺;*Kitl* ^{Δ ex7/ Δ ex7} and germline *Kitl* ^{Δ ex7/ Δ ex7} models, which have deletion of *mKitl*, it was found that there was a significant disruption of the association between HSCs and ECs in the BM. This is consistent with previous

findings, which have highlighted the role of Kitl in anchoring via cell-cell contact during haematopoiesis^{172,278}. These previously reported studies did not distinguish between mKitl and sKitl, although it was postulated that the transmembrane form of Kitl directly binds to the c-kit receptor on HSCs and helps anchor these cells in the BM niche. The findings of the work reported here would appear to indicate that it is mKitl that has a specific role in anchoring HSCs within the BM microenvironment. However one observation appeared unexpected, namely that there was an apparent reduction in the number of HSCs associated with ECs in BM sections from *LepRCre⁺;Kitl^{Δex7/Δex7}* mice. This would not have been predicted given that in these mice, *mKitl* expression in ECs should be unaffected. I speculate that one is looking at the delocalisation of HSCs from LepR⁺ PVCs, which are in very close association with ECs and therefore give the appearance of altered positioning relative to ECs. It is not possible to confirm this hypothesis at this time however, work to simultaneously stain BM sections with Abs against LepR, and EC and HSC markers is ongoing.

In the *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice there was no change in the HSC cell cycle status, whilst in *LepRCre⁺;Kitl^{Δex7/Δex7}* mice there was a significant reduction in the frequency of HSCs in G₀ and a resultant increase in G₁, which was comparable to that seen in the dKO mice. This effect in *LepRCre⁺;Kitl^{Δex7/Δex7}* mice was even more noteworthy given that *mKitl* was only deleted ~20-53 % from LepR⁺ cells. Thus the deletion of *mKitl* in two different stromal cell populations produces markedly different effects on HSC cell cycle status. It would appear therefore that *mKitl*-expressing LepR⁺ PVCs might have a greater role in maintaining HSCs in quiescence than ECs. This may be related to the greater reported local expression of *mKitl* in LepR⁺ PVCs compared to ECs in BM⁸⁹. Unlike in the dKO *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* mice, the change in cell cycle status of HSCs in *LepRCre⁺;Kitl^{Δex7/Δex7}* mice appeared not to

translate into any haematopoietic effects in either blood parameters or MPC numbers. This may suggest that in these sKO mice, the *mKitl* in the EC cells and the systemic levels of sKitl, which were maintained at WT levels, compensate and help to maintain normal HSC numbers. These combined data may also indicate that cell-specific expression of *mKitl* is differentially important in quiescence and HSC maintenance *in vivo*.

Both young (4-week old) and aged (12-13 months) *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* mice revealed haematopoietic defects. These mice had significant reductions in their Hgb levels and RBCs, indicative of anaemia, which was slightly more prominent in aged mice, in addition to changes in HCT, MCV and MCH. Only the older dKO mice showed a significant reduction in LSK CD48⁻CD150⁺ HSCs compared to aged WT littermates. There was a trend however, in the sKO aged mice to show a decline in HSCs, particularly in *LepRCre⁺;Kitl^{Δex7/Δex7}* mice, but this was not statistically significant. Young and aged dKO mice both exhibited marked effects on MPCs, although some differences in the cell populations affected were seen (notably in Pre GMs and Pre CFU-Es). These data may be indicative of a changing requirement over time for mKitl by HSCs. This is supported by data from viable *c-Kit* null 'Vickid' mutants, which exhibit a progressive loss of B and T cells with age²⁸², indicating Kitl/c-Kit signalling may become increasingly important to haematopoiesis with time. However, the normal changes in haematopoiesis that occur during ageing, such as the increase in the number of HSCs, which are preferentially biased towards myeloid differentiation^{139,142} needs to be taken into consideration when evaluating the differences between WT and these dKO aged mice. Thus, despite the absence of *mKitl* from ECs and its reduced expression in LepR⁺ PVCs, if one compares 4-week and 12-13 month dKO *LepRCre⁺;Kitl^{Δex7/Δex7}* mice, HSC numbers still increased

markedly from ~3,000 to ~11,000 cells / femur and tibia, but less so than the corresponding increase of HSCs in young and aged WT mice, which was from ~3,600 to ~27,000 cells / femur and tibia. One can speculate, but it is difficult to confirm whether these differences arise from an inability to maintain HSCs (given Kitl/c-Kit signalling's anti-apoptotic capabilities in combination with BCL-2⁵³) or a reduced capacity to expand the HSC pool in aged *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* mice, resulting from down-regulation of *mKitl* expression in ECs & LepR⁺ PVCs and markedly lower systemic levels of sKitl.

Overall these findings indicated that the combined presence of mKitl on ECs and LepR⁺ PVCs, localised sKitl and potentially systemic sKitl are important for maintaining haematopoiesis within the adult BM HSC niche. However, a limitation of these mouse models is that they delete *mKitl* or total *Kitl* from ECs and LepR⁺ PVCs not only in the BM, but also potentially in these cells in other tissues. One would assume that the majority of the changes in haematopoiesis seen in these models, arose from the deletion of *mKitl* or total *Kitl* from BM stromal cells, however some contribution to the phenotypes observed, resulting from their deletion in these cells in other tissues cannot be eliminated. Therefore, in an attempt to further dissect out the role of the Kitl in experiments in which *mKitl* or total *Kitl* expression was deleted from BM stromal cells only in a model in which systemic sKitl levels were unaffected, kidney subcapsule explantation experiments were conducted with E15.5 femurs from *Tie2Cre⁺;Kitl^{Δex7/Δex7}* and *Tie2Cre⁺;Scf^{fl/fl}* embryos. Bones at this stage of embryogenesis are known to possess no HSCs, which only migrate to the BM at ~E17¹⁶.

KSE studies revealed that despite the near complete deletion (~99 %) of *mKitl* from the ECs of *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice, there was no difference seen in the BM cellularity, HSC or MPC numbers of their explanted embryonic femurs compared to those found in littermate control *Tie2Cre⁻;Kitl^{Δex7/Δex7}* explanted embryonic femurs. Thus, in the absence of *mKitl* expression in BM ECs of *Tie2Cre⁺;Kitl^{Δex7/Δex7}* explanted femurs, homing of HSCs to the BM readily appeared to still take place. In addition, this BM microenvironment was able to maintain comparable HSC numbers to *Tie2Cre⁻;Kitl^{Δex7/Δex7}* littermate controls, in the presence of essentially equivalent circulating sKitl levels. Therefore, in this explant model, there is no impact on BM cellularity or HSC numbers following the deletion of *mKitl* from ECs, which may reflect a contribution from circulating sKitl levels from the WT recipient and the greater involvement of other BM stromal cell populations expressing *mKitl*. However, if one compares and contrasts the results in the adult *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mice and those from corresponding foetal bone KSE studies, the number of HSCs is unaffected in the former, despite a significant reduction in the level of systemic sKitl. This may indicate the relative higher contribution of other stromal cell populations to HSC maintenance in this *Tie2Cre⁺;Kitl^{Δex7/Δex7}* mouse. The Morrison group has in fact previously indicated that *mKitl* is expressed at ~100 fold higher levels in LepR⁺ PVCs compared to VE-Cad⁺ ECs⁸⁹. This might explain why deletion of *mKitl* expression in ECs may cause a change in the localisation of haematopoietic progenitors relative to the ECs themselves, yet is insufficient to reduce numbers of MNCs, HSCs or MPCs. There is also one further consideration, which is that in this model, all stromal cells including ECs within the explanted BM should express sKitl locally and this will undoubtedly contribute to HSC regulation.

In vivo, deletion of total *Kitl* from ECs in 8-week old adult *Tie2Cre⁺;Scf^{fl/fl}* mice, resulted in a significant reduction in the number of HSCs and in the level of circulating sKitl. In contrast, there was no difference seen in the BM cellularity or HSC numbers between *Tie2Cre⁺;Scf^{fl/fl}* and *Tie2Cre⁻;Scf^{fl/fl}* explanted femurs into WT C57BL/6 recipients in which systemic sKitl levels were maintained. The only difference between the experiment in adult *Tie2Cre⁺;Scf^{fl/fl}* and that conducted with foetal femurs from this model is the reduced level of systemic sKitl in the adult (41 % reduction). This might suggest that the expression of *Kitl* by ECs and other cell types within the BM niche is less critical than the contribution of circulating levels of the soluble ligand in *Tie2Cre⁺;Scf^{fl/fl}* mice for HSC maintenance. This conclusion would be in conflict with that drawn from the work in the *Tie2Cre⁺;Kitl^{Δex7/Δex7}* model, which seemed to suggest the greater relative importance of certain stromal cells populations compared to circulating sKitl levels. However, this apparent conflict may arise because in the *Tie2Cre⁺;Scf^{fl/fl}* model, there is loss of local sKitl from the BM niche, albeit only from total *Kitl* deletion in ECs, which combined with reduced circulating sKitl levels may result in a fall in HSC numbers. To try and determine the contribution of LepR⁺ PVCs, studies were considered with dKO *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* and *Tie2Cre⁺;LepRCre⁺;Scf^{fl/fl}* embryonic femur explants, however, given the limited deletion efficiency in the LepR⁺ cells, it was decided that these experiments would not be able to adequately address the question.

To re-inforce the conclusions derived from the KSE data, it may be necessary to investigate the remote possibility that the lack of a haematopoietic phenotype results from the migration of WT ECs from the recipient to the explanted embryonic femurs. These WT ECs would express *Kitl*, which could then potentially ‘rescue’ the

phenotype. To confirm that this is or is not the case, it would be necessary to isolate and cell sort ECs from the explanted bones and determine their gene expression of *mKitl* and total *Kitl*. If the ECs were from the explanted, *Tie2Cre*⁺ bones, it would be expected that the levels of *mKitl* and total *Kitl* EC-gene expression would be very low (given the ~99 % deletion using this *Cre* model) if there was no migration of WT ECs.

Furthermore, to compare and contrast the role of *mKitl* and *sKitl*, a second biological niche was investigated, that in the testes. Within the spermatogonial niche, the expression and role of total *Kitl* has been documented^{291,296}. However, it remained to be determined whether the cell-specific loss of the membrane-bound isoform played a non-redundant role in spermatogenesis. To investigate this, *AmhCre*⁺;*Kitl*^{Δex7/Δex7} mice were generated to specifically delete *mKitl* from spermatogonial stromal Sertoli cells.

Analysis of these mice revealed several tissue-specific defects including smaller sized testes and increased numbers of SSCs and significant decreased numbers of their downstream SPCs. Investigation of other niches in these animals in the BM and thymus, demonstrated that *Kitl*/c-Kit signalling remained functional within these niches. These *AmhCre*⁺;*Kitl*^{Δex7/Δex7} mice were also shown to maintain levels of circulating *sKitl*, indicating that the phenotype seen in the testes was a localised niche effect. It has previously been reported that i) SSCs do not express c-Kit, unlike quiescent SPCs, which are c-Kit^{lo}^{302,303}, ii) the differentiation of SSCs into proliferating SPCs cells is defined by latter's higher c-Kit expression and iii) exposure to *Kitl* does not influence SSC self-renewal or their differentiation into SPCs³⁰⁴. Thus, SSC to SPC differentiation was claimed to occur independent of *Kitl* stimulation²⁹⁵. Ohta *et al.* summarised this as a requirement for *Kitl*/c-Kit signalling for the

maintenance and proliferation of differentiated c-Kit⁺ spermatogonia, but not for the initial step of spermatogonial cell differentiation²⁹⁵. The almost complete lack of SPCs seen in *AmhCre⁺;Kitl^{Δex7/Δex7}* mice would seem to result from Sertoli cell-specific deletion of *mKitl*. Therefore, within the spermatogonial niche, Sertoli cell expression of *mKitl* seems to have a key role in spermatogenesis, specifically in SPC maintenance, given that it can have no impact on SSC differentiation. The phenotype of *AmhCre⁺;Kitl^{Δex7/Δex7}* mice demonstrates that systemic sKitl cannot compensate for the loss of *mKitl* from Sertoli cells and therefore the latter is required in the spermatogonial niche. These findings, in a second niche, highlight the importance of mKitl in niche biology.

7 Conclusions

This work has attempted to provide new insights into the role of the two isoforms of *Kitl*, the membrane-bound and soluble forms.

Sl/Sl^d mice, the long-regarded ‘proof of principle’ for the requirement of m*Kitl*, which had a severe haematopoietic phenotype with significantly reduced phenotypic HSC numbers, have been confirmed to express no m*Kitl* and contrary to interpretation of previous findings, do essentially not express s*Kitl* *in vivo*. The phenotype seen in this naturally occurring mutant must arise from the absence of both m*Kitl* and s*Kitl*.

A number of *mKitl* and total *Kitl* KO models have been generated and investigated. Overall findings are summarised in **Tables 7.1** and **7.2**.

In *Kitl^{Δex7/Δex7}* mice, the germline deletion of *mKitl* and associated decline in systemic s*Kitl* levels resulted in anaemia and impaired HSC maintenance & erythropoiesis. Loss of *mKitl* compromised embryonic development, growth and viability, which are all typical *Sl* mutant features.

Deletion of *mKitl* from certain cells (ECs or LepR⁺ PVCs) in *Tie2Cre⁺;Kitl^{Δex7/Δex7}* or *LepRCre⁺;Kitl^{Δex7/Δex7}* mice did not impact upon HSC or MPC numbers but disrupted the localisation of HSPCs within the BM and in LepR⁺ PVCs also caused HSCs to enter G₁. These data are consistent with m*Kitl*’s role in anchoring and quiescence.

Deletion of *mKitl* from both of these cell populations simultaneously in *Tie2Cre⁺;LepRCre⁺;Kitl^{Δex7/Δex7}* mice resulted in additionally, reductions to RBC-associated parameters and fewer MPCs. This dKO resulted in more appreciable

losses with increased age, such as significantly fewer phenotypic HSCs. Thus, deletion of *mKitl* in this dKO model, resulting in a decline in circulating sKitl was associated with certain haematopoietic defects and further indicated the importance of both mKitl and sKitl in maintaining HSCs.

A marked reduction (as high as 61 %) in the level of systemic sKitl was one of the consequences of *mKitl* deletion. This may reflect the relative importance of the proteolytic cleavage of mKitl (KL2) to release sKitl, over the corresponding cleavage of KL1. Therefore the deletion of *mKitl* directly or indirectly from the lack of the protein able to be cleaved and give rise to sKitl, may be responsible for a number of the phenotypic features seen in a number of the models.

Loss of total *Kitl* from cell populations resulted in more marked effects, notably a reduction in phenotypic HSCs and a greater reduction in the level of circulating sKitl. This confirmed the contribution of sKitl to HSC maintenance.

By comparing all the adult and embryonic KSE data, it was concluded that within the BM, it is the combination of the action of mKitl, systemic and local sKitl, which all contribute to maintaining normal murine haematopoiesis in the BM niche.

This is not the case in the spermatogonial niche, where deletion of *mKitl* in the Sertoli cells of *AmhCre⁺;Kitl^{Δex7/Δex7}* mice results in a significant reduction in the number of SPCs which arise from SSCs. This effect is localised to the testes, as no other niches seem affected and the levels of systemic sKitl are maintained. In the testes, mKitl is specifically required for spermatogenesis.

Overall, the work reported here has for the first time demonstrated different requirements for the isoforms of the cytokine Kitl in spatially and functionally distinct niches *in vivo*.

Table 7.1 Summary of *Kitl* Models and Associated Haematopoietic Phenotypes

Key: Significant increase (↑), significant decrease (↓), indicates no significant difference (≈).

Strain	<i>Kitl</i> Deletion	PB	N° HSCs/MPCs	Systemic s <i>Kitl</i>	HS(P/C) Positioning	Cell Cycle
<i>Kitl</i> ^{Δex7/Δex7}	Germline <i>Kitl</i> exon 7	↓Hgb ↓RBC ↓HCT ↑MCV ↑MCH	↓HSCs ↓Pre CFU-Es ↓ProErys	↓~61% vs. WT	Loss of EC association / proximity	Not investigated
<i>RosaCre</i> ^{ERT2+} ; <i>Kitl</i> ^{Δex7/Δex7}	Inducible <i>Kitl</i> exon 7	↓WBC ↓Hgb ↓RBC ↓HCT ↓MCV ↓MCH ↑Pit	↓HSCs ↓LSKs ↓Pre GMs ↓MkPs ↓GMPs ↓Pre MegEs ↓Pre CFU-Es ↓CFU-Es ↓ProErys	↓~62% vs. WT	Not investigated	Not investigated
<i>Tie2Cre</i> ⁺ ; <i>Kitl</i> ^{Δex7/Δex7}	ECs <i>Kitl</i> exon 7	≈ WT	HSCs ≈ WT MPCs ≈ WT	↓~27% vs. WT	Loss of EC association / proximity	≈ WT
<i>LepRCre</i> ⁺ ; <i>Kitl</i> ^{Δex7/Δex7}	LepR ⁺ PVCs <i>Kitl</i> exon 7	≈ WT	HSCs ≈ WT MPCs ≈ WT	≈ WT	Loss of EC association / proximity	↓G ₀ ↑G ₁
<i>Tie2Cre</i> ⁺ ; <i>LepRCre</i> ⁺ ; <i>Kitl</i> ^{Δex7/Δex7}	ECs & LepR ⁺ PVCs <i>Kitl</i> exon 7	↓Hgb ↓RBC	HSCs ≈ WT ↓Pre GMs ↓MkPs ↓GMPs ↓CFU-Es ↓ProErys	↓~31% vs. WT	Loss of EC association / proximity	↓G ₀ ↑G ₁
<i>Tie2Cre</i> ⁺ ; <i>Scf</i> ^{fl/fl}	ECs total <i>Kitl</i>	≈ WT	↓HSCs MPCs ≈ WT	↓41% vs. WT	Not investigated	Not investigated
<i>LepRCre</i> ⁺ ; <i>Scf</i> ^{fl/fl}	LepR ⁺ PVCs total <i>Kitl</i>	↑MCV	HSCs ≈ WT MPCs ≈ WT	≈ WT	Not investigated	Not investigated
<i>Tie2Cre</i> ⁺ ; <i>LepRCre</i> ⁺ ; <i>Scf</i> ^{fl/fl}	ECs & LepR ⁺ PVCs total <i>Kitl</i>	↓Hgb ↓RBC ↑MCV	↓HSCs ↓MkPs ↓Pre MegEs ↓Pre CFU-Es ↓CFU-Es ↓ProErys	↓38% vs. WT	Not investigated	Not investigated

Table 7.2 Summary of *AmhCre⁺;Kitl^{Δex7/Δex7}* Model-Associated Phenotype

Key: Significant increase (↑), significant decrease (↓), no significant difference (≈), flow cytometry (FC), immunohistochemistry (IHC).

Strain	<i>Kitl</i> Deletion	Testes	N° Germ Cells (FC)	N° Germ Cells (IHC)	BM	Thymus	Systemic sKitl
<i>AmhCre⁺;Kitl^{Δex7/Δex7}</i>	Sertoli cell <i>Kitl</i> exon 7	↓Size ↓Weight	↓SPCs	↑SSCs ↓SPCs	Cellularity ≈ WT HSCs ≈ WT LSKs ≈ WT	Cellularity ≈ WT ETPs ≈ WT DN1s-DN4s ≈ WT	≈ WT

8 Addendum

Figure 3.1 – S.M.

Figure 3.2– S.M.

Figure 3.4 – Experiment performed by S.M., analysed by R.F.

Figure 3.10 – Experiment performed by S.M., analysed by R.F.

Figure 3.11 – Experiment performed by S.M and R.F, analysed by R.F.

Figure 3.15 – S.M.

Figure 4.1 – Experiment performed by S.M and R.F

Figure 4.5 – Experiment performed by T.L. and R.F, analysed by R.F.

Figure 4.6 – Data collected by T.L., analysed by R.F.

Figure 4.8a – S.M.

Figure 5.4 c-f– S.M.

Table 3.1– S.M.

Table 3.2 – Experiment performed by S.M and R.F, analysed by R.F.

9 Bibliography

- 1 Purton, L. E. & Scadden, D. T. The hematopoietic stem cell niche. *StemBook, Harvard Stem Cell Institute* (2008).
- 2 Lennartsson, J. & Rönstrand, L. Stem cell factor receptor/c-Kit: from basic science to clinical implications. *Physiol Rev.* **92**, 1619-1649 (2012).
- 3 Kiel, M. J. & Morrison, S. J. Uncertainty in the niches that maintain haematopoietic stem cells. *Nat Rev Immunol* **8**, 290-301 (2008).
- 4 Alexander, W. S. Knockout Mouse Models of Cytokine Action in Hematopoietic Stem Cell Regulation. *Madame Curie Bioscience Database, Landes Bioscience* (2000).
- 5 Eliasson, P. *et al.* Hypoxia mediates low cell-cycle activity and increases the proportion of long-term-reconstituting hematopoietic stem cells during in vitro culture. *Exp Hematol* **38**, 301-310 (2010).
- 6 Eliasson, P. & Jonsson, J. I. The hematopoietic stem cell niche: low in oxygen but a nice place to be. *J Cell Physiol* **222**, 17-22 (2010).
- 7 Adamo, L. *et al.* Biomechanical forces promote embryonic haematopoiesis. *Nature* **459**, 1131-1135 (2009).
- 8 Guzman, A. *et al.* Formation of micronucleated erythrocytes in mouse bone-marrow under conditions of hypothermia is not associated with stimulation of erythropoiesis. *Mutat Res.* **656**, 8-13 (2008).
- 9 Proulx, C., Dupuis, N., St-Amour, I., Boyer, L. & Lemieux, R. Increased megakaryopoiesis in cultures of CD34-enriched cord blood cells maintained at 39°C. *Biotechnol Bioeng* **88**, 675-680 (2004).
- 10 Wang, L. D. & Wagers, A. J. Dynamic niches in the origination and differentiation of haematopoietic stem cells. *Nat Rev Mol Cell Biol* **12**, 643-655 (2011).
- 11 Ikuta, K. & Weissman, I. L. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc Natl Acad Sci U S A* **89**, 1502-1506 (1992).
- 12 Till, J. E. & McCulloch, E. A. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* **14**, 213-222 (1961).
- 13 Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* **4**, 7-25 (1978).
- 14 Matsumoto, Y., Iwasaki, H. & Suda, T. Maintenance of Adult Stem Cells: Role of the Stem Cell Niche. *Editor: Phinney, D.G. Springer Science + Business Media*, 35-55 (2011).
- 15 Spangrude, G. J., Heimfeld, S. & Weissman, I. L. Purification and characterization of mouse hematopoietic stem cells. *Science* **241**, 58-62 (1988).
- 16 Dzierzak, E. & Speck, N. A. Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nat Immunol* **9**, 129-136 (2008).
- 17 Ottersbach, K., Smith, A., Wood, A. & Gottgens, B. Ontogeny of haematopoiesis: recent advances and open questions. *Br J Haematol* **148**, 343-355 (2010).
- 18 Palis, J., Robertson, S., Kennedy, M., Wall, C. & Keller, G. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* **126**, 5073-5084 (1999).
- 19 Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C. & Keller, G. A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725-732 (1998).
- 20 Huber, T. L., Kouskoff, V., Fehling, H. J., Palis, J. & Keller, G. Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* **432**, 625-630 (2004).
- 21 Godin, I., Dieterlen-Lièvre, F. & Cumano, A. Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. *Proc Natl Acad Sci U S A* **92**, 773-777 (1995).
- 22 Boisset, J. C. *et al.* In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* **464**, 116-120 (2010).

- 23 Cumano, A., Dieterlen-Lièvre, F. & Godin, I. Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell* **86**, 907-916 (1996).
- 24 de Bruijn, M. F., Speck, N. A., Peeters, M. C. & Dzierzak, E. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J.* **19**, 2465-2474 (2000).
- 25 Christensen, J. L., Wright, D. E., Wagers, A. J. & Weissman, I. L. Circulation and chemotaxis of fetal hematopoietic stem cells. *PLoS Biol* **2**, E75 (2004).
- 26 Adams, G. B. & Scadden, D. T. The hematopoietic stem cell in its place. *Nat Immunol* **7**, 333-337 (2006).
- 27 Medvinsky, A. & Dzierzak, E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897-906 (1996).
- 28 Wagers, A. J., Christensen, J. L. & Weissman, I. L. Cell fate determination from stem cells. *Gene Ther* **9**, 606-612 (2002).
- 29 Morrison, S. J. & Kimble, J. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* **441**, 1068-1074 (2006).
- 30 Morrison, S. J. & Weissman, I. L. The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. *Immunity* **1**, 661-673 (1994).
- 31 Morrison, S. J., Wandycz, A. M., Hemmati, H. D., Wright, D. E. & Weissman, I. L. Identification of a lineage of multipotent hematopoietic progenitors. *Development* **124**, 1929-1939 (1997).
- 32 Akashi, K., Traver, D., Miyamoto, T. & Weissman, I. L. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**, 193-197 (2000).
- 33 Kondo, M., Weissman, I. L. & Akashi, K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**, 661-672 (1997).
- 34 Martín, P. *et al.* Concept of lymphoid versus myeloid dendritic cell lineages revisited: both CD8alpha(-) and CD8alpha(+) dendritic cells are generated from CD4(low) lymphoid-committed precursors. *Blood* **96**, 2511-2519 (2000).
- 35 Kawamoto, H., Ohmura, K. & Katsura, Y. Direct evidence for the commitment of hematopoietic stem cells to T, B and myeloid lineages in murine fetal liver. *Int Immunol* **9**, 1011-1019 (1997).
- 36 Kawamoto, H., Ohmura, K., Fujimoto, S. & Katsura, Y. Emergence of T cell progenitors without B cell or myeloid differentiation potential at the earliest stage of hematopoiesis in the murine fetal liver. *J Immunol* **162**, 2725-2731 (1999).
- 37 Katsura, Y. & Kawamoto, H. Stepwise lineage restriction of progenitors in lympho-myelopoiesis. *Int Rev Immunol.* **20**, 1-20 (2001).
- 38 Mansson, R. *et al.* Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitors. *Immunity* **26**, 407-419 (2007).
- 39 Adolfsson, J. *et al.* Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* **121**, 295-306 (2005).
- 40 Mendez-Ferrer, S., Chow, A., Merad, M. & Frenette, P. S. Circadian rhythms influence hematopoietic stem cells. *Curr Opin Hematol* **16**, 235-242 (2009).
- 41 Sugiyama, T., Kohara, H., Noda, M. & Nagasawa, T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* **25**, 977-988 (2006).
- 42 Mendez-Ferrer, S., Lucas, D., Battista, M. & Frenette, P. S. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature* **452**, 442-447 (2008).
- 43 Burberry, A. *et al.* Infection mobilizes hematopoietic stem cells through cooperative NOD-like receptor and Toll-like receptor signaling. *Cell host & microbe* **15**, 779-791 (2014).
- 44 Papayannopoulou, T. & Scadden, D. T. Stem-cell ecology and stem cells in motion. *Blood* **111**, 3923-3930 (2008).
- 45 Levesque, J.-P., Hendy, J., Takamatsu, Y., Simmons, P. J. & Bendall, L. J. Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by G-CSF or cyclophosphamide. *J Clin Invest* **111**, 187-196 (2003).

- 46 Petit, I. *et al.* G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol* **3**, 687-694 (2002).
- 47 Wilson, A. & Trumpp, A. Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol* **6**, 93-106 (2006).
- 48 Lapidot, T., Dar, A. & Kollet, O. How do stem cells find their way home? *Blood* **106**, 1901-1910 (2005).
- 49 Mahmood, Z. & Shukla, Y. Death receptors: targets for cancer therapy. *Exp Cell Res* **316**, 887-899 (2010).
- 50 Guicciardi, M. E. & Gores, G. J. Life and death by death receptors. *FASEB J.* **23**, 1625-1637 (2009).
- 51 Kim, J. H. *et al.* Differential apoptotic activities of wild-type FOXL2 and the adult-type granulosa cell tumor-associated mutant FOXL2 (C134W). *Oncogene* **30**, 1653-1663 (2011).
- 52 Oguro, H. & Iwama, A. Life and death in hematopoietic stem cells. *Curr Opin Immunol* **19**, 503-509 (2007).
- 53 Domen, J. & Weissman, I. L. Hematopoietic stem cells need two signals to prevent apoptosis; BCL-2 can provide one of these, Kitl/c-Kit signaling the other. *J Exp Med* **192**, 1707-1718 (2000).
- 54 Domen, J., Cheshier, S. H. & Weissman, I. L. The role of apoptosis in the regulation of hematopoietic stem cells: Overexpression of Bcl-2 increases both their number and repopulation potential. *J Exp Med* **191**, 253-264 (2000).
- 55 Veis, D. J., Sorenson, C. M., Shutter, J. R. & Korsmeyer, S. J. Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* **75**, 229-240 (1993).
- 56 Opferman, J. T. *et al.* Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science* **307**, 1101-1104 (2005).
- 57 Okada, S. *et al.* *In vivo* and *in vitro* stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. *Blood* **80**, 3044-3050 (1992).
- 58 Cooper, D. D. & Spangrude, G. J. (R)evolutionary considerations in hematopoietic development. *Ann N Y Acad Sci* **872**, 83-93 (1999).
- 59 Adolfsson, J. *et al.* Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity* **15**, 659-669 (2001).
- 60 Osawa, M., Hanada, K., Hamada, H. & Nakauchi, H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. *Science* **273**, 242-245 (1996).
- 61 Yang, L. *et al.* Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood* **105**, 2717-2723 (2005).
- 62 Goodell, M. A., Brose, K., Paradis, G., Conner, A. S. & Mulligan, R. C. Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J Exp Med* **183**, 1797-1806 (1996).
- 63 Phillips, R. L., Reinhart, A. J. & Van Zant, G. Genetic control of murine hematopoietic stem cell pool sizes and cycling kinetics. *Proc Natl Acad Sci U S A* **89**, 11607-11611 (1992).
- 64 Li, C. L. & Johnson, G. R. Rhodamine123 reveals heterogeneity within murine Lin⁻, Sca-1⁺ hemopoietic stem cells. *J Exp Med* **175**, 1443-1447 (1992).
- 65 Spangrude, G. J. & Johnson, G. R. Resting and activated subsets of mouse multipotent hematopoietic stem cells. *Proc Natl Acad Sci U S A* **87**, 7433-7437 (1990).
- 66 Veillette, A. Immune regulation by SLAM family receptors and SAP-related adaptors. *Nat Rev Immunol* **6**, 56-66 (2006).
- 67 Kim, I., He, S., Yilmaz, O. H., Kiel, M. J. & Morrison, S. J. Enhanced purification of fetal liver hematopoietic stem cells using SLAM family receptors. *Blood* **108**, 737-744 (2006).
- 68 Kiel, M. J., Yilmaz, O. H., Iwashita, T., Terhorst, C. & Morrison, S. J. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* **121**, 1109-1121 (2005).

- 69 BD LSR II User's Guide. (2007).
- 70 BD FACSAria User's Guide. (2007).
- 71 Shapiro, H. M. Practical Flow Cytometry. *Wiley-Liss 4th Edition* (2003).
- 72 Deng, W. & Lin, H. Spectrosomes and fusomes anchor mitotic spindles during asymmetric germ cell divisions and facilitate the formation of a polarized microtubule array for oocyte specification in *Drosophila*. *Dev Biol* **189**, 79-94 (1997).
- 73 Xie, T. & Spradling, A. C. A niche maintaining germ line stem cells in the *Drosophila* ovary. *Science* **290**, 328-330 (2000).
- 74 Kiger, A. A., Jones, D. L., Schulz, C., Rogers, M. B. & Fuller, M. T. Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. *Science* **294**, 2542-2545 (2001).
- 75 Zhang, J. *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* **425**, 836-841 (2003).
- 76 Calvi, L. M. *et al.* Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* **425**, 841-846 (2003).
- 77 Nilsson, S. K., Johnston, H. M. & Coverdale, J. A. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. *Blood* **97**, 2293-2299 (2001).
- 78 Lam, B. S., Cunningham, C. & Adams, G. B. Pharmacologic modulation of the calcium-sensing receptor enhances hematopoietic stem cell lodgment in the adult bone marrow. *Blood* **117**, 1167-1175 (2011).
- 79 Suda, T., Arai, F. & Shimmura, S. Regulation of stem cells in the niche. *Cornea* **24**, S12-S17 (2005).
- 80 Levesque, J.-P. & Winkler, I. G. Hierarchy of immature hematopoietic cells related to blood flow and niche. *Curr Opin Hematol* **18**, 220-225 (2011).
- 81 Lo Celso, C. *et al.* Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature* **457**, 92-96 (2009).
- 82 Ugarte, F. & Forsberg, E. C. Haematopoietic stem cell niches: new insights inspire new questions. *EMBO J.* **32**, 2535-2547 (2013).
- 83 Mendez-Ferrer, S. *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* **466**, 829-834 (2010).
- 84 Arai, F. *et al.* Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**, 149-161 (2004).
- 85 Brunner, S., Theiss, H. D., Murr, A., Negele, T. & Franz, W. M. Primary hyperparathyroidism is associated with increased circulating bone marrow-derived progenitor cells. *Am J Physiol Endocrinol Metab.* **293**, E1670-1675 (2007).
- 86 Kollet, O. *et al.* Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells. *Nat Med.* **12**, 657-664 (2006).
- 87 Mansour, A. *et al.* Osteoclasts promote the formation of hematopoietic stem cell niches in the bone marrow. *J Exp Med.* **209**, 537-549 (2012).
- 88 Sacchetti, B. *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* **131**, 324-336 (2007).
- 89 Ding, L., Saunders, T. L., Enikolopov, G. & Morrison, S. J. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* **481**, 457-462 (2012).
- 90 Winkler, I. G. *et al.* Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood* **116**, 4815-4828 (2010).
- 91 Chow, A. *et al.* Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *J Exp Med.* **208**, 261-271 (2011).
- 92 Katayama, Y. *et al.* Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell* **124**, 407-421 (2006).
- 93 Yamazaki, S. *et al.* Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell* **147**, 1146-1158 (2011).

- 94 Naveiras, O. *et al.* Bone-marrow adipocytes as negative regulators of the haematopoietic microenvironment. *Nature* **460**, 259-263 (2009).
- 95 Kiel, M. J. & Morrison, S. J. Uncertainty in the niches that maintain haematopoietic stem cells. *Nat Rev Immunol* **8**, 290-301 (2008).
- 96 Lord, B. I., Testa, N. G. & Hendry, J. H. The relative spatial distributions of CFUs and CFUc in the normal mouse femur. *Blood* **46**, 65-72 (1975).
- 97 Xie, Y. *et al.* Detection of functional haematopoietic stem cell niche using real-time imaging. *Nature* **457**, 97-101 (2009).
- 98 Kohler, A. *et al.* Altered cellular dynamics and endosteal location of aged early hematopoietic progenitor cells revealed by time-lapse intravital imaging in long bones. *Blood* **114**, 290-298 (2009).
- 99 Greenbaum, A. *et al.* CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* **495**, 227-230 (2013).
- 100 Ding, L. & Morrison, S. J. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* **495**, 231-235 (2013).
- 101 Puri, M. C. & Bernstein, A. Requirement for the TIE family of receptor tyrosine kinases in adult but not fetal hematopoiesis. *Proc Natl Acad Sci U S A* **100**, 12753-12758 (2003).
- 102 Arai, F. & Suda, T. Maintenance of quiescent hematopoietic stem cells in the osteoblastic niche. *Ann N Y Acad Sci* **1106**, 41-53 (2007).
- 103 Cheshier, S. H., Morrison, S. J., Liao, X. & Weissman, I. L. *In vivo* proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc Natl Acad Sci U S A* **96**, 3120-3125 (1999).
- 104 Yoshihara, H. *et al.* Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell* **1**, 685-697 (2007).
- 105 Suda, T., Arai, F. & Hirao, A. Hematopoietic stem cells and their niche. *Trends Immunol* **26**, 426-433 (2005).
- 106 Yavropoulou, M. P. & Yovos, J. G. Osteoclastogenesis--current knowledge and future perspectives. *J Musculoskelet Neuronal Interact.* **8**, 204-216 (2008).
- 107 Tavassoli, M. Structure and function of sinusoidal endothelium of bone marrow. *Prog Clin Biol Res* **59B**, 249-256 (1981).
- 108 Kopp, H. G., Avecilla, S. T., Hooper, A. T. & Rafii, S. The bone marrow vascular niche: home of HSC differentiation and mobilization. *Physiology* **20**, 349-356 (2005).
- 109 Kiel, M. J., Radice, G. L. & Morrison, S. J. Lack of evidence that hematopoietic stem cells depend on N-cadherin-mediated adhesion to osteoblasts for their maintenance. *Cell Stem Cell* **1**, 204-217 (2007).
- 110 Yao, L., Yokota, T., Xia, L., Kincade, P. W. & McEver, R. P. Bone marrow dysfunction in mice lacking the cytokine receptor gp130 in endothelial cells. *Blood* **106**, 4093-4101 (2005).
- 111 Kobayashi, H. *et al.* Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol* **12**, 1046-1056 (2010).
- 112 Avecilla, S. T. *et al.* Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat Med* **10**, 64-71 (2004).
- 113 Shi, S. & Gronthos, S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res.* **18**, 696-704 (2003).
- 114 Shi, C. *et al.* Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands. *Immunity* **34**, 590-601 (2011).
- 115 Nombela-Arrieta, C. *et al.* Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nat Cell Biol* **15**, 533-543 (2013).
- 116 Suda, T., Takubo, K. & Semenza, G. L. Metabolic regulation of hematopoietic stem cells in the hypoxic niche. *Cell Stem Cell* **9**, 298-310 (2011).
- 117 Spencer, J. A. *et al.* Direct measurement of local oxygen concentration in the bone marrow of live animals. *Nature* **508**, 269-273 (2014).
- 118 Greco, V. & Guo, S. Compartmentalized organization: a common and required feature of stem cell niches? *Development* **137**, 1586-1594 (2010).

- 119 De Bruyn, P. P., Breen, P. C. & Thomas, T. B. The microcirculation of the bone marrow. *Anat Rec.* **168**, 55-68 (1970).
- 120 Abkowitz, J. L., Robinson, A. E., Kale, S., Long, M. W. & Chen, J. Mobilization of hematopoietic stem cells during homeostasis and after cytokine exposure. *Blood* **102**, 1249-1253 (2003).
- 121 Ando, J. & Yamamoto, K. Effects of shear stress and stretch on endothelial function. *Antioxid Redox Signal.* **15**, 1389-1403 (2011).
- 122 Hsieh, H. J., Li, N. Q. & Frangos, J. A. Shear stress increases endothelial platelet-derived growth factor mRNA levels. *Am J Physiol* **260**, H642-646 (1991).
- 123 Mitsumata, M., Fishel, R. S., Nerem, R. M., Alexander, R. W. & Berk, B. C. Fluid shear stress stimulates platelet-derived growth factor expression in endothelial cells. *Am J Physiol* **265**, H3-8 (1993).
- 124 Sterpetti, A. V. *et al.* Shear stress increases the release of interleukin-1 and interleukin-6 by aortic endothelial cells. *Surgery* **114**, 911-914 (1993).
- 125 Kosaki, K., Ando, J., Korenaga, R., Kurokawa, T. & Kamiya, A. Fluid shear stress increases the production of granulocyte-macrophage colony-stimulating factor by endothelial cells via mRNA stabilization. *Circ Res* **82**, 794-802 (1998).
- 126 Okuda, T., Nishimura, M., Nakao, M. & Fujita, Y. RUNX1/AML1: a central player in hematopoiesis. *Int J Hematol.* **74**, 252-257 (2001).
- 127 Chattopadhyay, N., Vassilev, P. M. & Brown, E. M. Calcium-sensing receptor: roles in and beyond systemic calcium homeostasis. *Biol Chem.* **378**, 759-768 (1997).
- 128 Adams, G. B. *et al.* Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. *Nature* **439**, 599-603 (2006).
- 129 Levesque, J.-P. *et al.* Hematopoietic progenitor cell mobilization results in hypoxia with increased hypoxia-inducible transcription factor-1 alpha and vascular endothelial growth factor A in bone marrow. *Stem Cells.* **25**, 1954-1965 (2007).
- 130 Mazumdar, J., Dondeti, V. & Simon, M. C. Hypoxia-inducible factors in stem cells and cancer. *J Cell Mol Med.* **13**, 4319-4328 (2009).
- 131 Levesque, J.-P., Winkler, I. G., Larsen, S. R. & Rasko, J. E. Mobilization of bone marrow-derived progenitors. *Handb Exp Pharmacol.*, 3-36 (2007).
- 132 Parmar, K., Mauch, P., Vergilio, J. A., Sackstein, R. & Down, J. D. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc Natl Acad Sci U S A* **104**, 5431-5436 (2007).
- 133 Takubo, K. *et al.* Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. *Cell Stem Cell* **7**, 391-402 (2010).
- 134 Wagner, W., Horn, P., Bork, S. & Ho, A. D. Aging of hematopoietic stem cells is regulated by the stem cell niche. *Exp Gerontol* **43**, 974-980 (2008).
- 135 Rossi, D. J. *et al.* Cell intrinsic alterations underlie hematopoietic stem cell aging. *Proc Natl Acad Sci U S A* **102**, 9194-9199 (2005).
- 136 Liang, Y., Van Zant, G. & Szilvassy, S. J. Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. *Blood* **106**, 1479-1487 (2005).
- 137 Xing, Z. *et al.* Increased hematopoietic stem cell mobilization in aged mice. *Blood* **108**, 2190-2197 (2006).
- 138 Ju, Z. *et al.* Telomere dysfunction induces environmental alterations limiting hematopoietic stem cell function and engraftment. *Nat Med* **13**, 742-747 (2007).
- 139 Sudo, K., Ema, H., Morita, Y. & Nakauchi, H. Age-associated characteristics of murine hematopoietic stem cells. *J Exp Med* **192**, 1273-1280 (2000).
- 140 Chen, J. Senescence and functional failure in hematopoietic stem cells. *Exp Hematol* **32**, 1025-1032 (2004).
- 141 Linton, P. J. & Dorshkind, K. Age-related changes in lymphocyte development and function. *Nat Immunol* **5**, 133-139 (2004).
- 142 Kim, M., Moon, H. B. & Spangrude, G. J. Major age-related changes of mouse hematopoietic stem/progenitor cells. *Ann N Y Acad Sci* **996**, 195-208 (2003).

- 143 Vanasse, G. J. & Berliner, N. Anemia in elderly patients: an emerging problem for the 21st century. *Hematology / the Education Program of the American Society of Hematology. American Society of Hematology. Education Program* **2010**, 271-275 (2010).
- 144 Buckley, S. M. & Verfaillie, C. *Blood and Bone Marrow Pathology*. 63-76 (Elsevier, 2011).
- 145 Zon, L. I. Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. *Nature* **453**, 306-313 (2008).
- 146 Lessard, J. & Sauvageau, G. Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. *Nature* **423**, 255-260 (2003).
- 147 Oguro, H. *et al.* Differential impact of Ink4a and Arf on hematopoietic stem cells and their bone marrow microenvironment in Bmi1-deficient mice. *J Exp Med* **203**, 2247-2253 (2006).
- 148 Bruggeman, S. W. *et al.* Ink4a and Arf differentially affect cell proliferation and neural stem cell self-renewal in Bmi1-deficient mice. *Genes Dev* **19**, 1438-1443 (2005).
- 149 Molofsky, A. V., He, S., Bydon, M., Morrison, S. J. & Pardal, R. Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senescence pathways. *Genes Dev* **19**, 1432-1437 (2005).
- 150 Tadokoro, Y., Ema, H., Okano, M., Li, E. & Nakauchi, H. *De novo* DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells. *J Exp Med* **204**, 715-722 (2007).
- 151 Probst, A. V., Dunleavy, E. & Almouzni, G. Epigenetic inheritance during the cell cycle. *Nat Rev Mol Cell Biol* **10**, 192-206 (2009).
- 152 Yamazaki, K. & Allen, T. D. Ultrastructural morphometric study of efferent nerve terminals on murine bone marrow stromal cells, and the recognition of a novel anatomical unit: the "neuro-reticular complex". *Am J Anat* **187**, 261-276 (1990).
- 153 Spiegel, A., Kalinkovich, A., Shviti, S., Kollet, O. & Lapidot, T. Stem cell regulation via dynamic interactions of the nervous and immune systems with the microenvironment. *Cell Stem Cell* **3**, 484-492 (2008).
- 154 Afan, A. M., Broome, C. S., Nicholls, S. E., Whetton, A. D. & Miyan, J. A. Bone marrow innervation regulates cellular retention in the murine haemopoietic system. *Br J Haematol* **98**, 569-577 (1997).
- 155 Alexander, W. S. Cytokines in hematopoiesis. *Int Rev Immunol*. **16**, 651-682 (1998).
- 156 Enver, T. & Jacobsen, S. E. Developmental biology: Instructions writ in blood. *Nature* **461**, 183-184 (2009).
- 157 Rieger, M. A., Hoppe, P. S., Smejkal, B. M., Eitelhuber, A. C. & Schroeder, T. Hematopoietic cytokines can instruct lineage choice. *Science* **325**, 217-218 (2009).
- 158 Sarrazin, S. *et al.* MafB restricts M-CSF-dependent myeloid commitment divisions of hematopoietic stem cells. *Cell* **138**, 300-313 (2009).
- 159 Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **103**, 211-225 (2000).
- 160 Massague, J. & Pandiella, A. Membrane-anchored growth factors. *Annu Rev Biochem* **62**, 515-541 (1993).
- 161 Heissig, B. *et al.* Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* **109**, 625-637 (2002).
- 162 Stein, J., Borzillo, G. V. & Rettenmier, C. W. Direct stimulation of cells expressing receptors for macrophage colony-stimulating factor (CSF-1) by a plasma membrane-bound precursor of human CSF-1. *Blood* **76**, 1308-1314 (1990).
- 163 Lyman, S. D. & Jacobsen, S. E. c-kit ligand and Flt3 ligand: stem/progenitor cell factors with overlapping yet distinct activities. *Blood* **91**, 1101-1134 (1998).
- 164 Huang, E. J., Nocka, K. H., Buck, J. & Besmer, P. Differential expression and processing of two cell associated forms of the kit-ligand: KL-1 and KL-2. *Mol Biol Cell* **3**, 349-362 (1992).
- 165 Stein, J. & Rettenmier, C. W. Proteolytic processing of a plasma membrane-bound precursor to human macrophage colony-stimulating factor (CSF-1) is accelerated by phorbol ester. *Oncogene* **6**, 601-605 (1991).
- 166 Pandiella, A. & Massague, J. Cleavage of the membrane precursor for transforming growth factor alpha is a regulated process. *Proc Natl Acad Sci U S A* **88**, 1726-1730 (1991).

- 167 Porteu, F. & Nathan, C. Shedding of tumor necrosis factor receptors by activated human neutrophils. *J Exp Med* **172**, 599-607 (1990).
- 168 Kohno, T. *et al.* A second tumor necrosis factor receptor gene product can shed a naturally occurring tumor necrosis factor inhibitor. *Proc Natl Acad Sci U S A* **87**, 8331-8335 (1990).
- 169 Gordon, M. Y. Hemopoietic growth factors and receptors: bound and free. *Cancer cells* **3**, 127-133 (1991).
- 170 Taichman, R. S. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. *Blood* **105**, 2631-2639 (2005).
- 171 Zhang, C. C. & Lodish, H. F. Cytokines regulating hematopoietic stem cell function. *Curr Opin Hematol.* **15**, 307-311 (2008).
- 172 Broudy, V. C. Stem cell factor and hematopoiesis. *Blood* **90**, 1345-1364 (1997).
- 173 Ku, H., Yonemura, Y., Kaushansky, K. & Ogawa, M. Thrombopoietin, the ligand for the Mpl receptor, synergizes with steel factor and other early acting cytokines in supporting proliferation of primitive hematopoietic progenitors of mice. *Blood* **87**, 4544-4551 (1996).
- 174 Ogawa, M. Differentiation and proliferation of hematopoietic stem cells. *Blood* **81**, 2844-2853 (1993).
- 175 Jelkmann, W. Erythropoietin after a century of research: younger than ever. *Eur J Haematol.* **78**, 183-205 (2007).
- 176 Israels, L. G. & Israels, E. D. *Erythropoiesis: an overview.* (Birkhäuser Verlag, 2003).
- 177 Zeigler, B. M., Vajdos, J., Qin, W., Loverro, L. & Niss, K. A mouse model for an erythropoietin-deficiency anemia. *Dis Model Mech.* **3**, 763-772 (2010).
- 178 Jelkmann, W. The role of the liver in the production of thrombopoietin compared with erythropoietin. *Eur J Gastroenterol Hepatol.* **13**, 791-801 (2001).
- 179 Zeigler, F. C. *et al.* *In vitro* megakaryocytopoietic and thrombopoietic activity of c-mpl ligand (TPO) on purified murine hematopoietic stem cells. *Blood* **84**, 4045-4052 (1994).
- 180 Methia, N., Louache, F., Vainchenker, W. & Wendling, F. Oligodeoxynucleotides antisense to the proto-oncogene c-mpl specifically inhibit *in vitro* megakaryocytopoiesis. *Blood* **82**, 1395-1401 (1993).
- 181 Solar, G. P. *et al.* Role of c-mpl in early hematopoiesis. *Blood* **92**, 4-10 (1998).
- 182 Guerriero, A. *et al.* Thrombopoietin is synthesized by bone marrow stromal cells. *Blood* **90**, 3444-3455 (1997).
- 183 Hodohara, K., Fujii, N., Yamamoto, N. & Kaushansky, K. Stromal cell-derived factor-1 (SDF-1) acts together with thrombopoietin to enhance the development of megakaryocytic progenitor cells (CFU-MK). *Blood* **95**, 769-775 (2000).
- 184 Qian, S., Fu, F., Li, W., Chen, Q. & de Sauvage, F. J. Primary role of the liver in thrombopoietin production shown by tissue-specific knockout. *Blood* **92**, 2189-2191 (1998).
- 185 Kimura, S., Roberts, A. W., Metcalf, D. & Alexander, W. S. Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proc Natl Acad Sci U S A* **95**, 1195-1200 (1998).
- 186 Alexander, W. S. Thrombopoietin and the c-Mpl receptor: insights from gene targeting. *Int J Biochem Cell Biol* **31**, 1027-1035 (1999).
- 187 Carver-Moore, K. *et al.* Low levels of erythroid and myeloid progenitors in thrombopoietin- and c-mpl-deficient mice. *Blood* **88**, 803-808 (1996).
- 188 Buza-Vidas, N. *et al.* FLT3 receptor and ligand are dispensable for maintenance and posttransplantation expansion of mouse hematopoietic stem cells. *Blood* **113**, 3453-3460 (2009).
- 189 McKenna, H. J. *et al.* Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. *Blood* **95**, 3489-3497 (2000).
- 190 Omatsu, Y. *et al.* The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity* **33**, 387-399 (2010).
- 191 Yarden, Y. *et al.* Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. *EMBO J.* **6**, 3341-3351 (1987).

- 192 Besmer, P. *et al.* A new acute transforming feline retrovirus and relationship of its oncogene *v-kit* with the protein kinase gene family. *Nature* **320**, 415-421 (1986).
- 193 Pawson, T. Regulation and targets of receptor tyrosine kinases. *Eur J Cancer*. **38 Suppl 5**, S3-10 (2002).
- 194 Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P. & Bernstein, A. The proto-oncogene *c-kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse *W* locus. *Nature* **335**, 88-89 (1988).
- 195 Longley, B. J., Reguera, M. J. & Ma, Y. Classes of *c-KIT* activating mutations: proposed mechanisms of action and implications for disease classification and therapy. *Leuk Res* **25**, 571-576 (2001).
- 196 Miettinen, M. & Lasota, J. *KIT* (CD117): a review on expression in normal and neoplastic tissues, and mutations and their clinicopathologic correlation. *Appl Immunohistochem Mol Morphol* **13**, 205-220 (2005).
- 197 Ronnstrand, L. Signal transduction via the stem cell factor receptor/*c-Kit*. *Cell Mol Life Sci*. **61**, 2535-2548 (2004).
- 198 Ashman, L. K. The biology of stem cell factor and its receptor *C-kit*. *Int J Biochem Cell Biol* **31**, 1037-1051 (1999).
- 199 Manova, K. *et al.* The expression pattern of the *c-kit* ligand in gonads of mice supports a role for the *c-kit* receptor in oocyte growth and in proliferation of spermatogonia. *Dev Biol* **157**, 85-99 (1993).
- 200 Simmons, P. J. *et al.* *c-kit* is expressed by primitive human hematopoietic cells that give rise to colony-forming cells in stroma-dependent or cytokine-supplemented culture. *Exp Hematol* **22**, 157-165 (1994).
- 201 Welham, M. J. & Schrader, J. W. Modulation of *c-kit* mRNA and protein by hemopoietic growth factors. *Mol Cell Biol* **11**, 2901-2904 (1991).
- 202 Sillaber, C. *et al.* IL-4 regulates *c-kit* proto-oncogene product expression in human mast and myeloid progenitor cells. *J Immunol* **147**, 4224-4228 (1991).
- 203 Keller, J. R., Jacobsen, S. E., Dubois, C. M., Hestdal, K. & Ruscetti, F. W. Transforming growth factor-beta: a bidirectional regulator of hematopoietic cell growth. *Int J Cell Cloning*. **10**, 2-11 (1992).
- 204 Jacobsen, F. W., Dubois, C. M., Rusten, L. S., Veiby, O. P. & Jacobsen, S. E. Inhibition of stem cell factor-induced proliferation of primitive murine hematopoietic progenitor cells signaled through the 75-kilodalton tumor necrosis factor receptor. Regulation of *c-kit* and p53 expression. *J Immunol* **154**, 3732-3741 (1995).
- 205 Taniguchi, S., Dai, C. H., Price, J. O. & Krantz, S. B. Interferon gamma downregulates stem cell factor and erythropoietin receptors but not insulin-like growth factor-I receptors in human erythroid colony-forming cells. *Blood* **90**, 2244-2252 (1997).
- 206 Yee, N. S., Langen, H. & Besmer, P. Mechanism of kit ligand, phorbol ester, and calcium-induced down-regulation of *c-kit* receptors in mast cells. *J Biol Chem* **268**, 14189-14201 (1993).
- 207 Yee, N. S., Hsiau, C. W., Serve, H., Vosseller, K. & Besmer, P. Mechanism of down-regulation of *c-kit* receptor. Roles of receptor tyrosine kinase, phosphatidylinositol 3'-kinase, and protein kinase C. *J Biol Chem* **269**, 31991-31998 (1994).
- 208 Miyazawa, K. *et al.* Ligand-dependent polyubiquitination of *c-kit* gene product: a possible mechanism of receptor down modulation in M07e cells. *Blood* **83**, 137-145 (1994).
- 209 Brizzi, M. F. *et al.* Protein kinase C-dependent release of a functional whole extracellular domain of the mast cell growth factor (MGF) receptor by MGF-dependent human myeloid cells. *Oncogene* **9**, 1583-1589 (1994).
- 210 Hirota, S. *et al.* Gain-of-function mutations of *c-kit* in human gastrointestinal stromal tumors. *Science* **279**, 577-580 (1998).
- 211 Furitsu, T. *et al.* Identification of mutations in the coding sequence of the proto-oncogene *c-kit* in a human mast cell leukemia cell line causing ligand-independent activation of *c-kit* product. *J Clin Invest* **92**, 1736-1744 (1993).

- 212 Sykora, K. W., Tomeczkowski, J. & Reiter, A. C-kit receptors in childhood malignant lymphoblastic cells. *Leuk Lymphoma* **25**, 201-216 (1997).
- 213 Huang, E. *et al.* The hematopoietic growth factor KL is encoded by the *Sl* locus and is the ligand of the c-kit receptor, the gene product of the *W* locus. *Cell* **63**, 225-233 (1990).
- 214 Zsebo, K. M. *et al.* Stem cell factor is encoded at the *Sl* locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* **63**, 213-224 (1990).
- 215 Zsebo, K. M. *et al.* Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. *Cell* **63**, 195-201 (1990).
- 216 Anderson, D. M. *et al.* Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell* **63**, 235-243 (1990).
- 217 Flanagan, J. G., Chan, D. C. & Leder, P. Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the *Sl^d* mutant. *Cell* **64**, 1025-1035 (1991).
- 218 Anderson, D. M. *et al.* Alternate splicing of mRNAs encoding human mast cell growth factor and localization of the gene to chromosome 12q22-q24. *Cell Growth Differ.* **2**, 373-378 (1991).
- 219 Rich, A. *et al.* Local presentation of Steel factor increases expression of c-kit immunoreactive interstitial cells of Cajal in culture. *Am J Physiol Gastrointest Liver Physiol* **284**, G313-320 (2003).
- 220 Martin, F. H. *et al.* Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell* **63**, 203-211 (1990).
- 221 Majumdar, M. K., Feng, L., Medlock, E., Toksoz, D. & Williams, D. A. Identification and mutation of primary and secondary proteolytic cleavage sites in murine stem cell factor cDNA yields biologically active, cell-associated protein. *J Biol Chem* **269**, 1237-1242 (1994).
- 222 Longley, B. J. *et al.* Chymase cleavage of stem cell factor yields a bioactive, soluble product. *Proc Natl Acad Sci U S A* **94**, 9017-9021 (1997).
- 223 Zou, J. *et al.* Catalytic activity of human ADAM33. *J Biol Chem* **279**, 9818-9830 (2004).
- 224 Vu, T. H. & Werb, Z. Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev* **14**, 2123-2133 (2000).
- 225 Arakawa, T. *et al.* Glycosylated and unglycosylated recombinant-derived human stem cell factors are dimeric and have extensive regular secondary structure. *J Biol Chem* **266**, 18942-18948 (1991).
- 226 Hsu, Y. R. *et al.* The majority of stem cell factor exists as monomer under physiological conditions. Implications for dimerization mediating biological activity. *J Biol Chem* **272**, 6406-6415 (1997).
- 227 Tajima, Y. *et al.* Role of dimerization of the membrane-associated growth factor kit ligand in juxtacrine signaling: the *Sl^{17H}* mutation affects dimerization and stability-phenotypes in hematopoiesis. *J Exp Med* **187**, 1451-1461 (1998).
- 228 Miyazawa, K. *et al.* Membrane-bound Steel factor induces more persistent tyrosine kinase activation and longer life span of c-kit gene-encoded protein than its soluble form. *Blood* **85**, 641-649 (1995).
- 229 Nishikawa, M. *et al.* Deletion mutagenesis of stem cell factor defines the C-terminal sequences essential for its biological activity. *Biochem Biophys Res Commun* **188**, 292-297 (1992).
- 230 Matous, J. V., Langley, K. & Kaushansky, K. Structure-function relationships of stem cell factor: an analysis based on a series of human-murine stem cell factor chimera and the mapping of a neutralizing monoclonal antibody. *Blood* **88**, 437-444 (1996).
- 231 Keshet, E. *et al.* Embryonic RNA expression patterns of the c-kit receptor and its cognate ligand suggest multiple functional roles in mouse development. *EMBO J.* **10**, 2425-2435 (1991).
- 232 Matsui, Y., Zsebo, K. M. & Hogan, B. L. Embryonic expression of a haematopoietic growth factor encoded by the *Sl* locus and the ligand for c-kit. *Nature* **347**, 667-669 (1990).

- 233 Hattangadi, S. M., Wong, P., Zhang, L., Flygare, J. & Lodish, H. F. From stem cell to red cell: regulation of erythropoiesis at multiple levels by multiple proteins, RNAs, and chromatin modifications. *Blood* **118**, 6258-6268 (2011).
- 234 Hassan, H. T. & Zander, A. Stem cell factor as a survival and growth factor in human normal and malignant hematopoiesis. *Acta haematologica* **95**, 257-262 (1996).
- 235 Linnekin, D. Early signaling pathways activated by c-Kit in hematopoietic cells. *Int J Biochem Cell Biol.* **31**, 1053-1074 (1999).
- 236 Wu, A. M., Till, J. E., Siminovitch, L. & McCulloch, E. A. A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. *J Cell Physiol* **69**, 177-184 (1967).
- 237 Wang, W., Horner, D. N., Chen, W. L., Zandstra, P. W. & Audet, J. Synergy between erythropoietin and stem cell factor during erythropoiesis can be quantitatively described without co-signaling effects. *Biotechnol Bioeng.* **99**, 1261-1272 (2008).
- 238 Mayer, T. C. & Green, M. C. An experimental analysis of the pigment defect caused by mutations at the *W* and *Sl* loci in mice. *Dev Biol* **18**, 62-75 (1968).
- 239 Silvers, W. K. The Coat Colors of Mice: A Model for Gene Action and Interaction. *Springer-Verlag, New York* (1979).
- 240 Mintz, B. & Russell, E. S. Gene-induced embryological modifications of primordial germ cells in the mouse. *J Exp Zool* **134**, 207-237 (1957).
- 241 Russell, E. S. Hereditary anemias of the mouse: a review for geneticists. *Adv Genet* **20**, 357-459 (1979).
- 242 Ruscetti, F. W., Boggs, D. R., Torok, B. J. & Boggs, S. S. Reduced blood and marrow neutrophils and granulocytic colony-forming cells in *Sl/Sl^d* mice. *Proc Soc Exp Biol Med* **152**, 398-402 (1976).
- 243 Kitamura, Y., Go, S. & Hatanaka, K. Decrease of mast cells in *W/W^v* mice and their increase by bone marrow transplantation. *Blood* **52**, 447-452 (1978).
- 244 Kitamura, Y. & Go, S. Decreased production of mast cells in *Sl/Sl^d* anemic mice. *Blood* **53**, 492-497 (1979).
- 245 Wolf, N. S. Dissecting the hematopoietic microenvironment. Stem cell lodgment and commitment, and the proliferation and differentiation of erythropoietic descendants in the *Sl/Sl^d* mouse. *Cell Tissue Kinet* **7**, 89-98 (1974).
- 246 Russell, E. S., and Lawson, F.A. Selection and inbreeding for longevity of a lethal type. *J. Hered.* **50**, 19-25 (1959).
- 247 Geissler, E. N., Ryan, M. A. & Housman, D. E. The dominant-white spotting (*W*) locus of the mouse encodes the c-kit proto-oncogene. *Cell* **55**, 185-192 (1988).
- 248 Sarvella, P. A. & Russell, L. B. Steel, a new dominant gene in the mouse. *J Hered* **47**, 123-128 (1956).
- 249 Waskow, C. & Rodewald, H. R. Lymphocyte development in neonatal and adult c-Kit-deficient (*c-Kit^{W/W}*) mice. *Adv Exp Med Biol* **512**, 1-10 (2002).
- 250 Thorén, L. A. *et al.* Kit regulates maintenance of quiescent hematopoietic stem cells. *J Immunol.* **180**, 2045-2053 (2008).
- 251 Kimura, Y. *et al.* c-Kit-mediated functional positioning of stem cells to their niches is essential for maintenance and regeneration of adult hematopoiesis. *PLoS One* **6**, e26918 (2011).
- 252 Bedell, M. A., Cleveland, L. S., O'Sullivan, T. N., Copeland, N. G. & Jenkins, N. A. Deletion and interallelic complementation analysis of *Steel* mutant mice. *Genetics* **142**, 935-944 (1996).
- 253 Cattanaach, B. M., Burtenshaw, M. D., Raspberry, C. & Evans, E. P. Large deletions and other gross forms of chromosome imbalance compatible with viability and fertility in the mouse. *Nat Genet.* **3**, 56-61 (1993).
- 254 Huang, E. J. *et al.* The murine steel panda mutation affects kit ligand expression and growth of early ovarian follicles. *Dev Biol* **157**, 100-109 (1993).
- 255 Brannan, C. I. *et al.* Developmental abnormalities in *Steel^{17H}* mice result from a splicing defect in the steel factor cytoplasmic tail. *Genes Dev* **6**, 1832-1842 (1992).
- 256 Wehrle-Haller, B. & Imhof, B. A. Stem cell factor presentation to c-Kit: Identification of a basolateral targeting domain. *J Biol Chem* **276**, 12667-12674 (2001).

- 257 Graw, J., Loster, J., Neuhauser-Klaus, A., Pretsch, W. & Schmitt-John, T. Molecular analysis of two new *Steel* mutations in mice shows a transversion or an insertion. *Mamm Genome* **7**, 843-846 (1996).
- 258 Graw, J., Neuhauser-Klaus, A. & Pretsch, W. Detection of a point mutation (A to G) in exon 5 of the murine *Mgf* gene defines a novel allele at the *Steel* locus with a weak phenotype. *Mutat Res* **382**, 75-78 (1997).
- 259 Brannan, C. I. *et al.* Steel-Dickie mutation encodes a c-kit ligand lacking transmembrane and cytoplasmic domains. *Proc Natl Acad Sci U S A* **88**, 4671-4674 (1991).
- 260 McCulloch, E. A., Siminovitch, L., Till, J. E., Russell, E. S. & Bernstein, S. E. The cellular basis of the genetically determined hemopoietic defect in anemic mice of genotype *Sl/Sl^d*. *Blood* **26**, 399-410 (1965).
- 261 Badea, T. C., Wang, Y. & Nathans, J. A noninvasive genetic/pharmacologic strategy for visualizing cell morphology and clonal relationships in the mouse. *J Neurosci* **23**, 2314-2322 (2003).
- 262 Kisanuki, Y. Y. *et al.* Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis *in vivo*. *Dev Biol* **230**, 230-242 (2001).
- 263 DeFalco, J. *et al.* Virus-assisted mapping of neural inputs to a feeding center in the hypothalamus. *Science* **291**, 2608-2613 (2001).
- 264 Holdcraft, R. W. & Braun, R. E. Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids. *Development* **131**, 459-467 (2004).
- 265 Su, H., Mills, A. A., Wang, X. & Bradley, A. A targeted X-linked CMV-Cre line. *Genesis* **32**, 187-188 (2002).
- 266 Srinivas, S. *et al.* Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* **1**, 4 (2001).
- 267 Liu, P., Jenkins, N. A. & Copeland, N. G. A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res* **13**, 476-484 (2003).
- 268 Bernstein, S. E. New mutants: steel dickie. *Mouse News Lett* **22**, 33-34 (1960).
- 269 Hendrix, M. J. *et al.* Expression and functional significance of VE-cadherin in aggressive human melanoma cells: role in vasculogenic mimicry. *Proc Natl Acad Sci U S A* **98**, 8018-8023 (2001).
- 270 Schwenk, F., Baron, U. & Rajewsky, K. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucleic Acids Res* **23**, 5080-5081 (1995).
- 271 Pronk, C. J. *et al.* Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. *Cell stem cell* **1**, 428-442 (2007).
- 272 Hameyer, D. *et al.* Toxicity of ligand-dependent Cre recombinases and generation of a conditional Cre deleter mouse allowing mosaic recombination in peripheral tissues. *Physiological genomics* **31**, 32-41 (2007).
- 273 Metcalf, D. Hematopoietic cytokines. *Blood* **111**, 485-491 (2008).
- 274 Orr-Urtreger, A. *et al.* Developmental expression of c-kit, a proto-oncogene encoded by the *W* locus. *Development* **109**, 911-923 (1990).
- 275 Besmer, P. *et al.* The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis. *Dev Suppl*, 125-137 (1993).
- 276 Pirih, F. Q. *et al.* Parathyroid hormone mediates hematopoietic cell expansion through interleukin-6. *PLoS One* **5**, e13657 (2010).
- 277 Murone, M., Carpenter, D. A. & de Sauvage, F. J. Hematopoietic deficiencies in c-mpl and TPO knockout mice. *Stem cells* **16**, 1-6 (1998).
- 278 Broudy, V. C., Lin, N. L., Priestley, G. V., Nocka, K. & Wolf, N. S. Interaction of stem cell factor and its receptor c-kit mediates lodgment and acute expansion of hematopoietic cells in the murine spleen. *Blood* **88**, 75-81 (1996).
- 279 Li, J. Quiescence regulators for hematopoietic stem cell. *Exp Hematol* **39**, 511-520 (2011).
- 280 Cheng, T. *et al.* Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* **287**, 1804-1808 (2000).

- 281 Scholzen, T. & Gerdes, J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol* **182**, 311-322 (2000).
- 282 Waskow, C., Paul, S., Haller, C., Gassmann, M. & Rodewald, H. R. Viable c-Kit(W/W) mutants reveal pivotal role for c-kit in the maintenance of lymphopoiesis. *Immunity* **17**, 277-288 (2002).
- 283 Warnock, G. L. *et al.* Viable purified islets of Langerhans from collagenase-perfused human pancreas. *Diabetes* **38**, 136-139 (1989).
- 284 Lo, D. & Sprent, J. Identity of cells that imprint H-2-restricted T-cell specificity in the thymus. *Nature* **319**, 672-675 (1986).
- 285 Mangoushi, M. A. Contiguous allografts of male and female gonadal primordia in the rat. *J Anat* **123**, 407-413 (1977).
- 286 Varas, F., Grande, T., Ramirez, A. & Bueren, J. A. Implantation of bone marrow beneath the kidney capsule results in transfer not only of functional stroma but also of hematopoietic repopulating cells. *Blood* **96**, 2307-2309 (2000).
- 287 Robertson, N. J., Fairchild, P. J. & Waldmann, H. in *Immunological Tolerance* Vol. 380 *Methods in Molecular Biology™* (ed P.J. Fairchild) Ch. 21, 347-353 (Humana Press, 2007).
- 288 Chan, C. K. *et al.* Endochondral ossification is required for haematopoietic stem-cell niche formation. *Nature* **457**, 490-494 (2009).
- 289 Barker, N. *et al.* Identification of stem cells in small intestine and colon by marker gene *Lgr5*. *Nature* **449**, 1003-1007 (2007).
- 290 Oatley, M. J., Racicot, K. E. & Oatley, J. M. Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. *Biol Reprod.* **84**, 639-645 (2011).
- 291 Ohta, H., Yomogida, K., Dohmae, K. & Nishimune, Y. Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. *Development* **127**, 2125-2131 (2000).
- 292 Rossi, P., Albanesi, C., Grimaldi, P. & Geremia, R. Expression of the mRNA for the ligand of c-kit in mouse Sertoli cells. *Biochem Biophys Res Commun* **176**, 910-914 (1991).
- 293 Sorrentino, V., Giorgi, M., Geremia, R., Besmer, P. & Rossi, P. Expression of the c-kit proto-oncogene in the murine male germ cells. *Oncogene* **6**, 149-151 (1991).
- 294 Hobbs, R. M., Seandel, M., Falciatori, I., Rafii, S. & Pandolfi, P. P. *Plzf* regulates germline progenitor self-renewal by opposing mTORC1. *Cell* **142**, 468-479 (2010).
- 295 Ohta, H., Tohda, A. & Nishimune, Y. Proliferation and differentiation of spermatogonial stem cells in the *W/W^v* mutant mouse testis. *Biol Reprod.* **69**, 1815-1821 (2003).
- 296 Sette, C., Dolci, S., Geremia, R. & Rossi, P. The role of stem cell factor and of alternative c-kit gene products in the establishment, maintenance and function of germ cells. *Int J Dev Biol* **44**, 599-608 (2000).
- 297 Li, C. L. & Johnson, G. R. Stem cell factor enhances the survival but not the self-renewal of murine hematopoietic long-term repopulating cells. *Blood* **84**, 408-414 (1994).
- 298 Rajaraman, S. *et al.* An allelic series of mutations in the Kit ligand gene of mice. Effects of ethylnitrosourea-induced *Kitl* point mutations on survival and peripheral blood cells of *Kitl(Steel)* mice. *Genetics* **162**, 341-353 (2002).
- 299 Russell, E. S. Developmental studies of mouse hereditary anemias. *Am J Med Genet* **18**, 621-641 (1984).
- 300 Heldin, C. H. & Westermark, B. Mechanism of action and *in vivo* role of platelet-derived growth factor. *Physiol Rev.* **79**, 1283-1316 (1999).
- 301 Jenkinson, W. E., Rossi, S. W., Parnell, S. M., Jenkinson, E. J. & Anderson, G. PDGFR α -expressing mesenchyme regulates thymus growth and the availability of intrathymic niches. *Blood* **109**, 954-960 (2007).
- 302 Shinohara, T., Avarbock, M. R. & Brinster, R. L. β 1- and α 6-integrin are surface markers on mouse spermatogonial stem cells. *Proc Natl Acad Sci U S A.* **96**, 5504-5509 (1999).
- 303 Kubota, H., Avarbock, M. R. & Brinster, R. L. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. *Proc Natl Acad Sci U S A.* **100**, 6487-6492 (2003).

- 304 Kubota, H., Avarbock, M. R. & Brinster, R. L. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. *Biol Reprod.* **71**, 722-731 (2004).