# The role of hypoxia signalling pathways in normal and leukaemic haemopoiesis

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# Abstract

Although haemopoietic stem cells (HSCs) represent one of the best-defined stem cell systems, the pathways regulating HSC development and maintenance are not fully understood. HSCs reside in the hypoxic niche and maintain intracellular hypoxia. Hypoxia and hypoxia signalling pathways are thought to play a vital role in HSC maintenance. Hypoxia inducible factors (Hifs) are evolutionarily conserved and are the key regulators of hypoxia. Hifs consist of an unstable, oxygen-dependent $\alpha$-subunit and an oxygen-independent stable ß-subunit. The two main isoforms of Hif-$\alpha$, namely *Hif-1$\alpha$* and *Hif-2$\alpha$*, are critical for the response to hypoxia. Hif-mediated pathways have been extensively studied and have been shown to regulate metabolic adaptation and to influence various cellular mechanisms, including cell growth, survival, differentiation and apoptosis, erythropoiesis and angiogenesis. *Hif-1$\alpha$* has been shown to be essential for maintenance of HSC functions under stressful conditions of serial transplantation and aging, but the role of *Hif-2$\alpha$* and the interplay between *Hif-1$\alpha$* and *Hif-2$\alpha$* in regulating HSC functions and their niche is not known. Hence, in this study, I have investigated the role of Hif-$\alpha$ in HSC functions. Furthermore, published evidence suggested that leukaemic stem cells (LSC) share the hypoxic properties with HSCs. *Cited2*, a hypoxia-inducible *Hif-1$\alpha$* and *Hif-2$\alpha$* target gene, is critical for embryonic and adult haemopoiesis and possesses oncogenic properties. I have investigated the role of *Cited2* in AML generation.

The results demonstrate that *Hif-2$\alpha$* is not essential for maintenance of HSC functions in a cell-autonomous manner under steady state and stressful conditions of serial transplantation and aging. It is also evident that HSCs lacking *Hif-2$\alpha$* together with *Hif-1$\alpha$* successfully maintain normal haemopoiesis. However, the data in this thesis show that *Hif-2$\alpha$* is essential for non-cell-autonomous maintenance of HSC functions, particularly in males and current work also indicate that a previously unappreciated complex interplay between *Hif-1$\alpha$*- and *Hif-2$\alpha$*-dependent signalling is required for adult HSC maintenance in a non-cell-autonomous manner.

Additionally, the data demonstrate that haemopoietic stem and progenitor cells (HSPCs) lacking *Cited2* display reduced transformation potential and failure to generate transplantable AML *in vivo*. Overexpression of *Mcl-1 (*an anti-apoptotic gene), in Cited2$^{\Delta/\Delta}$ cells bypassed their defective transformation potential forming transformed colonies *in vitro*. Hence, the data in this thesis provide evidence that *Cited2* is essential for leukaemic transformation at least in part via *Mcl-1* regulation.

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## Related publication

Guitart AV, **Subramani C**, Armesilla-Diaz A, Smith G, Sepulveda C, Gezer D, Vukovic M, Dunn K, Pollard P, Holyoake TL, Enver T, Ratcliffe PJ & Kranc KR. 2013. Hif-2alpha is not essential for cell-autonomous haemopoietic stem cell maintenance. Blood 122(10):1741-5.

This thesis is dedicated to my parents, Subramani and Latha. I am sure my dad is taking pride at my achievement from afar. Mom, you are my best friend and the strongest woman I have ever known. Thank you for inspiring me and encouraging me at every walk of life.

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# Author's Declaration

This work represents original work carried out by the author and has not been submitted in any form to any other University.

Chithra Subramani

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# Definitions/Abbreviations

2-HG – 2-Hydroxygluterate

5-FU – 5-Flurouracil

ADM – Adrenomedullin

AGM – Aorta-gonad mesonephros

ALL – Acute lymphobastic leukaemia

AML – Acute myeloid leukaemia

AML1 – Acute myelogenous leukaemia-1

Ang-1 – Angiopoietin-1

ARNT – Aryl hydrocarbon nuclear translocator

bHLH – Basic Helix-loop-helix

BM – Bone marrow

CAD – C-terminal transactivation domain

CAR – Cxcl12-abundant reticular cells

CEBPA – CCAAT/enhancer binding protein alpha

CFU-S – Colony-forming unit - Spleen

CH1-3 – Cysteine-histidine-rich domain 1-3

CITED – CBP/p300 interacting transactivators with glutamic acid (E) and aspartic acid (D)-rich tail

Cited2 – CBP/p300 interacting transactivators with glutamic acid (E) and aspartic acid (D)-rich tail 2 (Murine)

CITED2 – CBP/p300 interacting transactivators with glutamic acid (E) and aspartic acid (D)-rich tail 2 (Human)

CKO – Conditional knockout

CLL – Chronic lymphocytic leukaemia

CLP – Common lymphoid progenitor

CML – Chronic myeloid leukaemia

CMP – Common myeloid progenitor

CODDD – Carboxy-terminal oxygen-dependent degradation domain

dHSC – Definitive haemopoietic stem cells

DMEM – Dulbecco's Modified Eagle's Medium

Epo – Erythropoietin

EpoR – Erythropoietin receptor

ETO – Eight Twenty One

Ets – E- twenty six

Evi1 – Ecotropic proviral integration site 1

FAB – French-American-British classification

FACS – Fluorescence-activated cell sorting

FBS – Feotal bovine serum

FDG – Fluorescein di[β-D-galactopyranoside]

FIH – Factor inhibiting HIF

Flt3 – FMS-like tyrosine kinase 3

G-CSF – Granulocyte colony-stimulating factor

GEM – Genetically engineered mice models

Gfi1 – Growth Factor independent 1

GLUT1 – Glucose transporter 1

GMP – Granulocyte/macrophage progenitor

HAF – Hypoxia-associated factor

HAT – Histone acetyl transferase

HDAC – Histone deacetylase

HIF – Hypoxia inducible factor (Human)

Hif – Hypoxia inducible factor (murine)

HK1, HK2 – Hexokinase 1 and 2

Hox – Homeobox

HRE – Hypoxia responsive element

HSC – Haemopoietic stem cell

HSPCs – Haemopoietic stem and progenitor cells

iCre- Improved Cre recombinase

IDH1/2 – Isocitrate dehydrogenase1/2

IL – Interleukin

IMDM – Iscove's modified Dulbecco's medium

IRES – Internal-ribosome-entry-site

ITD – Internal tandem mutation

JHDM – Jumonji histone lysine demethylases

LDHA – Lactate dehydrogenase A

LIF – Leukaemia initiating factor

LMPP – Lymphoid-primed multipotent progenitors

LSC – Leukaemic stem cell

LSK – Lineage$^-$Sca-1$^+$c-Kit$^+$

LT-HSC – Long-term haemopoietic stem cell

LTC-IC – Long-term culture initiating cell

LTR – Long terminal repeat

Ly-bi – Lymphoid-biased

MDS – Myelodysplastic syndrome

MEFs – Mouse embryonic fibroblasts

Meis – Myeloid ectropic insertion site

MEP – Megakaryocyte/erythroid progenitors

mHSC – Murine haemopoietic stem cell

MLL – Mixed lineage leukaemia

MPN – Myeloproliferative neoplasm

MSC – Mesenchymal stem cell

MSCV – Murine stem cell virus

My-bi – Myeloid-biased

NAD – N-terminal transactivation domain

NO – Nitric oxide

NOD-SCID – Non-obese diabetes-severe combined immunodeficient mice

NODDD – Amino-terminal (N) oxygen-dependent degradation domain

O2 – Oxygen

OB – Osteoblasts

OC – Osteoclasts

ODDD – Oxygen-dependent degradation domain

P300/CBP – p300/Creb-binding protein

PAS – Per-ARNT-Sim domain

PB – Peripheral blood

PBX – pre-B-cell leukaemia homeobox

PDK1 – Pyruvate dehydrogenase kinase 1

PGK1 – Phosphoglycerate kinase 1

PHD – Prolyl hydroxylase

Pimo - Pimonidazole

PLZF – Promyelocytic leukaemia zinc finger

pO2 – Partial pressure of oxygen

PPR – Parathyroid hormone/parathyroid hormone related peptide receptor

pVHL – von-Hippel-Lindau

RBC – Red blood cells

ROS – Reactive oxygen species

Runx1 – Runt-related transcription factor 1

Scf – Stem cell factor

Scl/Tal1 - Stem cell leukaemia gene

SEP – Super elongation complex

Sirt – Sirtuins

ST-HSC – Short-term-haemopoietic stem cell

TAD – Transactivation domain

TBP – TATA-binding protein

TET2 – Ten-eleven-translocation gene 2

TF – Transcription factor

TGF-ß – Transforming growth factor- ß

TKD – Tyrosine kinase domain

Vegf – Vascular endothelial growth factor

WHO – World health organisation

WT – Wild type

WT1 – Wilm's tumour 1

$\alpha$- KG – alpha keto-gluterate

# Chapter 1

# Introduction

## 1.1 Haemopoietic stem cells (HSCs)

Haemopoiesis is the process of formation and maintenance of the blood system and serves as a model for the understanding of the biology and fates of other tissue stem cells (Jacobson et al. 1951; Till and Mc 1961; Wu et al. 1967; Kondo et al. 2003; Orkin and Zon 2008). Haemopoiesis is conserved in vertebrates and occurs throughout one's lifetime to replenish short-lived mature blood cells (Orkin and Zon 2008). Haemopoiesis critically depends on rare multipotent stem cells called HSCs (Jacobson et al. 1951; Lorenz et al. 1952; Till and Mc 1961; Tanaka 1966; Wu et al. 1967; Wu et al. 1968). Indeed, HSCs were demonstrated to be capable of rescuing the fatal BM failure in lethally irradiated mice upon transplantation, confirming their multipotent ability (Jacobson et al. 1951; Lorenz et al. 1952; Till and Mc 1961; Tanaka 1966; Wu et al. 1967; Wu et al. 1968). Till and McCulloch were the pioneers in establishing the ability of the BM cell fraction to form colony-forming unit Spleen (CFU-S) in irradiated mice (Till and Mc 1961). In 1963, Becker et al first reported the clonal origin of HSCs from the BM that generated mixed lineage CFU-S in irradiated host mice (Becker et al. 1963). However, it was later shown that CFU-S were formed by committed progenitors and not HSCs (Schofield 1978). These experiments opened up the field of adult stem cells and exploration of their self-renewal and multipotent characteristics.

### 1.1.1 Properties of HSCs

HSCs are localised within the hypoxic BM and have a unique capability to self-renew, sustaining a critical pool of HSCs that can subsequently differentiate to form multipotent progenitors or self-renew again (Jacobson et al. 1951; Lorenz et al. 1952; Till and Mc 1961; Tanaka 1966; Wu et al. 1967; Wu et al. 1968; Kondo et al. 1997; Akashi et al. 2000; Orkin 2000; Adolfsson et al. 2001; Reya et al. 2001; Adolfsson et al. 2005; Parmar et al. 2007; Simsek et al. 2010; Takubo et al. 2010). HSCs regulate haemopoietic homeostasis by maintaining a strict balance between fate choices of self-renewal, quiescence, differentiation and apoptosis (Orkin and Zon 2008). *In vitro* studies showed that HSCs undergo symmetric or asymmetric cell division to maintain the HSC pool and differentiate into mature blood

lineages (Morrison and Kimble 2006; Wu et al. 2007). During early embryogenesis, following injury or transplantation, HSCs divide symmetrically to produce two HSCs or two progenitors (Morrison et al. 1995; Bodine et al. 1996; Morrison et al. 1997b; Wright et al. 2001; Morrison and Kimble 2006). Alternatively, HSCs divide asymmetrically to produce two daughter cells destined to different fate choices; one that retains the characteristics of the mother HSC and the other that commits to differentiate (Suda et al. 1984; Takano et al. 2004; Giebel et al. 2006; Ting et al. 2012) (Figure 1.1). Cell intrinsic (Beckmann et al. 2007; Ting et al. 2012) and cell extrinsic factors influence the choices of symmetrical or asymmetrical cell division, thus signifying the role of the BM niche in regulating HSC fate choices (Osawa et al. 1996; Takano et al. 2004; Wu et al. 2007).

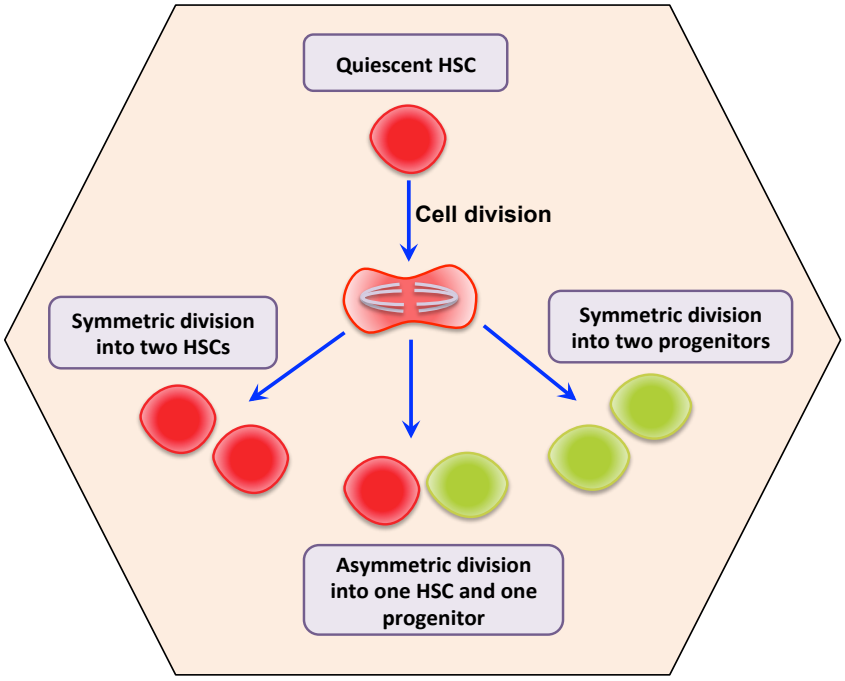



**Figure1. 1: Model of properties of HSCs.**

HSCs undergo symmetric cell division to produce identical daughter cells (both HSCs or both progenitor cells) or asymmetric cell division to produce two daughter cells that are destined to different fate choices. One daughter cell retains the characteristics of the mother HSC and the other daughter cell commits to differentiate.

## 1.1.2 Embryonic haemopoiesis

Early haemopoiesis in mammals occurs sequentially at multiple sites giving rise to less lineage-committed progenitors during early haemopoiesis (Green 1966; Moore and Metcalf 1970; Johnson and Moore 1975; Silver and Palis 1997; Palis et al. 1999). The yolk sac supports the production of an initial wave of blood cells, termed primitive haemopoietic cells, that are mainly erythrocytes at E7.5 (embryonic day 7.5) followed by early myeloid cells at E8.25 (Silver and Palis 1997; Palis et al. 1999). At E9.5, primitive progenitors with the potential of reconstitution are restricted to neonatal murine embryos (Moore and Metcalf 1970; Cumano et al. 2001). The primitive haemopoiesis is later replaced by definitive haemopoiesis (Medvinsky and Dzierzak 1996). Definitive HSC (dHSCs) activity is established in the aorta-gonad mesonephros (AGM) region at E10.5 (Muller et al. 1994; Medvinsky and Dzierzak 1996), although there is evidence that they can also be found in other regions, such as the yolk sack and placenta (Samokhvalov et al. 2007; Rhodes et al. 2008). The dHSCs from the AGM region migrate to the foetal liver that is the predominant site of haemopoiesis by E12.5 (Kumaravelu et al. 2002). Subsequently, at around E16-E17, foetal liver HSCs colonise the BM, making it the ultimate haemopoietic site (Morrison et al. 1995; Wright et al. 2001).

The embryonic origin of murine adult haemopoietic system is controversial. Some studies suggest that the common precursor population for both primitive and dHSCs arises in the yolk sac and then colonises the AGM and foetal liver regions, forming adult HSCs (Moore and Metcalf 1970; Johnson and Moore 1975; Samokhvalov et al. 2007). Other studies provide proof for the embryonic origin of dHSCs through endothelial precursors (Dieterlen-Lievre 1975; Dzierzak and Medvinsky 2008). Early studies of the origin of haemopoiesis suggest that HSCs arise from endothelial cells termed haemangioblasts (Maximow 1909; Sabin 1920). In concordance with this, studies utilising mouse and zebra fish models, recent imaging and lineage tracing experiments, have gathered direct evidence for the existence of endothelial precursors of haemopoietic cells (Dieterlen-Lievre 1975; Zovein et al. 2008; Lancrin et al. 2009; Beerman et al. 2010; Bertrand et al. 2010; Rybtsov et al. 2011). Research involving human embryos provided evidence

of haemopoiesis in the AGM region, followed by yolk sac, foetal liver and placenta (Ivanovs et al. 2011).

### 1.1.3 Hierarchy of differentiation of HSCs

Various studies have focused on the multipotent differentiation potential of HSCs by evaluating the expression of cell surface markers and performing functional assays (Spangrude et al. 1988; Morrison and Weissman 1994; Uchida et al. 1994; Adolfsson et al. 2001; Weissman et al. 2001; Kiel et al. 2005; Kim et al. 2006b; Kiel et al. 2007). These studies suggest that self-renewal and multipotency potential is progressively lost as cells proceed down the haemopoietic hierarchy (Papathanasiou et al. 2009; Beerman et al. 2010; Morita et al. 2010; Oguro et al. 2013). The resultant data suggest that haemopoietic progeny are defined by the formation of Long-term HSCs (LT-HSCs) that undergo division to give rise to multipotent short-term HSCs (ST-HSCs) with reduced self-renewal potential (Adolfsson et al. 2001; Reya et al. 2001; Adolfsson et al. 2005). ST-HSCs divide asymmetrically to form lymphoid-primed multipotent progenitors (LMPPs) (Akashi et al. 2000; Adolfsson et al. 2005) and common myeloid progenitors (CMPs) (Adolfsson et al. 2005). Further down the hierarchy, LMPPs generate committed granulocyte/macrophage progenitors (GMPs) and common lymphoid progenitors (CLPs) (Adolfsson et al. 2001; Adolfsson et al. 2005; Oguro et al. 2013). CMPs give rise to committed GMPs and megakaryocyte/erythroid progenitors (MEPs) (Akashi et al. 2000; Adolfsson et al. 2005; Nutt et al. 2005). However, the existence of LMPPs that are $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ $Flt3^{+}$ (LSK $Flt3^{+}$) is controversial (Forsberg et al. 2006; Boyer et al. 2011). The lineage restricted GMPs, CLPs and MEPs differentiate into mature blood cells, including myeloid cells (granulocytes, monocytes), lymphocytes (B- and T-cells, natural killer cells), dendritic cells, erythrocytes, megakaryocytes and platelets, respectively (Figure 1.2). LT-HSCs also generate MEPs (Pronk et al. 2007) and a recent publication showed that platelet-biased stem cells reside at the top of the HSC hierarchy adding to the evidence of heterogeneity of HSCs (Moignard et al. 2013).

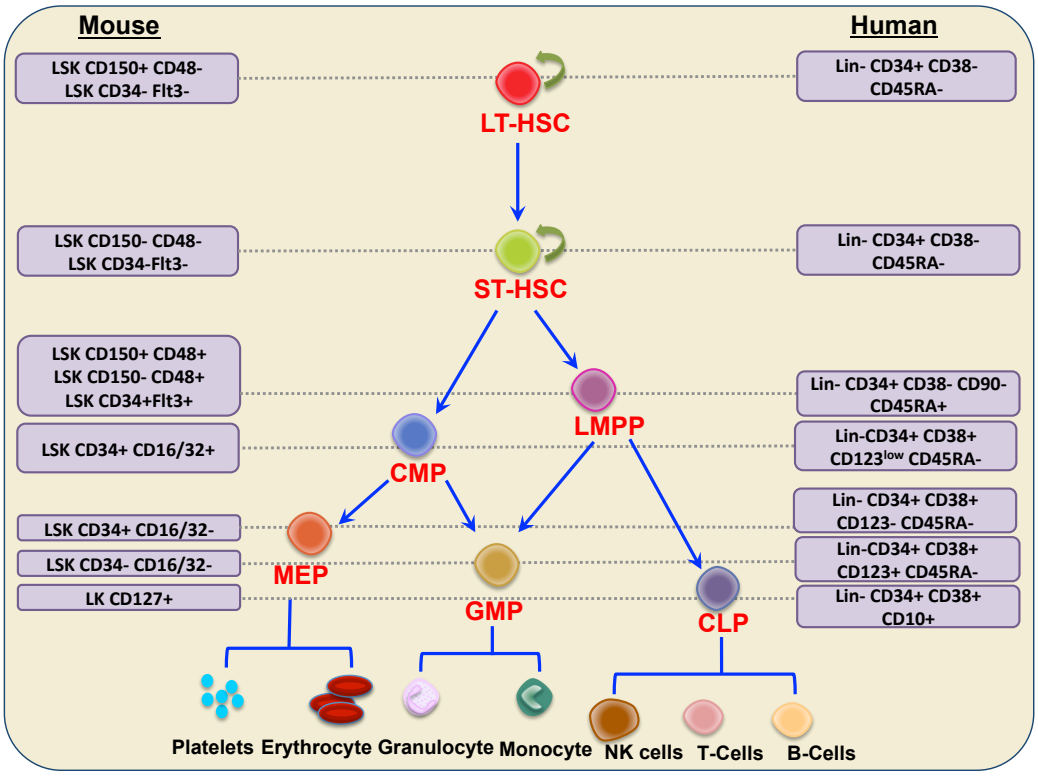



**Figure 1. 2: Schematic representation of haemopoietic hierarchy.**

LT-HSCs sit at the top of the differentiation hierarchy and maintain adult haemopoiesis. LT-HSCs self-renew and undergo division to give rise to downstream progenitors and eventually terminally differentiate to mature blood lineages. Immunophenotypic markers have been developed to identify HSCs and downstream hierarchical populations. LT-HSC-Long-term HSC; ST-HSC-Short-term HSC; LMPP-Lymphoid primed multipotent progenitor; CMP-Common myeloid progenitor; CLP-Common lymphoid progenitor; GMP-Granulocyte/macrophage progenitor; MEP-Megakaryocyte/erythroid progenitor; NK cells-Natural killer cells; CD-Cluster of differentiation; Lin-Lineage; LSK-Lineage$^-$Sca-1$^+$c-Kit$^+$ and LK-Lineage$^-$ c-Kit$^+$.

## 1.1.4 Heterogeneity of HSCs

Previous studies involving transplantation of limiting doses of BM and analysis of purified individual HSCs revealed functional heterogeneity in self-renewal, differentiation and repopulation potential (Abkowitz et al. 2000; Muller-Sieburg et al. 2002; Uchida et al. 2003; Takano et al. 2004; Sieburg et al. 2006; Dykstra et al. 2007). In addition, HSCs have been shown to possess heterogeneity at the molecular level with regard to gene expression (Glotzbach et al. 2011; Moignard et al. 2013). Muller-Sieburg et al defined the different subtypes of HSCs based on their contribution to donor-derived mature cells as myeloid-biased (My-bi), lymphoid-biased (Ly-

bi) or balanced (Bala) (Muller-Sieburg et al. 2012). Another study classified the HSCs based on their differentiation potential to become circulating mature blood cells, and resultantly termed HSCs as lymphoid-deficient (α), myeloid-deficient (γ and δ) or balanced (β) (Dykstra et al. 2007). Serial transplantation experiments showed that α- and β-HSCs retained long-term reconstitution potential and their distinctive differentiation properties (Dykstra et al. 2007; Benz et al. 2012). It has been reported that secondary clonal progeny resulting from α- and β-HSCs maintain the unique differentiation pattern (Dykstra et al. 2007; Dykstra et al. 2011; Muller-Sieburg et al. 2012). But in some instances, primary α-clones generate secondary β-clones and vice versa as shown by Benz et al (Benz et al. 2012). Recent evidence identified a distinct fraction of HSCs that expresses a megakaryocyte gene termed von Willebrand factor that are primed to generate platelets (Sanjuan-Pla et al. 2013). The study reported that platelet-primed HSCs are at the apex of the cellular hierarchy with self-renewal potential and ability to generate myeloid biased and lymphoid biased HSCs (Sanjuan-Pla et al. 2013). Based on the expression of cell surface marker-CD150, Morita et al showed that $CD150^{high}$ HSCs maintained long-term self-renewal properties and upon transplantation, generated heterogeneous sub-fractions of HSCs: $CD150^{med}$ and $CD150^{neg}$ (Morita et al. 2010). This study also showed that reduced CD150 expression was correlated with reduced erythroid/megakaryocyte differentiation (Morita et al. 2010).

Molecular mechanisms that influence the fate choice and clonal origin of different subtypes of HSCs were examined by high-throughput single cell gene expression analysis (Challen et al. 2010; Moignard et al. 2013). Studies revealed both cell-intrinsic and cell-extrinsic cues that differentially influence HSC subtypes (Challen et al. 2010; Benz et al. 2012; Moignard et al. 2013). TGF-β1 (Transforming growth factor- β1) exhibited differential effects on the HSC subtypes as it enhanced the proliferation of My-bi HSCs, but had an inhibitory effect on Ly-bi HSCs (Challen et al. 2010). A recent publication provided more insight into the differential gene expression profile of 18 key haemopoietic transcription factors (TFs) (Moignard et al. 2013). This study, with profiling of 597 single cells, showed that genes like *Erg, Lmo2* and *Meis1,* were differentially expressed in HSCs and progenitors,

while *Runx1* and *Fli1* had similar expression profiles across different cell types (Moignard et al. 2013). In addition, Moignard et al established a strong negative correlation between *Gfi1* and *Gata2* that was specific to HSCs (Moignard et al. 2013). In GMPs and CLPs, *Gfi1b* and *Gata1* negatively correlated with *Pu.1, Gfi1, Lyl1* and *Lmo2,* but not in primitive progenitors and MEPs (Moignard et al. 2013). This dynamic regulatory pathway illustrates the distinct correlation between TFs among HSCs and progenitor cells that are vital for cell fate transition. The BM microenvironment is suggested to play a role in favouring the enrichment of α-HSCs over ß-HSCs (Benz et al. 2012). Another study revealed that the DNA damage responsive gene, *Btaf* aided maintenance of α-HSCs compared to ß-HSCs, suggesting the role of epigenetic regulators in HSC heterogeneity (Wang et al. 2012). Together these studies suggest that the heterogeneity of HSC is determined by cell-intrinsic factors that are influenced by cell-extrinsic cues. However, more research is essential to understand the mechanisms regulating heterogeneity of the HSC population and its lineage commitment properties.

## 1.1.5 Immunophenotypic characterisation of HSCs and primitive progenitors

### 1.1.5.1 Murine HSCs and downstream progenitors

HSCs are identified by cell surface makers and their self-renewing capability (Spangrude et al. 1988; Ikuta and Weissman 1992; Morrison and Weissman 1994; Uchida et al. 1994; Morrison et al. 1997a; Kondo et al. 2003; Adolfsson et al. 2005; Yang et al. 2005). Fluorescence-activated cell sorting (FACS) is widely used to identify and isolate HSCs and progenitors based on their expression of surface markers, or cluster of differentiation molecules (CDs). Studies showed that heterogeneous populations of HSCs and primitive progenitors capable of self-renewal and long-term reconstitution were confined within the Thy-1.1$^{low}$ and Lineage$^{low/-}$Sca-1$^{+}$c-Kit$^{+}$ (LSK) compartment in the BM (Spangrude et al. 1988; Ikuta and Weissman 1992; Uchida et al. 1994). To further purify HSCs, several cell surface markers have been employed. Primarily, Thy-1.1$^{low}$ LSKs were purified as HSCs that lacked the expression of Mac-1 and CD4, while multipotent progenitors expressed low levels of Mac-1 and CD4 (Morrison and Weissman 1994;

Morrison et al. 1997a). Using Flt3 expression, LSKs categorised as LSK $Flt3^-$ with extensive self-renewal potential were identified (Christensen and Weissman 2001; Kondo et al. 2003; Adolfsson et al. 2005; Yang et al. 2005). Further, LSKs were demonstrated to acquire the expression of Flt3 (LSK $Flt3^+$) and differentiate to generate LMPPs with limited self-renewal potential (Christensen and Weissman 2001; Adolfsson et al. 2005). Also, chst 33342 (Ho), a fluorescent dye that stains live cells by DNA binding was used to differentiate the LSK side population (SP LSK) enriched for HSCs (Goodell et al. 1996). Murine HSCs were demonstrated to lack or express very low levels of CD34 (Osawa et al. 1996). Slam family markers, such as CD150, CD224 and CD48, were used to further classify LSKs into LT-HSCs ($CD150^+CD224^-CD48^-$), multipotent primitive progenitor ($CD150^-CD224^+CD48^-$) and committed progenitors ($CD150^+CD224^+CD48^+$ and $CD150^-CD224^+CD48^+$) (Kiel et al. 2005; Kim et al. 2006b). Additionally, cells with declining expression of CD150 exhibited gradual loss of self-renewal and reconstitution ability (Kiel et al. 2005; Papathanasiou et al. 2009; Morita et al. 2010; Oguro et al. 2013). Collectively, current literature describes the purification of HSCs and multipotent progenitors from LSKs by using combinations of either CD34 and Flt3 (Osawa et al. 1996; Kranc et al. 2009; Takubo et al. 2010) or CD150 and CD48 (Kiel et al. 2005; Yilmaz et al. 2006; Nakada et al. 2010; Buza-Vidas et al. 2011; Magee et al. 2012). Additional cell surface markers, such as CD244.2, CD229, CD127 and CD16/32, have been used and have shown that both HSCs and primitive progenitors possess heterogeneous populations with varying quiescence, self-renewal and differentiation potential (Kiel et al. 2007; Kent et al. 2009; Morita et al. 2010) (Figure 1.2).

#### 1.1.5.2 Human HSCs and downstream progenitors

Human HSCs, similar to murine HSCs (mHSCs), are classified based on their self-renewal and differentiation potential, using cell surface markers and functional assays (Civin et al. 1984; McCune et al. 1988; Baum et al. 1992; Krause et al. 1996). Early studies demonstrated that a heterogeneous population of HSCs expressing the CD34 cell surface marker displayed multipotency (Civin et al. 1984; Krause et al. 1996). More recent publication showed that self-renewing $Lin^-CD34^-CD38^-CD93^{hi}$ cells were placed above the

haemopoietic hierarchy (Anjos-Afonso et al. 2013). Isolating the lineage negative population and additionally considering cell surface markers CD38, CD90, Baum and colleagues showed that HSCs were enriched within $Lin^-CD34^+CD38^-CD90^+$ cells (Baum et al. 1992). In addition, HSCs expressing $Lin^-CD34^+CD38^-CD90^+$ cell surface markers included long-term culture-initiating cells (LTC-IC) and cells with repopulation potential in non obese diabetic-severe combined immunodeficient (NOD-SCID) mice (Baum et al. 1992; Huang and Terstappen 1994; Larochelle et al. 1996; Bhatia et al. 1997). Furthermore, the functional heterogeneity of $CD34^+CD38^-CD90^+$ HSCs was studied using xenotransplant models, which showed that the repopulation potential varied between clonal stem cells, and they also displayed extensive variability in the lifespan and proliferative capacity (Guenechea et al. 2001; McKenzie et al. 2006). Using CD45RA (an isoform of CD45 with repressive regulatory effects on some classes of cytokines), Majeti et al identified a multipotent progenitor population downstream of HSCs with restricted self-renewal properties ($Lin^-CD34^+CD38^-CD90^-CD45RA^-$) (Majeti et al. 2007). Moreover, Goardan et al identified a population of progenitor cells that functionally resembled LMPPs of mHSCs expressing $Lin^-CD34^+CD38^-CD90^-CD45RA^+$ (Goardon et al. 2011). More committed progenitors ($CD34^+CD38^+$) were differentiated based on CD10, CD123 and CD45RA into CMPs ($Lin^-CD34^+CD38^+CD123^{low}CD45RA^-$), MEPs ($Lin^-CD34^+CD38^+CD123^-CD45RA^-$), GMPs ($Lin^-CD34^+CD38^+CD123^+CD45RA^-$) and CLPs ($Lin^-CD34^+CD38^+CD10^+$) (Ogawa 1993; Galy et al. 1995; Manz et al. 2002) (Figure 1.2).

## 1.2 Haemopoietic TFs

Haemopoietic TFs play a key role in HSC fate decisions from self-renewal to differentiation and apoptosis (Pimanda et al. 2007; Challen et al. 2010; Pimanda and Gottgens 2010; Wilson et al. 2010; Moignard et al. 2013). TFs are connected to the wider regulatory network and several studies have explored the interaction between TFs and co-factors by an analysis of global expression of TFs (Pimanda et al. 2007; Pimanda and Gottgens 2010; Wilson et al. 2010; Novershtern et al. 2011). A recent publication on single cell gene analysis profiles of haemopoietic stem and progenitor cells (HSPCs) revealed more insight into TF regulatory pathways and their characteristic

expression in different cell types, contributing to the heterogeneity of HSCs and primitive progenitors (Moignard et al. 2013). The section below describes TFs that are key to haemopoiesis.

### 1.2.1 Homeobox TFs

*Hox* genes belong to the homeodomain-containing family of TFs and are important during embryogenesis (Gellon and McGinnis 1998; Wilson et al. 2010). The *Hox* gene cluster in mammals consists of 39 genes divided into four clusters (A-D) based on the chromosomal location (Amores et al. 1998). Specificity and selectivity of Hox TFs are modulated by binding to DNA co-factors, such as pre-B-cell leukaemia (Pbx) and myeloid ectopic insertion site (Meis) families (Moens and Selleri 2006). *Hox* gene expression is restricted to HSCs and progenitor cells and is important in HSC homeostasis (Argiropoulos and Humphries 2007) and Hox clusters display a lineage-restricted expression pattern (Giampaolo et al. 1994; Pineault et al. 2002). *HOXA* is expressed in the myeloid lineage, while *HOXB* is expressed in the erythroid and *HOXC* in lymphoid lineages (Giampaolo et al. 1994; Pineault et al. 2002). Irrespective of haemopoietic specific expression of *Hox* genes, loss of *Hoxb4* or the entire *Hoxb* cluster only mildly affected the self-renewal ability of HSCs and their lineage commitment (Thorsteinsdottir et al. 2002). This could be explained by the functional redundancy of *Hox* genes that have overlapping roles in HSC functions. However, *Hoxa9* is highly expressed in HSCs and genetic knockout studies showed defective myeloid and lymphoid lineages, along with severely impaired reconstitution ability, both in adult and foetal liver HSCs (Bijl et al. 2006; Magnusson et al. 2007). Additional studies revealed that loss of other *Hox* genes, like *Hoxa5, Hoxa7* and *Hoxc3,* led to defective committed progenitor compartments down the HSC hierarchy (Crooks et al. 1999; So et al. 2004). Gain-of-function studies showed that overexpression of *Hox* genes (*Hoxb3, Hoxb6, HOXA5, HOXA10*) resulted in expansion of HSCs and progenitor cells and led to block in differentiation (Sauvageau et al. 1997; Buske et al. 2001; So et al. 2004). Overexpression of *Hoxa9* led to an increase in proliferation of HSCs and myeloid progenitor cells with a partial block in pre-B cell development, eventually resulting in the development of leukaemia (Thorsteinsdottir et al. 2002).

Pbx and Meis families are important Hox cofactors and have three amino acid loop extension proteins that play an important role in embryonic development and haemopoiesis (Thorsteinsdottir et al. 2001; Moens and Selleri 2006). Pbx1 null mice are embryonic lethal and were shown to have severe anaemia due to haemopoietic defects, hence Pbx1 is required for dHSCs maintenance in the foetal liver (DiMartino et al. 2001). Conditional deletion of *Pbx1* in adult mice resulted in the progressive loss of LT-HSCs, due to reduced quiescence and defective self-renewal properties, possibly via a defective TGF-beta signalling pathway (Ficara et al. 2008).

Meis1 belongs to the TALE subfamily of homeobox proteins and interacts with Hox TFs, modulating their DNA binding ability. A loss-of-function approach reported that Meis1 deficient mice were embryonic lethal with defective early haemopoiesis causing haemorrhage and liver hypoplasia (Hisa et al. 2004). In *Meis1* deficient embryos, HSCs were decreased in the foetal liver with defective repopulation potential (Azcoitia et al. 2005). The conditional deletion of *Meis1* in adult mice resulted in loss of quiescence and repopulation potential of HSCs mediated through reactive oxygen species (ROS) (Kocabas et al. 2012).

### 1.2.2 Ets TFs

The Ets family of TFs are vital in regulating haemopoiesis and HSC functions and include TFs such as *Fli-1, Tel/Etv6, Pu.1, Elf4, GABP*$\alpha$ and Erg (Hock et al. 2004b; Lacorazza et al. 2006; Loughran et al. 2008; Kruse et al. 2009; Yu et al. 2011). Pu.1 (*Sfpi-1*) expression is restricted to HSCs and mature monocytes, granulocytes, B-cells, with low or no expression in CLPs, CMPs and T-cells (Nutt et al. 2005). The transcriptional activity of Pu.1 and *Pu.1*-dependent cell fate choices are guided by cell-specific expression of *Pu.1* and interaction of *Pu.1* with various TFs, including *GATA-1, GATA-2, Runx1, c-Jun, C/EBP*$\alpha$ and *C/EBPβ,* (Rekhtman et al. 1999; Zhang et al. 1999; Walsh et al. 2002; Hoogenkamp et al. 2007; Yeamans et al. 2007). Deletion of *Pu.1* is embryonic lethal with loss of mature lineages as well as reduced HSCs and progenitor cells (Scott et al. 1994; Kim et al. 2004). The conditional ablation of *Pu.1* in mice showed a reduction in monocyte and lymphoid lineages and an increase in granulocytic cells, revealing a *Pu.1* dose-dependent

regulation of cell fate choice in progenitor cells (Iwasaki et al. 2005). Additionally, an 80% decrease in the expression of Pu.1 protein, by deleting a 14kb URE element upstream of its promoter region, resulted in the development of AML (acute myeloid leukaemia) and defective cytokine response in HSCs and myeloid progenitors (Rosenbauer et al. 2004; Rosenbauer et al. 2006). These studies provide evidence that altered levels of TFs regulate HSC functions to cause detrimental effects on haemopoiesis, leading to leukaemia.

### 1.2.3 Core binding TF – *Runx1*

*Runt-related transcription factor 1* (*RUNX1*), also known as *acute myeloid leukaemia-1* (*AML1*), regulates the expression of various haemopoietic-specific genes, by binding to their consensus sequences TGTGGT or TGCGGT (Meyers et al. 1993). *Runx1* has been shown to be critical for embryonic haemopoiesis (Okuda et al. 1996; Chen et al. 2009; Boisset et al. 2010), but continuous expression of *Runx1* is dispensable for the maintenance of adult haemopoiesis, as shown by conditional knockout (CKO) studies (Ichikawa et al. 2004; Chan et al. 2009). However, contradictory studies showed that *Runx1*-conditional deletion resulted in HSC expansion and an increase in their long-term reconstitution potential (Motoda et al. 2007; Ichikawa et al. 2008). In agreement, another study showed that expression of a truncated isoform of Runx1 promoted HSC engraftment, while overexpression of full-length Runx1 resulted in the HSC loss (Tsuzuki et al. 2007). On the other hand, a recent study demonstrated that haemopoietic specific deletion of Runx1 had minimal effects on LT-HSCs (Cai et al. 2011). However, *Runx1* is one of the commonly mutated genes in AML (M0 FAB classification and myelodysplastic syndrome) and forms chimeric gene fusions that cause leukaemia (Osato et al. 1999; Imai et al. 2000; Yan et al. 2006; Ding et al. 2009). Further research is essential to enhance our understanding of the role of Runx1 in normal haemopoiesis.

### 1.2.4 Zinc finger TFs

A wide range of TFs, including *Gata2, Gata3, Evi-1, Klf4, Ikaros, Gfi1, Gfi1b, Sall4, Zfx* and *Prdm16,* contain zinc finger domains. The zinc finger

TFs play a critical role in regulating HSC homeostasis (Tsai et al. 1994; Orlic et al. 1995; Hock et al. 2004a; Zeng et al. 2004; Rodrigues et al. 2005; Galan-Caridad et al. 2007; Goyama et al. 2008; Ng et al. 2009; Yang et al. 2011; Ku et al. 2012).

*Growth Factor independent 1 (Gfi1*) is a transcriptional repressor that has been shown to maintain quiescence, self-renewal and reconstitution potential of HSCs (Hock et al. 2004a; Zeng et al. 2004). Gfi1$^{-/-}$ HSCs displayed enhanced proliferation and defective reconstitution during serial transplantation (Hock et al. 2004a). *Gfi1* and its paralogue *Gfi1b* exhibit complimentary expression patterns in haemopoietic lineages, but both TFs were expressed in HSCs (Yucel et al. 2004; Vassen et al. 2005). While *Gfi1* was shown to be important for development of lymphoid and myeloid lineages, *Gfi1b* was important for erythroid and megakaryocyte development (Moroy 2005; Hock and Orkin 2006; Anguita et al. 2010). Analysis of Gfi1b$^{-/-}$ mice demonstrated that HSCs lacking *Gfi1b* lost their quiescent state, but maintained the ability to self-renew and differentiate (Khandanpour et al. 2010). However, deletion of both *Gfi1* and *Gfi1b* led to a complete loss of HSCs (Khandanpour et al. 2010).

*Gata2* is expressed in HSCs, myeloid progenitors and mast cells and regulates the functions of HSCs and progenitor cells (Tsai et al. 1994; Orlic et al. 1995; Rodrigues et al. 2005). Gata2$^{-/-}$ mice are embryonic lethal with defective haemopoiesis and severe anaemia (Tsai et al. 1994; Tsai and Orkin 1997). Deletion of one allele of *Gata2* in Gata2$^{+/-}$ mice led to reduced HSCs with an increase in their apoptosis and increased quiescence (Rodrigues et al. 2005). Gata2$^{+/-}$ mice also showed specific reduction of GMPs with defective functionality, but no effect on other committed progenitors, such as CMPs or CLPs (Rodrigues et al. 2008). Overexpression of *Gata2* led to severe loss of HSCs and differentiation defects (Heyworth et al. 1999; Persons et al. 1999).

*Evi-1 (Ecotropic proviral integration site 1*) is a TF expressed specifically in HSCs with long-term reconstitution potential and has been shown to be important in regulating haemopoiesis (Mucenski et al. 1988; Goyama et al. 2008; Zhang et al. 2011). Evi1$^{-/-}$ mice were embryonic lethal with severe

defects in dHSCs (Yuasa et al. 2005). Conditional deletion of *Evi-1* in adult mice resulted in reduced HSCs, with loss of their quiescence and reconstitution potential, coupled with an increase in the number of cycling HSCs (Zhang et al. 2011). Expression levels of *Evi-1* are of prime importance in regulating HSC self-renewal (Goyama et al. 2008). Heterozygous deletion of *Evi-1* led to the partial loss of HSCs, whereas overexpression of *Evi-1* resulted in enhanced HSC self-renewal with reduced differentiation potential (Goyama et al. 2008). Evi1 acts as an oncoprotein and elevated expression of *Evi1* has been reported at high frequency in AML and in myelodysplasia (Buonamici et al. 2004; Jin et al. 2007). Evi1$^{-/-}$ transformed cells displayed loss of proliferation potential (Goyama et al. 2008). Thus, *Evi1* is a common regulator of normal and leukaemic HSCs.

### 1.2.5 Basic helix-loop-helix (bHLH) TFs

Stem cell leukaemia gene (*Scl*/*Tal1*) is a member of the bHLH family of TFs that play a vital role in the development of the vascular and central nervous systems and regulate haemopoiesis (Begley et al. 1989; Robb et al. 1995; Shivdasani et al. 1995). Gene profiling of HSCs revealed that increased expression of *Scl* and *Cdkn1a* was a characteristic feature of quiescent HSCs (Venezia et al. 2004). *Scl* is expressed at high levels in LT-HSCs and has been shown to impede G0-G1 transition in HSCs (Lacombe et al. 2010). In foetal liver and adult haemopoiesis, *Scl* forms a regulatory complex with TFs *Gata2* and *E2A*, along with co-factors *Lmo2* and *Ldb1* that are essential for normal HSC functions (Yamada et al. 1998; Li et al. 2011). *Scl* also regulates the short-term reconstitution potential of HSCs, but has no effect on their long-term reconstitution ability (Mikkola et al. 2003; Curtis et al. 2004). Conditional deletion of *Scl* impaired the differentiation potential, particularly of myeloid lineages (Mikkola et al. 2003). These studies suggest that Scl is critical in regulating HSC and progenitor functions. In human cells, low expression levels of *Scl* impaired the reconstitution property of HSCs when transplanted to SCID recipients (Brunet de la Grange et al. 2006).

*Lyl1*, a paralogue of *Scl,* is expressed in HSCs, myeloid and B-cell lineages (Capron et al. 2006). *Lyl1* deficient HSCs displayed defects in lymphoid lineage formation, but normal myeloid differentiation upon transplantation

(Capron et al. 2006). Conditional deletion of both *Lyl1* and *Scl* resulted in the loss of repopulation potential of HSCs and an increase in apoptotic HSCs and progenitor cells (Souroullas et al. 2009). This study revealed a genetic interaction between *Scl* and *Lyl1* and their partial overlapping roles in the maintenance of HSC functions (Souroullas et al. 2009). Additionally, bHLH E-proteins, E47 and E12 have been shown to be important in regulating HSC functions (Semerad et al. 2009).

Myc family members, *c-Myc*, *N-Myc* and *L-Myc,* are oncogenes (DePinho et al. 1987). Myc proteins play an important role in various biological processes, including proliferation, cellular growth, angiogenesis, differentiation and apoptosis, as well as in the reprogramming of adult fibroblasts to iPS cells (Eisenman 2001; Murphy et al. 2005; Lewitzky and Yamanaka 2007). Deletion of *c-Myc* or *N-myc* resulted in embryonic lethality, while overexpression of *Myc* promoted tumourigenesis (Charron et al. 1992; Pelengaris et al. 2002; Dubois et al. 2008). Conditional deletion of *c-Myc* in the adult BM led to impaired self-renewal properties of HSCs and resulted in their accumulation with defective differentiation (Wilson et al. 2004). The forced localisation of HSCs in the BM niche caused by the increased expression of adhesion receptors resulted in the accumulation of these mutant HSCs (Wilson et al. 2004). Deletion of both *c-Myc* and *N-myc* resulted in rapid lethality due to haemopoietic failure, including loss of HSCs (Laurenti et al. 2008). Hence, bHLH TFs are essential for normal functioning of HSCs.

## 1.3 Hypoxia

The placenta, respiratory and circulatory systems collectively contribute to the complex oxygen-regulating system in multicellular organisms (Bunn and Poyton 1996; Webb et al. 2009). During hypoxia, defined as the reduced availability of oxygen to cells, tissue or organism, the cells undergo adaptive mechanisms, such as altered expression of TFs or posttranslational modifications of proteins in order to survive the hypoxic conditions (Kaelin and Ratcliffe 2008; Simon and Keith 2008). The existence of low oxygen tension was revealed by the direct measurement of partial pressure of oxygen in the tissues during embryonic development (Mitchell and Yochim

1968). Physiological hypoxia in organs like thymus, kidney medulla and BM is defined as 1% (7.2mm Hg) or less partial pressure of oxygen ($pO_2$) compared to normoxic conditions with $pO_2$ ranging between 2-9% (14.4-64.8 mm Hg (Brahimi-Horn and Pouyssegur 2007; Simon and Keith 2008). The role of hypoxia in embryogenesis was first demonstrated by Morriss et al in 1979 (Morriss and New 1979). Hypoxia occurs during embryonic development as well as in adults (Schofield 1978; Cipolleschi et al. 1993; Braun et al. 2001; Mohyeldin et al. 2010). Hypoxic conditions are characteristic of specialised microenvironments, such as the niche, which harbours stem cells (Schofield 1978; Cipolleschi et al. 1993; Braun et al. 2001; Mohyeldin et al. 2010). The increase in characteristic features of stem cells, such as self-renewal and quiescence, under hypoxic culture conditions provides evidence that the $O_2$ gradient in the stem cell niche regulates stem cell dynamics (Krohn et al. 2008; Mohyeldin et al. 2010; Takubo et al. 2010; Winkler et al. 2010). However, recent studies have challenged the BM $O_2$ gradient in regulating HSC functions and showed that the hypoxic nature of HSCs was a cell-intrinsic phenomenon (Nombela-Arrieta et al. 2013). Hypoxia is also reported in pathological conditions, such as tissue inflammation and ischaemia and is associated with tumour formation (Simon and Keith 2008).

### 1.3.1 Hypoxic stem cell niche

The stem cell niche is composed of distinct cell types, blood vessels, glycoproteins and an intricate three-dimensional architecture that provides a complex microenvironment in which stem cells can function (Schofield 1978; Calvi et al. 2003; Zhang et al. 2003; Kiel et al. 2005; Scadden 2006; Morrison and Spradling 2008; Wang and Wagers 2011). The cellular and acellular components of the niche contribute to the maintenance of stem cell abilities of self-renewal and multilineage differentiation (Scadden 2006; Jones and Wagers 2008). Several years of intense research have been undertaken to characterise the HSC niche and its role in regulating HSC fate decisions (Schofield 1978; Calvi et al. 2003; Zhang et al. 2003; Kiel et al. 2005; Scadden 2006; Morrison and Spradling 2008; Wang and Wagers 2011). HSCs reside in the specialised niches, that are broadly classified as endosteal, vascular and perivascular niches, based on different cell types including osteoblasts (OBs), osteoclasts (OCs), monocytes and macrophages,

endothelial cells, mesenchymal stem cells (MSCs) and pericytes (Gong 1978; Schofield 1978; Calvi et al. 2003; Zhang et al. 2003; Kiel et al. 2005; Nilsson et al. 2005; Scadden 2006; Morrison and Spradling 2008; Ellis et al. 2011; Wang and Wagers 2011) (Figure 1.3).

#### 1.3.1.1 Endosteal niche

Several studies have supported the idea of localisation of primitive haemopoietic cells in close proximity to OBs (Gong 1978; Zhang et al. 2003; Nilsson et al. 2005; Ellis et al. 2011). Transgenic murine studies with targeted activation of OBs led to the expansion and increased mobilisation of HSPCs (Calvi et al. 2003; Zhang et al. 2003). Early studies reported that ablation of OBs resulted in haemopoietic defects, including reduced BM cellularity and HSPCs with induction of extramedullary haemopoiesis in the spleen, while expansion of OBs resulted in elevated HSPC numbers (Zhang et al. 2003; Visnjic et al. 2004). These studies support the osteoblastic localisation of HSPCs. Other studies demonstrated that the HSC homeostasis was supported by immature OBs and not the mature OBs, hence providing evidence of MSC activity as a regulator of HSC functions (Calvi et al. 2012). However, several publications showed that impairment of OBs alone was not sufficient to disrupt HSC functions (Lymperi et al. 2008; Ma et al. 2009; Schepers et al. 2012), suggesting that the effect of OBs on HSCs was influenced by additional factors, including the specific stage of OB differentiation and their mode of activation (Nakamura et al. 2010). Live imaging studies of the calvarium following induced activation of OBs showed closer association of HSC-enriched cells with the endosteum compared to the controls (Lo Celso et al. 2009). Together, these studies provide evidence of endosteal localisation of HSCs.

OCs that belong to the osteolineage cell type have a complex role in the maintenance of HSCs (Mansour et al. 2012). Kollet and colleagues reported that an increase in OC activity by G-CSF administration resulted in HSPC mobilisation (Kollet et al. 2006). However, in contradiction, a recent study showed that increased osteocalcination by serial treatment with G-CSF reduced the HSPC mobilisation (Miyamoto et al. 2011). Hence, more research investigating OCs' role within the HSC niche is essential.

Macrophages and monocytes in the endosteal niche are associated with HSPC mobilisation in response to the G-CSF administration, thus contributing to the localisation of HSCs within their niche (Winkler et al. 2010). Recent investigation discovered that BM macrophages expressing $\alpha$-smooth muscle actin are involved in the maintenance of HSCs in the endosteal niche (Ludin et al. 2012).

#### 1.3.1.2 Vascular niche

Endothelial cells have been shown to play an important role in developmental haemopoiesis and in regulating HSC functions (Medvinsky and Dzierzak 1996). Kiel et al showed that HSCs were in close proximity to BM sinusoids (Kiel et al. 2005). Additional studies reported that depletion of endothelial cells impaired the transplantability of HSCs (Hooper et al. 2009; Butler et al. 2010). Transgenic mice studies showed that the interactions of endothelial cells and HSCs were mediated by notch signalling (Duncan et al. 2005). Co-culture of HSCs with immortalised endothelial cells enhanced their expansion (Soki et al. 2013). Altogether, these studies supported the hypothesis of endothelial cells forming the unique HSC niche.

Perivascular stromal cells are defined by the heterogeneous population of cells, including Nestin+ mesenchymal cells, *Scf (Stem cell factor)*-expressing perivascular cells and Cxcl12-abundant reticular cells (CAR) (Barker 1997; Sugiyama et al. 2006; Mendez-Ferrer et al. 2010; Ding et al. 2012). Gene expression studies showed that the *Scf*-expressing perivascular cells had high levels of *Cxcl12, alkaline phosphatase, Vcam1, Pdgfra,* and *Pdgfrb* expression, thus attributing to their origin from mesenchymal and stromal cells (Ding et al. 2012). Gene deletion studies in murine models showed that the ablation of *Scf* expression from $Tie2^{+}$ endothelial and $Lepr^{+}$ perivascular cells specifically impaired HSC functions (Ding et al. 2012). *CxCl12* is expressed in all stromal cells in the BM niche (Greenbaum et al. 2013). However deletion of *Cxcl12,* specifically from early mesenchymal progenitor cells (negative for nestin expression), led to detrimental loss of HSCs with reduced quiescence and defective long-term reconstitution ability (Greenbaum et al. 2013). Deletion of *CxCl12* from OBs, CAR cells or endothelial cells had no or modest effect on HSC functions (Greenbaum et

al. 2013). Thus, it is evident that the perivascular niche is heterogeneous and has a varied effect on HSC maintenance based on the cell type.

MSCs are implicated as a component of the HSC niche (Masuda et al. 2009; Mendez-Ferrer et al. 2010). Published evidence suggests that nestin$^+$ MSCs regulate HSC maintenance genes, like *Cxcl12, c-Kit ligand, angiopoietin-1 (Ang-1), interleukin-7 (IL-7),* and *vascular cell adhesion molecule-1* (Mendez-Ferrer et al. 2010). The depletion of nestin$^+$ MSCs resulted in reduced HSCs and displayed extramedullary haemopoiesis in mice (Mendez-Ferrer et al. 2010). Additionally, purified HSCs when transplanted were shown to home in close proximity to nestin$^+$ MSCs in lethally irradiated mice (Mendez-Ferrer et al. 2010). Human HSPCs co-cultured with MSCs showed enhanced self-renewal and better engraftment potential in non-human primates (Masuda et al. 2009).

#### 1.3.1.3 Periendosteum-perivascular HSC niche

Advanced live-imaging studies of mouse calvarium have revealed the co-existence of both endosteal and vascular cells forming the periendosteal-perivascular HSC niche (Lo Celso et al. 2009). Another recent publication by Nombela-Arrieta et al showed the vascular architecture of the HSC niche to be highly vascularised at proximal bone regions by laser scanning microscopy and confocal imaging techniques (Nombela-Arrieta et al. 2013). This study also reported enriched HSPCs in the endosteal zone with close association to the BM perivasculature region (Nombela-Arrieta et al. 2013). Altogether, these studies provide evidence that the endosteal and vascular cells co-exist forming the specialised HSC niche.

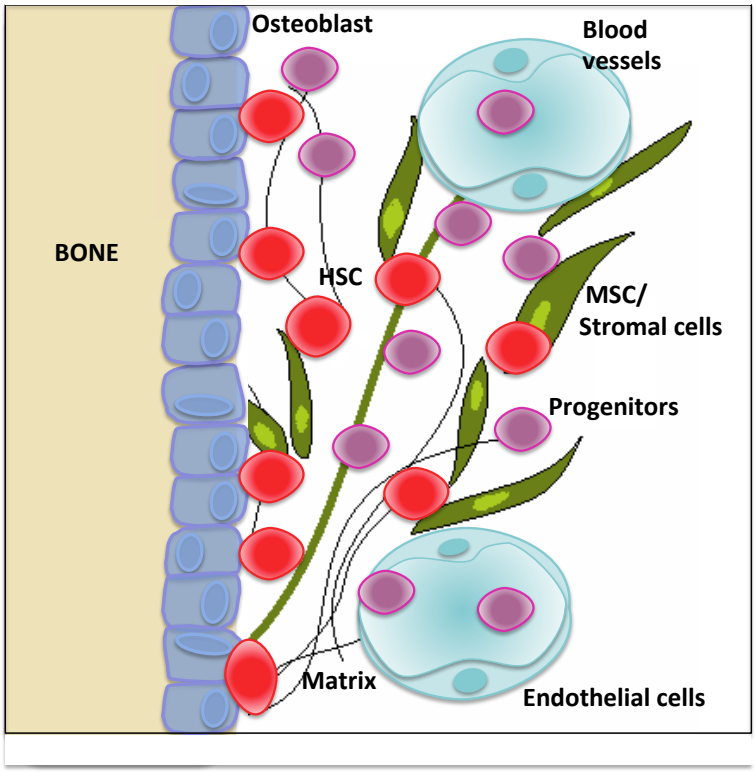



**Figure 1. 3: The HSC niche.**

HSPCs are localised in the specialised BM microenvironment referred to as the HSC niche. The HSC niche consists of various cell types including osteoblasts, osteoclasts, mesenchymal stem cells (MSCs) and endothelial cells along with blood vessels.

## 1.3.2 Hypoxia and HSCs

HSCs are localised in the niche within the BM, which exhibits low $pO_2$ compared to other tissues (Harrison et al. 2002; Takubo and Suda 2012). The HSC niche is characterised by the endosteal zone, vascular and perivascular zones, with several cell types, including OBs, OCs, endothelial, MSCs and reticular cells (Gong 1978; Zhang et al. 2003; Kiel et al. 2005; Lo Celso et al. 2009; Mendez-Ferrer et al. 2010; Omatsu et al. 2010; Ellis et al. 2011; Suda et al. 2011; Nombela-Arrieta et al. 2013) (Figure 1.3). The three dimensional vascular organisation of the BM examined with the scanning electron microscope revealed an enlarged canonical arrangement of arterial capillaries in the sinusoids enabling easy movement of HSCs (Draenert and Draenert 1980). The arterial capillaries were shown to arise from the arterioles that were formed by the branching of the smaller arteries from the metaphysis region in the BM niche (Draenert and Draenert 1980). This

unique vascularisation of the endosteal niche in the BM attributed to the limited perfusion and low $pO_2$ in the endosteal region of the BM (Draenert and Draenert 1980). Additionally, due to abundant haemopoietic cells in the BM the blood flow is coupled with high $O_2$ consumption (Chow et al. 2001). Live animal imaging of the BM in the calvarium of mice showed that the transplanted HSC-enriched cells migrate deep into the BM that is believed to be hypoxic (Lo Celso et al. 2009). Furthermore, the localisation of transplanted HSPCs closer to the endosteum improved their engraftment potential (Lo Celso et al. 2009). These data suggested the importance of niche components in regulating HSC functions (Lo Celso et al. 2009). Further studies in humans showed that the average $pO_2$ in BM aspirates of healthy individuals measured 55mmHg ± 0.95 and mean $O_2$ saturation of 87.5% ± 1.1% (Harrison et al. 2002). Simulation studies of $O_2$ diffusion in the BM suggested a 90% drop in the $pO_2$ in close proximity of 100μm from the BM vasculature (Chow et al. 2001). A more recent publication demonstrated that the peri-sinusodial niche is the most hypoxic with lower $pO_2$ value compared to the endosteal niche in the calvarium of the mice (Spencer et al. 2014). These studies provide evidence supporting the hypoxic nature of the HSC niche.

The hypoxic nature of BM haemopoietic cells was analysed by injecting mice with a perfusion dye, pimonidazole (Pimo) and nucleic acid dye Ho (Parmar et al. 2007; Simsek et al. 2010; Takubo et al. 2010). Pimo is a 2-nitroimidazole-hypoxia probe, that when reduced in a hypoxic environment, forms hydroxylamine that covalently binds to thiol-containing proteins in hypoxic cells. The protein adducts can be detected by immunostaining (Varia et al. 1998). These experiments showed that the lowest end of the Ho perfusion dye gradient was enriched with HSCs and these low perfused cells highly retained the Pimo suggesting the hypoxic nature of the HSCs (Parmar et al. 2007; Simsek et al. 2010; Takubo et al. 2010). HSCs that were localised in the low-oxygenated niche remained quiescent and expressed high levels of *Notch1, N-cadherin*, telomerase and cell cycle inhibitor *p21,* compared to cells that were close to the well-oxygenated vasculature (Jang and Sharkis 2007). In agreement with the above study, human $CD34^+CD38^-$ cord blood cells cultured in 1% $O_2$ re-acquired the hypoxic property before

induction of quiescence and exhibited improved reconstitution potential with enhanced expression of *p21* when transplanted into immunodeficient mice (Danet et al. 2003; Shima et al. 2009; Shima et al. 2010). Also, under extreme hypoxic conditions (0.1%), $CD34^+$ cells acquired a quiescent state (Hermitte et al. 2006). ROS generated through $O_2$ mediated mitochondrial electron transport chain or NADPH oxidase during cytosolic or phagosomal activity regulates stem cell functions (Jang and Sharkis 2007). The hypoxic niche favours the immature state of HSCs by limiting the production of ROS to low levels (Jang and Sharkis 2007; Simsek et al. 2010). A more detailed description of the hypoxic nature of HSCs and their niche is included in Chapter 4.

## 1.4 Hypoxia inducible factors (Hifs)

Hypoxia inducible factor (Hifs) are evolutionarily conserved TFs that are the key regulators of oxygen homeostasis in all mammalian cells (Kaelin and Ratcliffe 2008; Majmundar et al. 2010). The elevated levels of *erythropoietin* (*EPO*) production in renal fibroblasts at high altitude or during anaemia led to the discovery of a protein complex that bound at the 3' end of the *EPO* gene, specifically during hypoxia, and was named *HIF-1* (Semenza and Wang 1992). Hifs consist of two subunits, an $\alpha$– subunit that is highly unstable and dependent on oxygen concentration and a stable, oxygen-independent ß-subunit (Wang et al. 1995). In mammals, there are three isoforms of Hifs, namely *Hif-1*$\alpha$, *Hif-2*$\alpha$ and *Hif-3*$\alpha$ that form dimers with the *Hif-1ß* paralogue, Arnt1, Arnt2 and Arnt3 (Ema et al. 1997; Flamme et al. 1997; Hogenesch et al. 1997; Tian et al. 1997; Gu et al. 1998; Semenza 2000; Wenger 2002). Unlike the less-studied *Hif-3*$\alpha$, *Hif-1*$\alpha$ and *Hif-2*$\alpha$ have been found to be critical for the response to changing oxygen concentrations (Kaelin and Ratcliffe 2008). Although, the role of *Hif-3*$\alpha$ is not well understood, it has been reported that alternative splice forms of *Hif-3*$\alpha$ bind to *Hif-1*$\alpha$ and block its transcriptional activity (Makino et al. 2001; Wenger 2002). Hif-mediated pathways have been extensively studied and have been shown to regulate metabolic adaptation and influence various cellular mechanisms, including cell growth, survival, differentiation

and apoptosis, erythropoiesis and angiogenesis (Weidemann and Johnson 2008; Majmundar et al. 2010).

### 1.4.1 Structure of Hif heterodimers

HIFs are heterodimers with a DNA binding protein complex composed of a basic helix-loop-helix (bHLH) domain and a PAS (PER-ARNT-SIM) protein domain that are associated with DNA binding and dimerisation (Wang et al. 1995). The heterodimer consists of a 150 kDa hypoxia-inducible, unstable $\alpha$-subunit and a stable nuclear ß-subunit of size 91-94 kDa (Aryl hydrocarbon nuclear translocator, ARNT). Both $\alpha$ and ß subunits bind to DNA of the target genes at the specific core sequence (G/ACGTG), termed the hypoxia responsive element (HRE) (Wang et al. 1995). Hif-$\alpha$ isoforms contain two independently functioning central oxygen-dependent degradation domains (ODDD) namely, the amino-terminal ODD domain (NODDD) and carboxy-terminal ODD (CODDD) domain. Hif-1$\alpha$ and Hif-2$\alpha$ also possess an N-terminal transactivation domain (NAD) and C-terminal transactivation domain (CAD), while the Hif-3$\alpha$ isoform lacks the CAD (Jiang et al. 1997; Pugh et al. 1997; Gu et al. 1998; Huang et al. 1998; O'Rourke et al. 1999; Hara et al. 2001). Hif-3$\alpha$ is less closely related with N-terminal bHLH and PAS domains, but lacks TAD (transactivation domain) (Makino et al. 2001). Both Hif-1$\alpha$ and Hif-2$\alpha$ share a degree of sequence homology and a similar structural domain with subtle variances in the transactivation domain that contribute to their unique characteristics (Wiesener et al. 2003; Dayan et al. 2006; Hu et al. 2007). Hif-1$\alpha$ and Hif-2$\alpha$ differ in the NAD region that determines the expression of specific target genes of the two isoforms (Hu et al. 2007). However, CAD is homologous in both Hif-1$\alpha$ and Hif-2$\alpha$ and is involved in the binding of transcriptional co-factors, such as p300/CBP, activating transcription of common target genes (Hu et al. 2007).

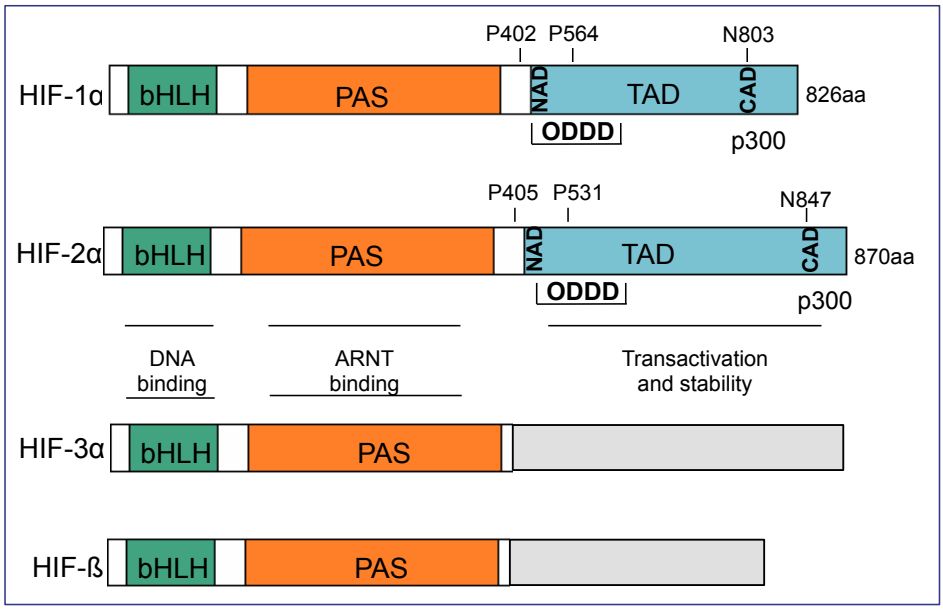



**Figure 1. 4: Structure of the Hif protein-α and β subunits of Hifs.**

Hifs are heterodimers with oxygen sensitive, highly unstable Hif-α subunits and a stable Hif-ß subunit. Both the isoforms have a DNA binding domain however, Hif-3α and Hif-ß subunits lack a transactivation domain. bHLH-basic helix-loop-helix; PAS-Per-Arnt-Sim; ODDD- oxygen dependent degradation domain; NAD- N-terminal transactivation domain; CAD-C-terminal transactivation domain; aa-amino acid.

## 1.4.2 Mechanism of regulation of Hif signalling

The Hif-α subunit is transcribed constitutively and the translated protein is highly unstable in normoxia with a half-life of 5-8 minutes (Huang et al. 1996; Berra et al. 2001). Despite the differences, both Hif-1α and Hif-2α isoforms share similar regulatory mechanisms that are reflected in their similar domain structures (Schofield and Ratcliffe 2004) (Figure 1.4). Hif-α protein levels are strictly regulated by both oxygen-dependent and oxygen-independent mechanisms.

### 1.4.2.1 Oxygen-dependent mechanisms of Hif-α regulation

Under well-oxygenated conditions, Hif-α subunits undergo proteasomal degradation by the ubiquitin-dependent pathway (Schofield and Ratcliffe 2004). In normoxia, Hif-α is hydroxylated by prolyl hydroxylase domain family (PHDs) members at conserved proline residues (in Hif-1α, Pro402 and Pro564 and in Hif-2α, Pro405 and Pro531) within the ODDD, positioned in the

NAD region (Epstein et al. 2001; Ivan et al. 2001; Jaakkola et al. 2001; Masson et al. 2001; Schofield and Ratcliffe 2004). Hydroxylation of specific prolyl residues by PHDs prompts the binding of von-Hippel-Lindau (pVHL) (a tumour suppressor protein) and other components of the ubiquitin ligase complex, including elongin B, elongin C, pVHL, RBX-1 and Cul2. The binding of the ubiquitin ligase complexes to Hif-$\alpha$ subunits results in polyubiquitination and proteasomal degradation of Hif-$\alpha$ (Maxwell et al. 1999; Ohh et al. 2000; Ivan et al. 2001; Jaakkola et al. 2001; Masson et al. 2001) (Figure 1.5). Multiple isoforms of PHDs (PHD1, PHD2 and PHD3) have been identified and the activities of these dioxygenases are dependent on the availability of oxygen and 2-oxoglutarate, as well as co-factors like iron (Fe-II) and ascorbate (Bruick and McKnight 2001; Epstein et al. 2001).

Hif-$\alpha$ stability is also mediated by nitric oxide (NO) and Krebs cycle metabolic intermediates, such as fumarate, succinate and ROS that inhibit PHDs (Brunelle et al. 2005; Kaelin 2005; Koivunen et al. 2007; Kaelin and Ratcliffe 2008). Under hypoxic conditions, E3 ubiquitin ligases, Siah1/2 mediate the proteasomal degradation of PHDs, thereby stabilising Hif-$\alpha$ expression (Nakayama et al. 2004) (Figure 1.5).

Factor inhibiting HIF (FIH1), another member of the Fe(II) and 2-OG-dependent dioxygenase family regulates Hif-$\alpha$ stability by hydroxylation of conserved single asparaginyl residue in the CAD domain of Hif-$\alpha$ (Mahon et al. 2001; Hewitson et al. 2002; Lando et al. 2002) (Figure 1.5). The hydroxylation at the CAD domain of Hif-$\alpha$ prevents the binding of the essential co-activator p300/Creb-binding protein (CBP/p300) and inhibits the transcription of CAD dependent genes that regulate adaptive cellular responses to hypoxia (Mahon et al. 2001; Hewitson et al. 2002; Lando et al. 2002). FIH1, unlike PHDs, is active in low-oxygen concentration and hence regulates Hif-$\alpha$ protein degradation that survived hydroxylation by PHDs (Koivunen et al. 2004; Dayan et al. 2006). Also, Hif-1$\alpha$ isoform is relatively less resistant to FIH1-mediated hydroxylation compared to Hif-2$\alpha$ (Yan et al. 2007). Hence factors including depth of hypoxia and the requirement of different isoforms of Hif-$\alpha$ determine the activation of HIF-responsive genes.

Under hypoxic conditions, or during loss of PHDs or pVHL, Hif-α stabilises and relocates to the nucleus where the Hif-ß isoform dimerises with Hif-α by binding to the HRE element in its promoter region (Wenger et al. 2005). The Hif-α and Hif-ß dimerisation is coupled with the binding of transcriptional co-factor, p300/CBP to the CAD domain of the Hif-heterodimer that activates the transcription of a specific subset of genes involved in angiogenesis, erythropoiesis, energy metabolism and autophagy (Firth et al. 1994; Ebert et al. 1995; Firth et al. 1995; Gleadle et al. 1995; Liu et al. 1995; Arany et al. 1996) (Figure 1.5).

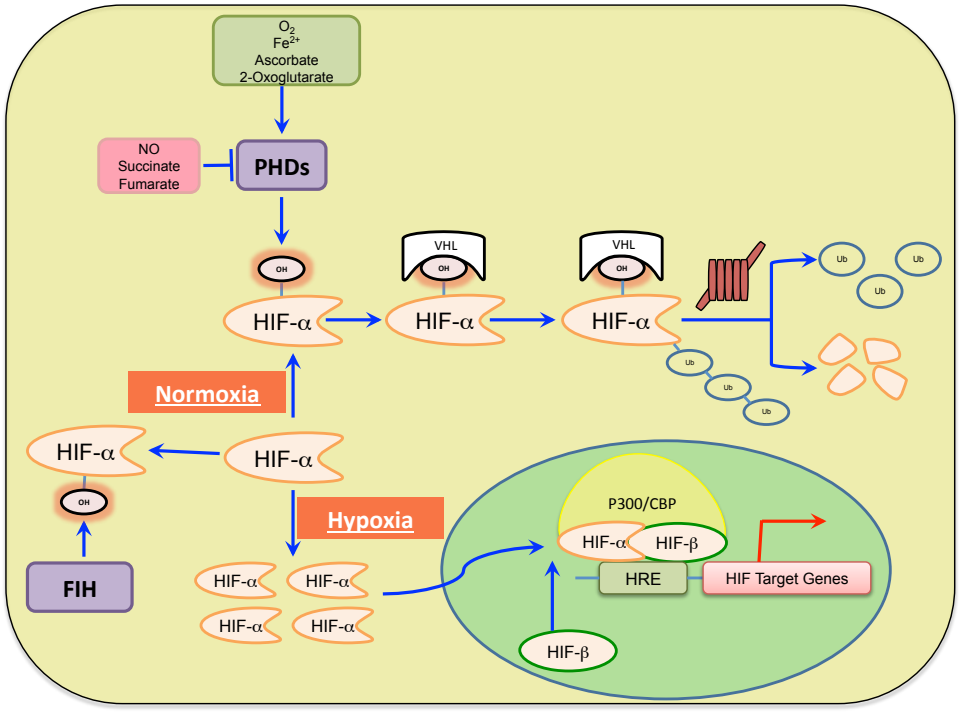



**Figure 1. 5: Oxygen-dependent regulation of Hif-α protein and downstream target genes.**

Under normoxic conditions, Hif-α subunit undergoes proteolitic degradation mediated by Hif prolyl hydroxylases (PHDs) and factor inhibiting Hifs (FIH). Whereas during hypoxia, stabilised Hif-α is translocated to the nucleus. Inside the nucleus, the two isoforms of Hif-α and Hif-1β form heterodimers along with p300/CBP and activate the transcription of various target genes involved in vital developmental pathways. HIF=Hypoxia inducible factor; VHL=von-Hippel-Lindau protein; OH=Hydroxylase; NO=Nitric oxide; $O_2$=Oxygen; $Fe_2^+$=Iron; Ub=Ubiquitin; HRE=Hypoxia response element.

### 1.4.2.2 Other mechanisms of Hif-α regulation

Several pVHL-independent pathways regulate Hif-α stability (Isaacs et al. 2002; Kong et al. 2006; Luo et al. 2010). Inhibitors of Hsp90 (an extracellular heat shock protein) and histone acetylase inhibitors promote pVHL-independent Hif-α protein degradation (Isaacs et al. 2002; Kong et al. 2006). Evidence also exists that Hsp70/CHIP regulates degradation of Hif-1α, but not Hif-2α in hypoxia (Luo et al. 2010).

Hif-1α-specific E3 ligase co-factor, termed hypoxia-associated factor (HAF), exhibits a unique domain-specific binding to Hif-α isoforms, hence differentially regulates the two Hif-α isoforms. The binding of HAF (654-800 aa) and Hif-1α (298-400 aa) leads to Hif-1α protein degradation, but binding of Hif-2α (604-750 aa) and HAF (300-500 aa) results in increased transactivation of Hif-2α (Koh et al. 2011). Additional studies using cell lines derived from normal and tumour tissues reveal that the binding of HAF and Hif-1α results in the ubiquitination and proteasomal degradation of Hif-1α (Koh et al. 2008).

Additionally, Hif-α can be sumoylated, which has been shown to be associated with both increased and decreased Hif-α stability (Carbia-Nagashima et al. 2007; Cheng et al. 2007). Hif-α may undergo sumoylation by binding to hypoxia induced small ubiquitin like modifier (SUMO) feeding to the oxygen-dependent pVHL-mediated degradation pathway in a hydroxylation-independent manner (Cheng et al. 2007). It has been shown that SUMO1 is induced by hypoxia and that hypoxic responses are modified by sumoylation of Hif-α (Comerford et al. 2003). Additional studies using mouse models lacking SUMO-specific protease 1 (SENP1) display a lack of stabilised Hif-α causing anaemia (Cheng et al. 2007).

Sirtuins (Sirts) are histone deacetylases that belong to the nicotinamide adenine dinucleotide ($NAD^+$)-dependent stress responsive family (Haigis and Sinclair 2010). Dioum et al showed that Sirt1 modulated the expression of Hif-2α by deacetylation of a lysine residue in Hif-2α protein (Dioum et al. 2009). Sirt1 has been shown to be involved in the regulation of *Epo*

expression in the hepatic and renal systems mediated via *Hif-2α* (Dioum et al. 2009). Sirt1 has been reported to interact with the *Epo* promoter and *Hif*-2α resulting in the increased transcription of Hif-2α (Dioum et al. 2009). On the other hand, Sirt6 has been shown to negatively regulate *Hif-1α* stability and protein synthesis (Zhong et al. 2010). Although a weak interaction of Hif-1α and Sirt6 has been established, their interaction mechanisms are yet to be defined (Zhong et al. 2010).

### 1.4.3 Tissue expression and transcriptional targets of Hifs

Irrespective of the similarities in the structure and regulatory mechanisms of the Hif-α isoforms, their tissue specific expression patterns are distinct (Ema et al. 1997; Tian et al. 1997; Wiesener et al. 2003; Gordan et al. 2007; Mole and Ratcliffe 2008). *Hif-1α* is ubiquitously expressed, while the expression of *Hif-2α* is more restricted to kidney, heart, lung and small intestine (Ema et al. 1997; Tian et al. 1997; Wiesener et al. 2003; Gordan et al. 2007). A recent study showing the microarray analysis of gene expression in human embryonic stem cells (ESCs), HSCs, and MSCs discovered *HIF-1α* as the only TF common in these stem cells (Kim et al. 2006a). Expression of *Hif-1α* and *Hif-2α* in some organs like kidney are cell-type specific (Rosenberger et al. 2002). *Hif-2α* is expressed in kidney endothelial cells and Epo-producing interstitial fibroblasts, while *Hif-1α* is expressed only in tubular epithelial cells (Rosenberger et al. 2002). In the lung, under hypoxic conditions *Hif-1α* is undetectable, while *Hif-2α* is stabilised in type II pneumocytes and pulmonary endothelial cells (Compernolle et al. 2002; Wiesener et al. 2003). *Hif-1α* is expressed at very high levels in adult HSCs compared to more differentiated populations (Simsek et al. 2010; Takubo et al. 2010). *Hif-2α* is expressed in HSCs and progenitor cells with high levels of expression in the lineage depleted population (Takubo et al. 2010).

Hif-α subunits regulate the expression of hundreds of downstream genes involved in angiogenesis, proteolysis, metabolism, proliferation and survival, autophagy and pH regulation (Lee et al. 2004; Ke and Costa 2006; Mazure and Pouyssegur 2010). *Hif-1α* specifically stimulates transcription of glycolytic enzymes, such as phosphoglycerate kinase and lactate

dehydrogenase-A, carbonic anhydrase-9 and *Bnip-3* (pro-apoptotic gene), glucose transporters and glycolytic inducing factors like *Cripto* (Hu et al. 2003; Hu et al. 2006; Miharada et al. 2011). Several *Hif-1α* target genes are known to be critical in HSC function and metabolism, such as *Vegf, ADM, SDF1, Scf, Ang2, p53, Foxos* and *Cited2* (Bhattacharya and Ratcliffe 2003; Freedman et al. 2003; Chen et al. 2007; Kranc et al. 2009; Takubo et al. 2010; Chou et al. 2012; Du et al. 2012; Zhang et al. 2012; Zhang and Sadek 2013). Also, TFs like SDF1, p21, Foxos and Cripto, co-ordinate HSC quiescence in the BM niche (Cheng et al. 2000; Miyamoto et al. 2007; Nie et al. 2008; Thoren et al. 2008; Miharada et al. 2011; Zheng et al. 2011). Under hypoxia, *Hif-2α* stimulates the expression of embryonic TF Oct-4, Cyclin D1, TWIST1, transforming growth factor-α (TGF-α) and Epo (Raval et al. 2005; Covello et al. 2006; Patel and Simon 2008). Both *Hif-1α* and *Hif-2α* have unique target genes, but also jointly regulate *Vegf, adrenomedullin (Adm)* and *Glucose transporter* (*Glut-1*) (Wiesener et al. 1998; Hu et al. 2003).

## 1.4.4 Differential functions of Hif- α isoforms

### 1.4.4.1 Hif regulatory system in embryonic development and early haemopoiesis

Hypoxia and Hif signalling play crucial roles in embryonic development and haemopoiesis during ontogeny (Maltepe et al. 1997; Iyer et al. 1998; Ryan et al. 1998; Tian et al. 1998; Adelman et al. 1999; Peng et al. 2000; Compernolle et al. 2002; Yoon et al. 2006). A null mutation of *Hif-1ß* isoform in mice was found to be embryonic lethal at E10.5 with defective angiogenesis and haemopoiesis (Maltepe et al. 1997; Adelman et al. 1999). Furthermore, *in vitro* colony-forming assay from Hif-1ß$^{-/-}$ embryoid bodies showed a significant reduction in haemopoietic progenitor cells that failed to respond to hypoxia (Adelman et al. 1999). Hif-1ß$^{-/-}$ ESCs displayed a lack of hypoxic responses and *Hif-1ß* deficient embryos had decreased haemopoietic progenitors in the yolk sac (Maltepe et al. 1997; Adelman et al. 1999). Adelman et al reported that defective haemopoietic progenitors in the yolk sac were due to a decease in cell-extrinsic *Hif-1ß*-dependent *Vegf* expression (Adelman et al. 1999; Schuh et al. 1999). Similar defects

were observed in the Flk-1 (*Vegf* receptor) null mice with reduced number of ESCs and an expansion of haemopoietic progenitors (Schuh et al. 1999).

It was also demonstrated that Hif-1$\alpha$ knockout mice displayed multiple defects, including neural tube malformation, cardiovascular defects and haemopoietic defects, with enhanced tissue hypoxia resulting in embryonic lethality at E10.5 (Iyer et al. 1998; Ryan et al. 1998; Yoon et al. 2006). The analysis of yolk sac at E9.5 revealed a decrease in myeloid and erythroid progenitors, as well as impaired terminal erythroid differentiation (Yoon et al. 2006). Furthermore, Hif-1$\alpha^{-/-}$ embryos had reduced haemoglobin content in erythroid colonies (Yoon et al. 2006). In addition, gene expression analyses revealed dysregulated expression of *Epo, EpoR, Vegf* and iron regulatory proteins in the Hif-1$\alpha^{-/-}$ yolk sac and embryos (Yoon et al. 2006). Another study showed that Vhl-deficient embryos, an upstream regulator of *Hif-*$\alpha$, died at the age of E10.5 to E12 due to severe placental dysgenesis (Gnarra et al. 1997). Other studies showed that a heterozygous deletion of *Hif-1*$\alpha$ in mice resulted in impaired physiological responses to chronic hypoxia and displayed defective carotid body function (Yu et al. 1999; Kline et al. 2002).

To understand the role of *Hif-2*$\alpha$ during early development, knockout mouse models were created. Deletion of *Hif-2*$\alpha$ generated a range of phenotypes, from embryonic lethality to perinatal death, depending on the mouse genetic background (Peng et al. 2000). Global deletion of *Hif-2*$\alpha$ led to embryonic lethality between E9.5 and E12.5 due to defective vasculature in the yolk sac and embryos (Peng et al. 2000). Hif-2$\alpha$ knockout mice died at mid-gestation due to bradycardia and decreased noradrenaline levels (Tian et al. 1998). An exogenous substitution of a metabolic intermediate that is converted to noradrenaline were born though dint survive to the adulthood (Tian et al. 1998). The above study, along with a study by Norris et al, who showed *Hif-2*$\alpha$ expression in the neural crest derivatives, collectively suggested that *Hif-2*$\alpha$ was involved in the regulation of embryonic catecholamine synthesis in response to physiological hypoxia (Norris and Millhorn 1995; Tian et al. 1998). Another study reported respiratory distress

syndrome and perinatal death of mice lacking *Hif-2α* expression due to defects in lung maturation (Compernolle et al. 2002).

Under pathological conditions, *Hif-2α* appears to regulate blood vessel dynamics (Brusselmans et al. 2003). It was demonstrated that a heterozygous deletion of *Hif-2α* protected mice against pulmonary hypertension and right ventricular dysfunction induced by chronic hypoxia, possibly mediated by increased expression of *endothelin-1* (Brusselmans et al. 2003). Additionally, a gain-of-function approach of *Hif-2α* revealed early embryonic lethality due to increased *Oct-4* expression (Covello et al. 2006). Xenograft assays using Hif-2α knock-in ESCs derived from tumours demonstrated an elevated growth and increased angiogenesis. The increased fraction of undifferentiated ESCs was reversed by knockdown of *Oct-4* (Covello et al. 2006) (Table 1.1). Moreover, a *Hif-2α* knock-in mouse model that has the *Hif-1α* promoter driving the expression of *Hif-2α,* showed that increased expression of *Hif-2α* did not rescue the phenotype of the Hif-1α$^{-/-}$ mice (Covello et al. 2006). This demonstrated the non-redundant role of Hif proteins in embryonic development.

### 1.4.4.2 Hif regulatory system in adult haemopoiesis

#### 1.4.4.2.1 Role of Hif-1α in adult haemopoiesis

Studies mentioned in the previous section showed that deletion of *Hif-1α, Hif-2α* or *Hif-1ß* subunit in mice was embryonic lethal (Maltepe et al. 1997; Iyer et al. 1998; Ryan et al. 1998; Tian et al. 1998; Adelman et al. 1999; Peng et al. 2000; Compernolle et al. 2002; Yoon et al. 2006). Hence, a CKO approach was utilised to decipher the role of Hifs in adult haemopoiesis. This model demonstrated that *Hif-1α* expression was enriched in murine HSCs (Simsek et al. 2010; Takubo et al. 2010). An inducible cre (Mx1-Cre) transgene was employed to conditionally delete *Hif-1α* (Takubo et al. 2010). Within this model, the administration of pIpC (polyinosic-polycytidylic acid, synthetic double stranded RNA) activates the Mx1 promoter, thus driving the expression of Cre recombinase in various tissues, including kidney, liver, haemopoietic cells and their microenvironment (Takubo et al. 2010). Under steady state conditions, HSCs lacking *Hif-1α* were shown to be capable of

sustaining multilineage haemopoiesis (Takubo et al. 2010). However, under stressful conditions, like serial BM transplantation, treatment with chemotherapeutic agents (5-Flurouracil treatment) and aging, *Hif-1α* deletion in HSCs resulted in a reduced tolerance to stress and induced a loss of the HSC pool within the BM (Takubo et al. 2010). HSCs from conditional Hif-1α knockout mice lost the capability to reconstitute the BM of lethally irradiated mice during serial transplantation (Takubo et al. 2010). A gene expression analysis profile of transplanted HSCs lacking *Hif-1α* showed elevated levels of *p16Ink4a and p19Arf*, the products of Ink4a/Arf locus that contribute to cellular senescence (Takubo et al. 2010). Furthermore, suppression of *p16Ink4a* and *p19Arf* reinstated the BM reconstitution potential in the Hif-1α CKO HSCs (Takubo et al. 2010). Hence, *Hif-1α* is involved in the protection of HSCs from stress-induced senescence (Table 1.1).

Conditional deletion of Vhl, an E3 ubiquitin ligase mediating Hif-α protein degradation, gave more insight into the regulatory mechanisms of Hifs in HSCs (Takubo et al. 2010). Heterozygous deletion of Vhl protein and consequent over-stabilisation of *Hif-1α* resulted in enhanced quiescence in HSCs (Takubo et al. 2010). Haemopoietic progenitor cell fractions, which expressed very low levels of Hif-1α protein entered into unusual cell cycle quiescence from a rapid proliferative state with deletion of *Hif-1α* (Takubo et al. 2010). Furthermore, induced deletion of both alleles of *Vhl* maximised Hif-1α stabilisation and resulted in increased HSC quiescence (Takubo et al. 2010). However, over-quiescent HSCs from $Vhl^{\Delta/\Delta}$ mice displayed defective BM reconstitution ability (Takubo et al. 2010). Co-deletion of *Hif-1α* in the HSCs lacking *Vhl* expression reversed the abnormal increase in HSC quiescence and rescued the defective BM reconstitution phenotype (Takubo et al. 2010). Hence, the defective stem and progenitor phenotype in the Vhl deficient mice was Hif-1α-dependent (Takubo et al. 2010). These results suggested that Vhl/Hif-1α-mediated regulatory pathways play a key role in regulating the cell cycle quiescence of HSPCs (Table 1.1).

#### 1.4.4.2.2 Role of Hif-2α in adult haemopoiesis

A null deletion of *Hif-2α* proved to be embryonic or neonatal lethal (Peng et al. 2000; Compernolle et al. 2002). Hence, a conditional deletion approach was used to understand the role of *Hif-2α* in adult physiology (Gruber et al. 2007). A mouse model was generated, whereby, acute deletion of *Hif-2α* was achieved using an ubiquitously expressed Cre (Ubc) induced by tamoxifen (Gruber et al. 2007). Acute deletion of *Hif-2α* in adult mice between 6-8 weeks of age resulted in anaemia with reduced levels of Epo production demonstrating that *Hif-2α* is a major regulator of adult erythropoiesis (Gruber et al. 2007). Deletion of *Hif-2α* also impaired Epo production under stressful conditions in adult mice (Gruber et al. 2007). *Hif-2α* was implicated in regulation of Epo production in other organs, such as astrocytes of the central nervous system and was shown to play an important role in the survival of neurons during ischaemia (Chavez et al. 2006). Furthermore, hepatocyte-specific deletion of *Hif-2α,* using albumin-Cre, revealed *Hif-2α* dependent hepatic *Epo* production, underscoring the importance of *Hif-2α* in Epo regulation (Gruber et al. 2007; Rankin et al. 2007).

Another study generated a small number of viable adult mice that globally lacked the expression of *Hif-2α* by crossing Hif-2α knockout mice from different genetic backgrounds (Scortegagna et al. 2003a). Scrotegagna et al. took the approach of generating speed congenic, a marker-assisted strategy that recognises the simple sequence length polymorphism between donor and recipient to find the progeny with the highest percentage of recipient genome to be backcrossed to generate speed congenic strain (jaxcommunications 2001). The cross between heterozygous 129S6/SvEvTac Hif-2α knockout mice and heterozygous C57BL/6 (C57 black/6) Hif-2α knockout mice using speed congenics generated adult F1 hybrid Hif-2α knockout mice at one-fourth of the expected Medelian ratio (Scortegagna et al. 2003a). The F1 hybrid from this genetic background displayed significant perinatal mortality within the first two days of life, but bypassed the embryonic lethality that was observed when Hif-2α null mice were inbred within either of the strains (Scortegagna et al. 2003b). The surviving 20% of

the expected Hif-2$\alpha^{-/-}$ adult mice had lower body weights and a short life span of 1-2 months (Scortegagna et al. 2003a). Hif-2$\alpha^{-/-}$ adult mice exhibited multiorgan dysfunctions, such as pancytopenia, mitochondrial defects, overt cardiac hypertrophy, retinopathy and hepatosteatosis (Scortegagna et al. 2003a). A study of Hif-2$\alpha^{-/-}$ mice revealed biochemical abnormalities, suggesting the role of *Hif-2*$\alpha$ in regulating the expression of antioxidant enzymes, including catalase, glutathione and superoxidase dismutase (Scortegagna et al. 2003a). The study showed that Hif-2$\alpha^{-/-}$ juvenile mice had reduced haematocrits and lower blood cells counts with decreased WBC, RBC and platelet counts (Scortegagna et al. 2003b). Conversely, transplantation of Hif-2$\alpha^{-/-}$ BM cells successfully re-populated irradiated recipient mice and efficiently generated all the mature blood lineages, suggesting that the deletion of *Hif-2*$\alpha$ in non-haemopoietic cells is the cause of pancytopenia in Hif-2$\alpha^{-/-}$ mice (Scortegagna et al. 2003b). In this study, Scrotegagna et al reported a reduction in renal Epo mRNA, and a lack of *Epo* upregulation in response to intermittent hypoxia, contributing to the defective haemopoiesis (Scortegagna et al. 2003b) (Table 1.1). However, the strain specific disparate phenotypes observed in Hif-2$\alpha^{-/-}$ mice do not explain the differential requirement of *Hif-2*$\alpha$ in adult haemopoiesis.

| | KO mouse model | Hif expression | Phenotype | Reference |
|---|---|---|---|---|
| Embryonic | Arnt$^{-/-}$ | Increased expression of *Hif-α* | • Embryonic lethal at E10.5<br>• Defective yolk sac angiogenesis and decreased haemopoietic progenitors<br>• Increased expression of Vegf<br>• Lack of hypoxic response of ESCs<br>• No differences in the regulation of glycolytic genes in ESCs | (Maltepe et al. 1997)<br><br>(Adelman et al. 1999) |
| | Hif-1α$^{-/-}$ | Germline deletion of *Hif-1α* expression | • Embryonic lethal at E10.5<br>• Malformation of neural tube and cardiovascular defects<br>• Increased tissue hypoxia<br>• Haemopoietic defects including reduced myeloid and erythroid progenitors | (Yoon et al. 2006)<br><br>(Ryan et al. 1998)<br><br>(Iyer et al. 1998) |
| | Hif-2α$^{-/-}$ | Germline deletion of *Hif-2α* expression | • Embryonic lethal between E9.5-E12.5<br>• Bradycardia<br>• Reduced level of catecholamine<br>• Cardiac failure and impaired lung maturation | (Peng et al. 2000)<br>(Tian et al. 1998)<br>(Norris and Millhorn 1995; Tian et al. 1998)<br>(Compernolle et al. 2002) |
| | Hif-2α Knock-in | Increased expression of *Hif-2α* with background deletion of Hif-1α | • Embryonic lethality due to elevated Oct-4 expression<br>• ESCs derived tumours showed increased growth and angiogenesis | (Covello et al. 2006) |
| Adult | Hif-1α $^{\Delta/\Delta}$ (Mx-1 Cre) | Conditional deletion of *Hif-1α* | • Loss of HSC cell cycle quiescence<br>• Lower stress resistance of HSCs<br>• Extramedullary haemopoiesis<br>• ROS overproduction<br>• Elevated expression of P16Ink4a P19Arf cell senescent genes | (Simsek et al. 2010)<br>(Takubo et al. 2010) |
| | Vhl$^{+/\Delta}$ (Mx-1 Cre) | Slight increase in *Hif-1α* expression | • Enhanced cell cycle quiescence<br>• Resistant to aging<br>• Higher BM chimerism after BM transplantation<br>• ROS suppression | (Takubo et al. 2010) |

| | | | | |
|---|---|---|---|---|
| | $Vhl^{\Delta/\Delta}$ (Mx-1 Cre) | Over stabilisation of *Hif-1α* | • Aberrant suppression of cell cycle<br>• Homing defect of HSCs | (Takubo et al. 2010) |
| | Vegfα mutant (mutated HRE element) | Defective binding of *Hif-1α* to the HRE | • Increase in HSC numbers<br>• Defective BM repopulation potential of HSCs | (Miharada et al. 2011) |
| | Hif-$2\alpha^{-/-}$ | Global deletion of *Hif-2α* with different genetic background | • Significant perinatal death with 20% surviving till adulthood of 1-2 months<br>• Pancytopenia, mitochondrial defects, over cardiac hypertrophy<br>• Retinopathy and hepatosteatosis<br>• Reduced haematocrits, WBC, RBC and platelets<br>• Reduced Epo levels with defective Epo regulation during intermittent hypoxia | (Scortegagna et al. 2003a) |
| | Hif-$2\alpha^{\Delta/\Delta}$ (Ubc-Cre) | Induced global deletion of *Hif-2α* | • Anaemia with defective Epo production and erythropoiesis | (Gruber et al. 2007) |
| | $Meis1^{\Delta/\Delta}$ (Mx-1 Cre) | Decreased expression of *Hif-1α* and *Hif-2α* | • Loss of HSC quiescence and increased apoptosis<br>• Defective BM repopulation potential<br>• Elevated ROS production<br>• Increased mitochondrial oxidative phosphorylation | (Kocabas et al. 2012) |
| | $PHD1^{-/-}$ and $PHD3^{-/-}$ | Increased *Hif-2α* expression | • Erythrocytosis mediated by Epo pathway<br>• Elevated Epo expression | (Takeda et al. 2008) |
| | $PHD2^{\Delta/\Delta}$ (CD68 Cre) | Stabilisation of *Hif-1α* and *Hif-2α* | • Increased proliferation of haemopoietic progenitors mediated by TGF-β<br>• Reduced stress response in HSCs with defective reconstitution during BM transplantation due to increased self-renewal of HSCs | (Singh et al. 2013) |

**Table 1. 1: Summary of phenotypes of genetic knockout/knockdown models of Hif-regulatory genes.**

### 1.4.4.3 Role of Hifs in cellular metabolism

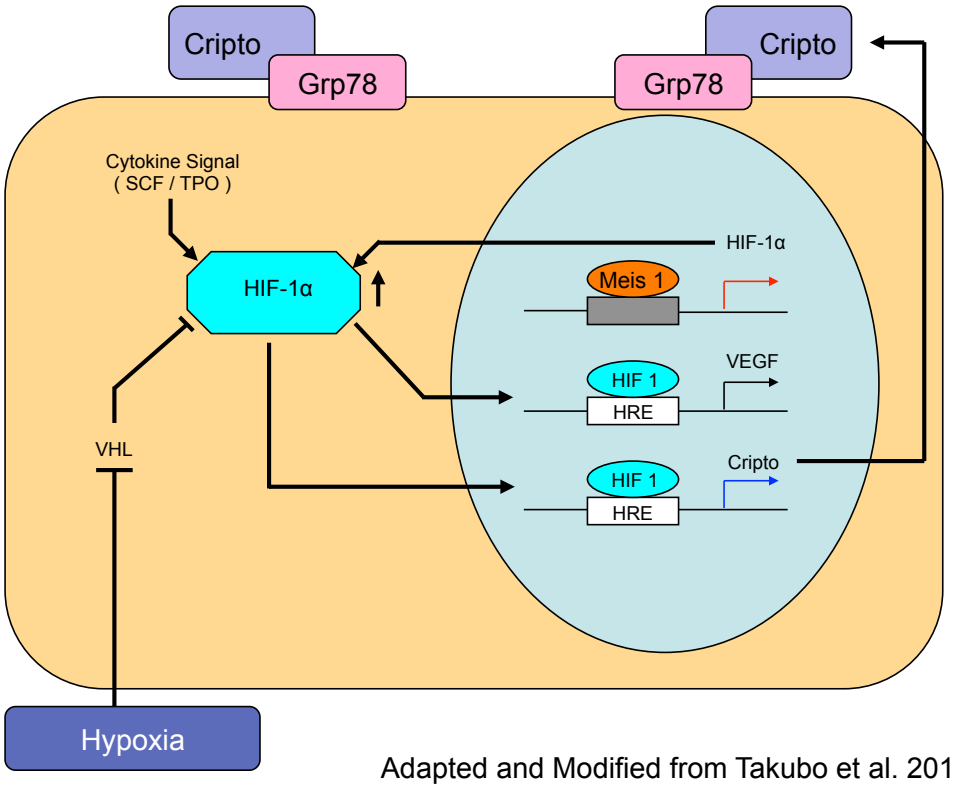
Under hypoxia, *Hif-1α* mediates the switch from mitochondrial oxidative respiration to glycolysis to maintain tissue homeostasis (Iyer et al. 1998). Cells display an adaptive mechanism by low $O_2$ consumption, reduction of mitochondrial respiration and an increase in glycolysis via active transcription of *Hif-1α*-dependent genes involved in the glycolytic pathway (Iyer et al. 1998). *Hif-1α* encodes enzymes regulating glycolysis and metabolism, such as lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1), respectively. *Hif-1α* regulates transcriptional activation of glucose transporters like Glut-1 (Iyer et al. 1998). A recent study demonstrated the effect of *Hif-1α* on HSC metabolism (Simsek et al. 2010; Kocabas et al. 2012). Conditional deletion of *Hif-1α* in HSCs resulted in decreased glycolysis and an increase in oxygen consumption rate with a shift towards oxidative metabolism (Kocabas et al. 2012).

*Meis1* has been shown to be essential for the transcriptional activation of *Hif-1α* in HSCs (Kocabas et al. 2012). The study also suggested that cell-intrinsic characteristics of HSCs included predominant anaerobic metabolism and increased *Hif-1α* expression (Kocabas et al. 2012). *Meis1* belongs to the Hox family of homeobox genes that encodes DNA-binding TFs and is expressed in the primitive haemopoietic subpopulation in both foetal liver and adult mice (Krumlauf 1994; Pineault et al. 2002; Azcoitia et al. 2005; Argiropoulos et al. 2007). Multiple studies showed that *Meis1* is essential for both embryonic and adult haemopoiesis (Pineault et al. 2002; Azcoitia et al. 2005; Argiropoulos et al. 2007). Kocabas et al reported that conditional deletion of *Meis1* resulted in decreased expression of both *Hif-1α* and *Hif-2α* in HSCs, resulting in increased mitochondrial oxidative phosphorylation and increased ROS production (Kocabas et al. 2012). Inducible deletion of *Meis1* led to dysregulated HSC function, including loss of quiescence and increased apoptosis, coupled with lack of their repopulation potential (Kocabas et al. 2012). The severe defects in HSC functions in Meis1 CKO were mediated through ROS, as systemic administration of ROS scavenger N-acetylcysteine restored the HSC quiescence and functions (Kocabas et al. 2012). Hence, HSC metabolism is governed by a transcriptional network, where *Meis1*

induced *Hif-1α* expression inhibited mitochondrial metabolism and stimulated glycolysis (Table 1.1).

Several downstream targets of *Hif-1α* are reported to play a role in *Hif-1α*-dependent maintenance of HSC quiescence and their metabolic properties (Miharada et al. 2011). *Vegf*, a well-known Hif-downstream target gene, was mutated at the HRE in its promoter region to generate a mutant mouse model (Miharada et al. 2011). This mutation interrupted the binding of the Hif-1 complex to the HRE causing down-regulation of *Vegfα* expression particularly in HSCs and not in the progenitor cells (Miharada et al. 2011). Loss of *Vegfα* expression in the Vegfα-mutated mouse model displayed a defective HSC phenotype, with an increase in the number of HSCs, but displayed defective BM reconstitution potential upon transplantation (Miharada et al. 2011) (Table 1.1).

Another study showed that *Hif-1α* induced Cripto/GRP78 signalling is essential for HSC maintenance (Miharada et al. 2011). Miharda et al found a sub-population of HSCs that expressed a heat shock protein, GRP78 (Miharada et al. 2011). $GRP78^+$ HSCs localised in the hypoxic endosteal region of the BM (Miharada et al. 2011). Miharda et al demonstrated that $GRP78^+$ HSCs were quiescent with lower mitochondrial potential compared to $GRP78^-$ HSCs (Miharada et al. 2011). Further, blocking GRP78 by neutralising antibody caused significant reduction in the $GRP78^+$ HSCs in the endosteal region (Miharada et al. 2011). Additionally, GRP78, together with its ligand Cripto, induced high glycolytic activity in HSCs (Miharada et al. 2011). Further evidence showed that *Cripto* is expressed in HSCs as well as in various niche cells, including OBs and MSCs (Miharada et al. 2011). Moreover, Hif-1α CKO mice demonstrated a decrease in the number of $GRP78^+$ HSCs and a reduction in the expression of *Cripto* in the endosteal niche cells (Miharada et al. 2011). Hence, the Cripto/GRP78 signalling pathway with *Hif-1α* as the intermediary regulates HSC quiescence and maintains HSCs in hypoxia (Table 1.1) (Figure 1.6).




Adapted and Modified from Takubo et al. 2012

**Figure 1. 6: Schematic representation of mechanism by which Hif-1α regulates cell metabolic pathway.**

*Hif-1α* transcriptional activity is induced by *Meis1* and also by hypoxia or cytokine signalling, including SCF or TPO, thus activating the *Hif-1α* downstream target genes, including *Cripto or Vegf*. *Cripto* accumulated from osteoblasts, MSCs or HSCs binds to GRP78 and plays a vital role in maintaining HSC quiescence.

### 1.4.4.4 Role of Hif-prolyl hydroxylases (PHDs) in haemopoiesis

Under normoxia, Hif-α are regulated by prolyl hydroxylase family members namely PHD-1, PHD-2 and PHD-3 (Epstein et al. 2001; Ivan et al. 2001; Masson et al. 2001; Yu et al. 2001). Of the three PHD isoforms, PHD2 majorly governs the HIF-prolyl hydroxylation in normoxic cells thereby maintaining normal levels of *Hif-α* expression (Berra et al. 2003; Minamishima et al. 2008; Takeda et al. 2008). *PHD3* is known to be relatively more efficient in *Hif-2α* suppression and hence might favour the role in maintaining balanced expression of *Hif-2α* than *Hif-1α* (Appelhoff et al. 2004). Deletion of *PHD2* resulted in dysregulated embryonic development, leading to embryonic lethality, similar to mice deficient of *pVhl* or *Hif-α* (Takeda et al. 2006). Somatic mutation of PHD2 resulted in

erythrocytosis and congestive cardiac failure (Takeda et al. 2008). PHD1 null mice and PHD3 null mice were viable with normal erythropoiesis, but mice lacking both *PHD1* and *PHD3* developed moderate erythrocytosis (Takeda et al. 2008). *Hif-2α* expression was elevated in the liver of mice lacking both *PHD1* and *PHD3,* suggesting that the Hif-2α/Epo pathway mediates erythrocytosis (Takeda et al. 2008). In mice deficient of *PHD2*, *Hif-1α,* but not *Hif-2α* levels were elevated in liver and kidney causing dramatic increase in *Epo* expression levels (Takeda et al. 2008). However, a recent study showed that conditional deletion of PHD2 using CD68-Cre, known to delete in the haemopoietic system and in a subset of epithelial cells, resulted in *Hif-2α*-dependent erythrocytosis (Franke et al. 2013). Under steady state, conditional deletion of *PHD2* using CD68-Cre demonstrated increased proliferation in primitive haemopoietic progenitors, but did not affect the quiescent HSC population (Singh et al. 2013). Loss of *PHD2* under physiological conditions resulted in increased cycling of HSCs mediated by inhibition of the TGF-ß pathway, due to stabilisation of *Hif-1α* (Singh et al. 2013). Under stressful conditions of transplantation, the reconstitution potential of HSCs lacking *PHD2* was hampered with significantly lower PB blood chimerism, however an increase in self-renewal properties of HSCs was reported (Singh et al. 2013). In contrast to the defective homing phenotype of HSCs with over-stabilised Hif-1α due to loss of Vhl, PHD2 lacking HSCs showed normal homing ability in the BM (Takubo et al. 2010; Singh et al. 2013). Bearing in mind the complexity of the Hif regulatory system, more insight into various hypoxia-regulated pathways is essential to understand the differential role of Hifs and its regulators in haemopoiesis (Table 1.1)

## 1.5 Cited2

*Cited2 (p35srj)* is one of the founding members of a family of transcriptional co-activators termed CITED (CBP/p300 interacting transactivators with glutamic acid (E) and aspartic acid (D)-rich tail) (Shioda et al. 1997; Bhattacharya et al. 1999). The CITED family was identified as the p300/CBP interacting protein and the conserved p300-binding domain defines the CITED family (Shioda et al. 1997; Bhattacharya et al. 1999). The CITED

family includes *CITED1* (*Msg-1*, melanocyte-specific gene 1) (Shioda et al. 1996), *CITED2* (Shioda et al. 1997; Bhattacharya et al. 1999), *CITED3* (Andrews et al. 2000) and *CITED4* (Braganca et al. 2002; Yahata et al. 2002). Human CITED family homologues have been found in other species but are restricted to vertebrates (Shioda et al. 1997; Bhattacharya et al. 1999). All CITED family members except *CITED3* are evolutionarily conserved in placental mammals (Shioda et al. 1997; Bhattacharya et al. 1999).

### 1.5.1 Tissue expression of *Cited2*

*Cited2* is ubiquitously expressed in murine tissues and has 95% homology between human and mouse (Shioda et al. 1997; Bhattacharya et al. 1999). *Cited2* expression is induced by hypoxia and multiple stimuli, such as serum, growth factors (granulocyte/macrophage colony-stimulating factor, interferon-γ, platelet-derived growth factor), cytokines IL-1$\alpha$, IL-2, IL-4, IL-6, IL-9 and IL-11), insulin and lipopolysaccharide (Sun et al. 1998; Bhattacharya et al. 1999; Xu et al. 2008). Cited2 functions as a transcriptional co-activator and also as an inhibitor of hypoxia-activated gene transcription by interrupting the binding of *Hif-1*$\alpha$ to p300/CBP (Bhattacharya et al. 1999; Bamforth et al. 2001; Braganca et al. 2003). *Cited2* plays a pivotal role in various biological and biochemical processes, including embryonic development, foetal and adult haemopoiesis and hypoxia-signalling pathways (Bhattacharya and Ratcliffe 2003; Chen et al. 2007; Kranc et al. 2009; Chou et al. 2012; Du et al. 2012).

### 1.5.2 Structure

*Mrg1* (Shioda et al. 1997) and *p35srj* (Bhattacharya et al. 1999) are two alternatively spliced isoforms encoded by the *CITED2* gene (Leung et al. 1999). Mrg1 is a cytokine-inducible factor with transformation activity (Sun et al. 1998) and p35srj is a nuclear protein that binds to p300/CBP, regulating the transcriptional response to hypoxia (Bhattacharya et al. 1999). The *CITED2* gene encodes both *Mrg1* and *p35Srj* (Leung et al. 1999) (Figure 1.7).

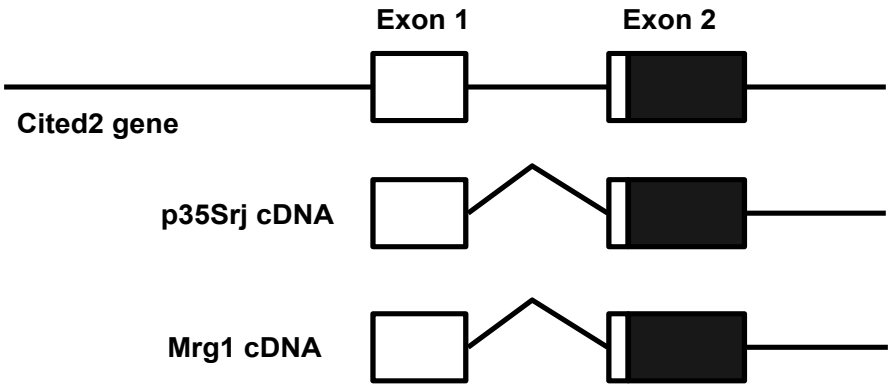



**Figure 1. 7: Structure of *CITED2* gene.**

*CITED2* gene is composed of three exons and two introns that encode both *Mrg1* and *p35Srj*. Exon-open boxes, Open reading frame (ORF) – Filled boxes.

CITED2 is an unstable nuclear protein with a half-life of 20 minutes (Bhattacharya et al. 1999). Sequence analysis of the CITED family revealed the presence of three conserved regions (CR), namely CR1, CR2 and CR3 (Shioda et al. 1996; Shioda et al. 1997; Sun et al. 1998; Bhattacharya et al. 1999). While CR2 is conserved in all CITED family members, the CR1 domain is present in CITED1, 2 and 3 and the CR3 motif is conserved in CITED2, 3 and 4 (Yahata et al. 2000; Bamforth et al. 2001; Braganca et al. 2002). The CITED2 protein encompasses 270 amino acid (aa) residues. It consists of TAD domain in the carboxy-terminal region with CR1 domain (residues 110-123) and the CR2 domain (residues 215-270) and CR3 domain (residues 1-11) that corresponds to the amino-terminal region. CITED2 consists of an unusual serine-glycine rich junction (Srj, residues 161-199) that is exclusive to CITED2 (Shioda et al. 1997; Bhattacharya et al. 1999). The functions of the CR1, CR3 and Srj domain are not known. Although point mutations and variant clustering has been reported in the Srj domain in congenital heart disease patients (Sperling et al. 2005; Yang et al. 2010), a recent study has shown that the Srj domain is dispensable for Cited2 function in mice and suggested that the Srj mutation is not the sole cause of the malformation reported in these patients (Chen et al. 2012). TAD of CITED2 functions as the p300/CBP-binding site, whereas the CR2 motif of CITED2 binds to the CH1 domain of p300/CBP that co-activates CITED2 (Bhattacharya et al. 1999; Freedman et al. 2003) (Figure 1.8).

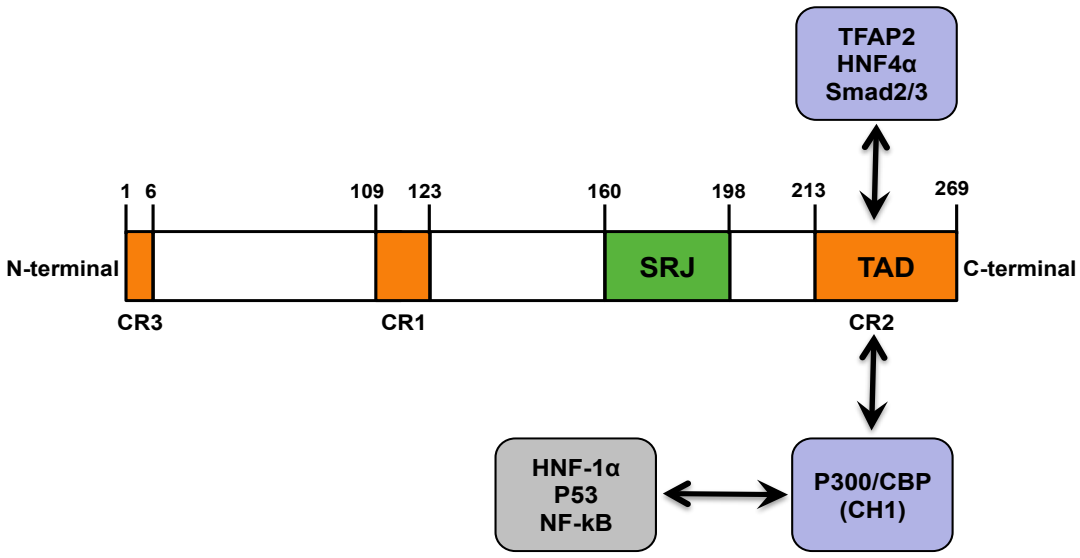



**Figure 1. 8: Structure and interaction partners of Cited2 protein.**

Cited2 consists of three conserved regions (CR1, CR2 and CR3). A unique SRJ domain is exclusive to Cited2 and TAD at the CR2 domain in C-terminal is the binding site of Cited2 interacting partners like TFAP2, HNF4, Smad2/3 and p300/CBP (that co-activates Cited2). CR1, CR2, CR3= Conserved region 1,2,3; SRJ = serine-glycine rich junction; TAD= transactivation domain.

### 1.5.3 *Cited2* as a negative regulator of *Hif-1α*

*Cited2* competes with *Hif-1α* for the conserved binding region in the CH1 domain of p300/CBP (Bhattacharya et al. 1999). *Cited2* inhibits the interaction of *Hif-1α* and *p300* resulting in the blockade of *Hif-1*-mediated transcription (Bhattacharya et al. 1999). Under normoxic conditions, *Hif-1α* undergoes hydroxylation of prolyl or asparagyl residues (Jaakkola et al. 2001; Lando et al. 2002). The asparagyl hydroxylation of *Hif-1α* prevents the interaction of *Hif-1α* with *p300* (Lando et al. 2002), while prolyl hydroxylation leads to proteasomal degradation mediated via Vhl ubiquitin ligase (Jaakkola et al. 2001). Under hypoxic conditions, stabile *Hif-1α* dimerises with its heterodimeric partner *Hif-1B* isoform in the nucleus and binds the DNA at the HRE region (Semenza 1999). The Hif-dimer complex recruits p300/CBP by binding to the CH1 domain, thus activating the transcription of various hypoxia target genes involved in cellular and systemic responses to hypoxia (Semenza 1999). *Cited2* expression is

activated by hypoxia via *Hif-1α* recognition sites in its promoter region (Bhattacharya et al. 1999). Additionally, binding of Cited2 to p300 is 33-fold stronger compared to Hif-1α favouring the inhibitory effect of Cited2 on Hif-1α-p300 complex formation (Freedman et al. 2003). Hence, Cited2 may function to auto-regulate Hif-1α transactivation by inhibiting Hif-1α-p300 interaction (Bhattacharya et al. 1999). The p300-CH1 complex either with Cited2 or with Hif-1α forms a very similar secondary structure and exhibits a degree of plasticity to accommodate different transactivation domains (Freedman et al. 2003). Hence, the CH1 domain of p300 functions as a scaffold supporting the folding of unstructured Cited2 and Hif-1α transactivation domains (Freedman et al. 2003).

Genetic evidence showed that loss of *Cited2* in murine embryonic tissues and cultured cells enhanced the expression of *Hif-1α* target genes, such as *Vegf, Glut-1* and *phosphoglycerate kinase (PGK-1)* (Yin et al. 2002). *Cited2*-deficiency results in embryonic lethality and develop various developmental defects, including cardiac defects, adrenal agenesis, haemopoietic defects, neural crest defects and excencephaly (Bamforth et al. 2001; Braganca et al. 2002; Yin et al. 2002; Schneider et al. 2003). The cardiac defects of outflow tract, interventricular septum, cardiac vasculature and hyposplenia in the Cited2$^{-/-}$embryo was rescued by *Hif-1α* haploinsufficiency (Xu et al. 2007). Also, heterozygous deletion of *Hif-1α* reverted the overexpression of *Vegfα* reported in Cited2$^{-/-}$ hearts, suggesting a negative feedback role of *Cited2* in regulating Hif-1 activity during cardiac developmental stages (Xu et al. 2007). Mouse embryonic fibroblasts (MEFs) from Cited2$^{-/-}$ embryos, when cultured in hypoxia, showed elevated levels of *Hif-1α* target genes, such as *Vegf, Glut-1* and *PGK-1* (Yin et al. 2002). Cited2$^{-/-}$ embryos displayed hyaloid hyper cellularity with abnormal vasculature in the eye (Huang et al. 2012). The abnormal hyaloid system (HVS) was in part due to *Cited2*-mediated overexpression of *Hif-1α* and *Vegf* (Huang et al. 2012). Indeed, deletion of *Hif-1α* or *Vegf* in the vasculature of eyes in *Cited2* knockout embryos partially rescued the abnormal HVS (Huang et al. 2012). Another recent publication reported that *Cited2* was essential for the maintenance of HSC quiescence at least partially in a *Hif-1α* dependent manner (Du et al. 2012). Deletion of *Cited2* in HSCs resulted in the loss of quiescence,

increased apoptosis and defective reconstitution potential upon transplantation (Kranc et al. 2009; Du et al. 2012). However, additional deletion of *Hif-1α* in *Cited2* CKO mouse models partially rescued the defective HSC quiescence and reconstitution capacity (Du et al. 2012). *Foxo3a*-stimulated expression of *Cited2* in fibroblasts and breast cancer cells resulted in reduced *Hif-1α*-induced apoptosis (Bakker et al. 2007). Also, a recent study demonstrated that *p57* and *Hes1* regulated HSC quiescence via *Cited2* (Du et al. 2012). The deletion of *Hif-1α* in *Cited2* CKO mice restored the expression of these genes to normal levels (Du et al. 2012). Collectively, it is plausible to conclude that *Cited2* acts as a negative regulator of *Hif-1α,* thus regulating hypoxia-responsive pathways.

## 1.5.4 p300/CBP, a transcriptional co-activator

p300 and its paralogue CREB-binding protein (CBP) are histone acetylases and transcriptional co-activators that bind various TFs to the core transcriptional machinery (Shiama 1997; Goodman and Smolik 2000; Bedford et al. 2010). p300 and CBP are ubiquitously expressed, evolutionarily conserved and play a fundamental role in cellular growth (Goodman and Smolik 2000). More than 400 interacting partners have been identified for p300 and CBP proteins and are essential for normal cardiovascular, neural and haemopoietic development. They consist of an enzymatic histone acetyl transferase (HAT) domain and three cysteine-histidine-rich domains (CH1, CH2 and CH3), a KIX-domain and a glutamine rich region. These conserved functional domains interact with multiple nuclear proteins and activate core transcriptional machinery. Cited2 was identified as a high affinity p300/CBP binding protein similar to hypoxia-induced Hif-1α (Bhattacharya et al. 1999; Semenza 1999). Cited2 and Hif-1α compete with each other to bind to the common conserved region in the CH1 domain of p300/CBP (Bhattacharya et al. 1999; Semenza 1999).

### 1.5.4.1 Functions of p300 and CBP during early development and cancer

Genetic evidence showed that embryos lacking both alleles of *CBP* were lethal between E9.5 to E10.5 due to anaemia, open neural tube defects and defective vasculature (Oike et al. 1999). Monoallelic deletion of *CBP* ($CBP^{+/-}$)

in mice resulted in splenomegaly, severe defects in multilineage differentiation and an increased incidence of haematological malignancies with age (Kung et al. 2000). Embryos lacking *p300* were lethal and died between E9 to E11.5 with various complications, including retarded growth, cardiac malformation and poor vasculature, but p300 heterozygous mice were born normally without any functional defects (Yao et al. 1998). Double heterozygocity of *p300* and *CBP* was embryonic lethal (Yao et al. 1998). Hence p300/CBP are crucial for early stages of embryonic development.

*p300* and *CBP* play a vital role in the maintenance of normal haemopoiesis by jointly regulating various critical haemopoietic TFs, including cJun, p53 and MyoD (Lill et al. 1997; Puri et al. 1997; Shiama 1997; Grossman 2001). *p300* and *CBP* individually interact with members of the Ets family, C/EBP family and *EKLF, AML1, c-Myb, GATA-1, E2F* (Lill et al. 1997; Puri et al. 1997; Shiama 1997; Grossman 2001). Heterozygous mice for *p300* and *CBP* showed that *CBP*, but not *p300,* was vital for self-renewal properties of HSCs. Additionally, *p300* was crucial for haemopoietic differentiation, but *CBP* was dispensable for haemopoietic differentiation (Rebel et al. 2002). Hence, *p300* and *CBP* have differential roles in regulating haemopoiesis. Lineage-specific conditional deletion of *p300* and *CBP* uncovered their role in B and T-cell development (Kasper et al. 2006; Xu et al. 2006). Expression of both *p300* and *CBP* were essential for B-cell homeostasis, while individual expression only mildly affected the B-cells (Kasper et al. 2006). *p300* and *CBP* are critical for T-cell development (Xu et al. 2006). A study by Kimbrel et al showed that mutant *p300* with a deleted CH1 domain or KIX domain resulted in severe defects in haemopoiesis, including loss of reconstitution ability (Kimbrel et al. 2009). Hence, p300 and CBP regulate haemopoiesis and act as activators for various TFs.

*p300* and *CBP* have also been shown to play a role in tumourigenesis. *CBP* is a tumour suppressor and patients with mutated *CBP* have Rubinsein-Taybi syndrome and are at high risk of development of tumours (Miller and Rubinstein 1995; Petrij et al. 1995). Consistent with this, *p300* and *CBP* are co-activators of *p53* (tumour suppressor) (Avantaggiati et al. 1997; Lill et al. 1997). MLL (Mixed Lineage leukaemia) and AML (M4 and M5 subtypes) are caused by somatic mutations in *p300* and *CBP* (Iyer et al. 2004). More recent

studies showed the frequent occurrence of mutations in *p300* and *CBP* in B-cell lymphoma and acute lymphoblastic leukaemia (ALL) (Mullighan et al. 2011; Pasqualucci et al. 2011). Thus, *p300* and *CBP* contribute to pathological conditions, including leukaemia.

## 1.5.5 Functions of *Cited2*

### 1.5.5.1 Role of *Cited2* during early development and embryonic haemopoiesis

*Cited2* plays a fundamental role in embryonic development (Bamforth et al. 2001; Braganca et al. 2002; Yin et al. 2002; Chen et al. 2007; Xu et al. 2007) and regulates various vital biological and biochemical functions (Freedman et al. 2003; Kranc et al. 2003; Chou and Yang 2006; Bakker et al. 2007; Qu et al. 2007; Kranc et al. 2009; Lou et al. 2011; Chou et al. 2012). The tissue-restricted expression of *Cited2* has been reported in the visceral endoderm before gastrulation and appears in the cranial neuroectoderm, neural crest, mesoderm and mesodermal tissues during gastrulation (Dunwoodie et al. 1998). During later stages of embryogenesis, *Cited2* is ubiquitously expressed (Dunwoodie et al. 1998). Deletion of *Cited2* resulted in embryonic lethality between E13.5 and E18.5 (Bamforth et al. 2001; Braganca et al. 2002). Embryos lacking *Cited2* displayed cardiac malformation, adrenal agenesis, neural crest defects, abnormal cranial ganglia and exencephaly (Bamforth et al. 2001; Braganca et al. 2002; Yin et al. 2002; Schneider et al. 2003). Cardiac defects in *Cited2* null embryos included defects in atrial and ventricular septum, cardiac outflow tract and aortic arch malformation (Bamforth et al. 2001). Loss of *Cited2* in murine embryos resulted in the malformation of anterio-posterior axis due to fusion of cranial ganglia and the cervical vertebrae (Bamforth et al. 2001; Bamforth et al. 2004). *Cited2* is also essential for formation of hyaloid vasculature, lens morphogenesis and development of liver and lungs (Qu et al. 2007; Chen et al. 2008; Xu et al. 2008). Hence, Cited2 regulates various developmental stages during embryogenesis. Murine ESC studies showed that *Cited2* overexpression maintained ESCs in an undifferentiated state in the absence of leukaemia initiating factor (LIF), a growth factor activating the gp130/Stat3 signalling pathway (Pritsker et al. 2006). Loss of *Cited2* did not affect the pluripotency of murine ESCs in the presence of LIF (Li et al. 2012). However,

in differentiating ESCs *Cited2* deletion resulted in delayed silencing of genes, including *Oct4, Klf4, Sox2* and *c-Myc,* that are involved in the maintenance of pluripotency and self-renewal (Li et al. 2012). Impaired *Oct4* expression, mediated in part through *Cited2,* resulted in defective cardiomyocyte, haemopoietic and neuronal differentiation in ESCs lacking Cited2 (Li et al. 2012). Loss of *Cited2* in MEFs resulted in impaired proliferation due to elevated expression of cell proliferation inhibitors $p16^{Ink4a}$ and *p19ARF* mediated partially via polycomb-group genes *Bmi1* and *Mel18* (Kranc et al. 2003). In MEFs, *Cited2* controls cell proliferation and *INK4a/ARF* expression (Kranc et al. 2003). This phenotype is in part mediated by *Bmi1* and *Mel18* as exogenous expression of *Bmi1* and *Mel18* enhanced the proliferation of $Cited2^{-/-}$ fibroblasts (Kranc et al. 2003). Taken together, previous publications collectively suggest the requirement of *Cited2* in the maintenance of primitive characteristics, such as self-renewal and pluripotency of stem cells.

Chen et al showed the requirement of *Cited2* for normal haemopoiesis in murine foetal liver (Chen et al. 2007). *Cited2* is highly expressed in the primitive cells and has low expression in differentiated cells (Chen et al. 2007; Kranc et al. 2009). Studies in the *Cited2* null mice demonstrated that foetal liver haemopoiesis was severely impaired with reduced numbers of LSKs (consisting of HSCs and haemopoietic progenitors) (Chen et al. 2007). Furthermore, foetal liver cells lacking *Cited2* failed to reconstitute the BM of lethally irradiated mice (Chen et al. 2007). Hence, *Cited2* plays a vital role in pathways regulating foetal liver HSC functions.

#### 1.5.5.2 Role of *Cited2* during adult haemopoiesis

*Cited2* has been shown to be essential in HSC function and maintenance (Kranc et al. 2009; Du et al. 2012). Conditional deletion of *Cited2* using an inducible cre, Mx1-Cre (activated by pIpC administration), resulted in severe HSC loss and fatal BM failure (Kranc et al. 2009; Du et al. 2012). HSCs lacking *Cited2* displayed defective multilineage repopulation potential during transplantation (Kranc et al. 2009; Du et al. 2012). The loss of HSCs in mice with acute deletion of *Cited2* was partially due to increased apoptosis resulting from elevated *p53* activity (Kranc et al. 2009). Deletion

of *p53*, a tumour suppressor, or *Ink4a/ARF* (encoding *p16Ink4a* and *p19ARF*), successfully restored HSC functions, including BM reconstitution (Kranc et al. 2009). Another study showed that induced deletion of *Cited2* resulted in loss of HSC quiescence and additional deletion of *Hif-1α* partially rescued defective HSC functions (Du et al. 2012). Additionally, an increase in quiescent HSCs and donor derived LSK chimerism was observed in cells lacking both *Cited2* and *Hif-1α* compared to cells from *Cited2*-deficient mice (Du et al. 2012). However, the defective apoptosis in *Cited2*-deficient mice was independent of *Hif-1α* (Du et al. 2012). Hence, *Cited2* regulates adult HSC functions via both *Hif-1α*–dependent and *Hif-1α*–independent pathways.

#### 1.5.5.3 Oncogenic properties of *Cited2*

*Cited2* has been shown to possess oncogenic properties and its role in cancer has not been fully elucidated. *Cited2* (*Mrg1*) expression is induced by serum, multiple growth factors, several cytokines and insulin (Sun et al. 1998; Chou et al. 2012). Previous studies showed that overexpression of *Cited2* in Rat1 cells led to tumour formation after injection in nude mice (Sun et al. 1998). These data suggested that over-expression of *Cited2* transformed cells encoding tumourigenic properties. In addition, solid tumour studies reported that in xenograft models, expression of *CITED2* promoted the growth of lung tumours. Additionally, co-expression of CITED2 with *m-Myc* or *E2F3* was associated with poor survival rate of lung cancer patients (Chou et al. 2012). On the other hand, several reports suggested that *Cited2* might act as tumour suppressor in some cell types (Bai and Merchant 2007). For example, knockdown of *Cited2 in vitro* increased the invasiveness of colon cancer, whilst exogenous expression of *Cited2* led to growth arrest in colon cancer cells (Bai and Merchant 2007). Similarly, in breast cancer patients, reduced *Cited2* expression was related with poor prognosis (van Agthoven et al. 2009). Taken together these studies suggest that *Cited2* behaves as a tumour suppressor or as an oncogene depending on the cell type and cancer. More investigation into the role of *Cited2* in cancer is therefore essential to understand its distinctive functions.

### 1.5.5.4 Molecular mechanisms regulated by *Cited2*

At a molecular level, dysregulated downstream targets of *Cited2* might contribute to some of the defective biological processes in Cited2$^{-/-}$ mice. *Cited2* is involved in transactivation of *Tfap2* (*transcription factor AP2*) (Bamforth et al. 2001). It was demonstrated that mice lacking *Tfap2* died perinatally as a result of severe cardiac malformation, defects in neural tubes and neural crests (Brewer et al. 2002; Kuckenberg et al. 2012). Hence defective functions of *Tfap2,* at least in part, may contribute to the malformations in cardiac, neural tubes and crests that are observed in Cited2$^{-/-}$ mice.

Also, *Cited2* competes with *Hif-1*$\alpha$ for binding to the CH1 domain of p300/CBP and thus negatively regulates *Hif-1*$\alpha$ transactivation. Deletion of *Hif-1*$\alpha$ and its target gene, *Vegf,* in the eye of Cited2 knockout mice, partially rescued the defects in hyaloid vasculature (Xu et al. 2007). Similarly, deletion of *Hif-1*$\alpha$ in murine adult HSCs lacking *Cited2* expression partially rescued their defective quiescence and reconstitution potential (Du et al. 2012) (Figure 1.9).

Under hypoxia, *Foxo3a* has been shown to stimulate the transcription of *Cited2* thereby reducing the expression of pro-apoptotic *Hif-1*$\alpha$ target genes *NIX* and *RTP801* and inhibiting *Hif-1*$\alpha$-induced apoptosis in fibroblast and breast cancer cells (Bakker et al. 2007). Loss of Cited2 in MEFs led to cessation of proliferation due to reduced expression of polycomb ring finger oncogenes, *Bmi1* and *Mel18,* resulting in low expression levels of *INK4a/ARF* (Kranc et al. 2003). Exogenous expression of *Bmi1* and *Mel18* increased the proliferation of MEFs (Kranc et al. 2003). Furthermore, deletion of *Cited2* in murine foetal liver cells was coupled with decreased expression of various genes involved in maintenance of HSC functions, such as *Bmi1* (Lessard and Sauvageau 2003), *GATA2* (Ling et al. 2004), *p53* (Liu et al. 2009), *Notch1* (Panepucci et al. 2010), *Mcl-1* (Campbell et al. 2010a) and *LEF-1* (Chen et al. 2007) (Figure 1.9). These studies provide hints of distinctive molecular mechanisms governed by Cited2 in various cell types. However, further dissection into system-specific regulatory pathways of Cited2 is essential.

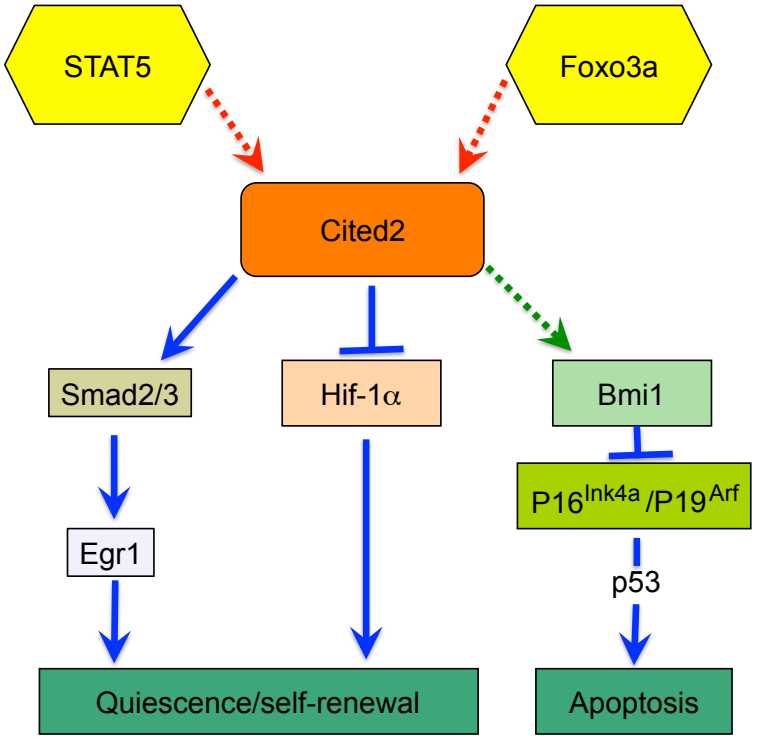



**Figure 1. 9: Schematic representation of regulation of *Cited2* at the molecular level.** *Cited2* has been shown to regulate various downstream target genes (*Hif-1, Bmi1 and Smad2/3*) by playing a key role in regulating HSC quiescence, self-renewal and apoptosis. *Foxo3a* and *STAT5* regulate transcription of *Cited2* in fibroblasts and Hela cells. Red arrow – Relationship shown in other systems yet to be tested in HSCs, Green arrow- Relationship that exists in other systems but not in HSCs.

## 1.6 Malignant haemopoiesis

Leukaemia is a clonal haemopoietic stem and progenitor cell malignancy characterised by excessive proliferation, survival and impaired differentiation (Jan and Majeti 2013). Based on the rapidity of the clinical course and cell of origin, leukaemia is broadly classified into chronic myeloid leukaemia (CML), chronic lymphocytic leukaemia (CLL), AML and acute lymphoblastic leukaemia (ALL) (Vardiman 2010; Campo et al. 2011). Acute leukaemia are characterised by more immature cells in the BM and peripheral blood (PB) that progress over weeks to months resulting in BM failure (Lowenberg et al. 1999; Estey and Dohner 2006). Chronic leukaemia is characterised by more mature cells in the BM and PB and is less aggressive. AML is the most common form of acute leukaemia in adults

accounting for 80% of cases, while ALL accounts for the majority of leukaemia cases in children (Bhayat et al. 2009; Jemal et al. 2010). Due to specific interest and relevance to my work, here I will be focusing on AML.

## 1.6.1 AML

AML is a heterogeneous clonal disorder characterised by various chromosomal translocations resulting in the formation of oncogenic fusion proteins (Rabbitts 1994; Look 1997; Greaves and Wiemels 2003). Over 160 non-random somatic chromosomal mutations are observed in patients contributing to the heterogeneity of the disease (Mrozek et al. 2001; Mrozek et al. 2004). The prognostic impact and disease pathogenesis of heterogeneous AML is intensely studied and distinct translocations are linked to subtypes of leukaemia (Grimwade et al. 1998; Wheatley et al. 1999; Bullinger et al. 2004; Valk et al. 2004; Leroy et al. 2005; Coenen et al. 2011).

### 1.6.1.1 Classification of AML

AML is classified based on morphological characteristics and extent of cell maturation by the French-American-British classification (FAB) system (Bennett et al. 1976). Using FAB classification, leukaemia is classified into subtypes from M0 through M7 (Bennett et al. 1976) (Table 1.2). Another system of classification by the world health organisation (WHO) is based on clinical data and biological characteristics encompassing morphology, genetics, immunological features and cytogenetics (Vardiman 2010). According to WHO classification, AML is sub-grouped into AML with recurrent genetic abnormalities, AML with multilineage dysplasia (MDS), therapy-related myeloid neoplasms, AML not otherwise specified, myeloid sarcoma; myeloid proliferations related to Down's syndrome; and blastic plasmacytoid dendritic cell neoplasm (Bennett et al. 1976) (Table 1.3).

| AML Classification | AML Subtypes |
|---|---|
| 1. AML with recurrent genetic abnormalities | t (8; 21) (q22; q22); RUNX1-RUNX1T1 |
| | Inv (16) (p13; 1q22) or t(16; 16) (p13.1; q22); CBFB-MYH11 |
| | APL with t (15; 17) (q22; q12); PML-RARA |
| | AML with t (9; 11) (p22; q23); MLLT3-MLL |
| | AML with t (6; 9) (p23; q34); DEK-NUP214 |
| | AML with inv3 (q21q26.2) or t (3; 3) (q21; q26.2); RPN1-EVI1 |
| | AML (megakaryoblastic) with t (1; 22) (p13; q13); RBM15-MKL1 |
| | Provisional entity: AML with mutated NPM1 |
| | Provisional entity: AML with mutated CEBPA |
| 2. AML with myelodysplasia-related changes | |
| 3. Therapy-related myeloid neoplasms | |
| 4. AML - not otherwise specified (NOS) | AML with minimal differentiation |
| | AML without maturation |
| | AML with maturation |
| | Acute myelomonocytic leukaemia |
| | Acute monoblastic/monocytic leukaemia |
| | Acute erythroid leukaemia<br>Pure erythroid leukaemia<br>Erythroleukaemia, erythroid/myeloid |
| | Acute megakaryoblastic leukaemia |

| | |
|---|---|
| | Acute basophilic leukaemia |
| | Acute panmyelosis with myelofibrosis |
| 5. Myeloid sarcoma | |
| 6. Myeloid proliferations related to Down's syndrome | Transient abnormal myelopoiesis |
| | Myeloid leukaemia associated with Down's syndrome |
| 7. Blastic plasmacytoid dendritic cell neoplasm | |
| 8. Acute leukaemia of ambiguous lineage | Acute undifferentiated leukaemia |
| | Mixed phenotype acute leukaemia with t (9; 22) (q34; q11.2); BCR-ABL |
| | Mixed phenotype acute leukaemia with t (v; 11q23); MLL rearranged |
| | Mixed phenotype acute leukaemia, B/myeloid, NOS |
| | Mixed phenotype acute leukaemia, T/myeloid, NOS |
| | Provisional entity: Natural killer (NK)–cell lymphoblastic leukaemia/lymphoma |

**Table 1. 2: WHO (World health organisation) classification of AML.**

| FAB subgroup | Type of leukaemia | Prognosis/ Frequency in AML | Morphology |
|---|---|---|---|
| M0 | Acute myeloblastic leukaemia without maturation | Worse/<5% | Immature myeloblasts, lacks definite myeloid differentiation by conventional morphology or cytochemical analyses |
| M1 | Acute myeloblastic leukaemia with minimal maturation | Average/20% | Immature myeloblasts predominate, <10% promyelocytes, myelocytes or monocytes |
| M2 | Acute myeloblastic leukaemia with maturation | Better/30% | Immature myeloblasts predominate, but more maturation than in M1 |
| M3 and M3v (variant) | Acute promyelocytic leukaemia | Best/10% | Promyelocytes predominate, marked granulation in more than 30% cells, granules not visible by light microscopy in M3v |
| M4 and M4EO | Acute myelomonocytic leukaemia | Average; better/25% | Mixture of abnormal monocytic cells (>20%) and myeloblasts/promyelocytes (>20%), 30% eosinophils in M4EO |
| M5a and M5b | Acute monocytic leukaemia | Average/10% | Monocytic cells predominate (>80%) in M5a, >80% nonerythoid cells are immature monoblasts, >20% are mature monocytes in M5b |
| M6 | Acute erythroleukaemia | Worse/<5% | Myeloblasts and erythroblasts predominate, abnormal multinucleated erythroblasts containing PAS-positive blocks |
| M7 | Acute megakaryoblastic leukaemia | Worse/<5% | Megakaryocytic cells as shown by platelet peroxidase activity on electron microscopy or by tests with platelet-specific antibodies, often myelofibrosis and increased BM reticulin |
| Others | Undifferentiated acute leukaemia, hypocellular AML | Not known | |

**Table 1. 3: AML classification based on the FAB (French-American-British classification) system.**

### 1.6.1.2 The cell of origin in AML

There is mounting evidence that AML is generated and sustained by a rare population of leukaemic stem cells (LSCs) that share common characteristics with normal HSCs. Like HSCs, LSCs follow a hierarchical organisation with quiescent LSCs at the apex, have the ability to self-renew and also to differentiate (Lapidot et al. 1994; Bonnet and Dick 1997; Appelbaum et al. 2001). Initial studies suggested that LSCs originated from HSCs and shared common immunophenotypic characteristics ($CD34^+$ $CD38^-$) (Bonnet and Dick 1997; Ishikawa et al. 2007). However, other research has challenged the HSC origin of LSCs (Cozzio et al. 2003; So et al. 2003; Huntly et al. 2004; Krivtsov et al. 2006; Taussig et al. 2008; Yoshimoto et al. 2009). Murine retroviral models provide more insight into the cell of origin. Studies conducted using retroviral models showed that fusion oncogenes (MLL-ENL, MOZ-TIF2 and MLL-AF9) reported in leukaemia successfully transformed committed progenitors (GMPs and LMPPs) to LSCs (Cozzio et al. 2003; So et al. 2003; Huntly et al. 2004; Krivtsov et al. 2006). It has also been demonstrated that retroviral transduction of MLL-fusion proteins (MLL-ENL and MLL-AF9) immortalised HSCs as well as committed progenitors that exhibit limited self-renewal potential (Akashi et al. 2000; (Na Nakorn et al. 2002; Cozzio et al. 2003). Also, studies conducted by Sommervaille et al showed that MLL-AF9 transformed and immortalised downstream progenitor cells generating LSCs (Somervaille and Cleary 2006; Kelly et al. 2007). Hence, primitive progenitors acquire mutations and inherit self-renewal properties and transformation potential. On the other hand, a study reported that progenitor cells expressing oncogenes, including *Flt3-ITD* and co-expressing *Meis1* and *Hoxa9,* failed to acquire self-renewal properties, thereby suggesting that the leukaemogenic properties of these oncogenes were confined to HSCs (Kvinlaug et al. 2011).

Evidence from xenotransplantation models further support the evolution of LSCs from both HSCs and progenitor cells. Primary human AML cells purified for HSCs ($CD34^+CD38^-$) and progenitor cells ($CD34^+CD38^+$) both displayed LSC activity (Taussig et al. 2008; Yoshimoto et al. 2009). LSC activity was reported with transplantable leukaemic potential in both $CD34^+$ and $CD34^-$ compartments from AML patients, suggesting LSCs possessed aberrant

immunophenotypic characteristics compared to HSCs (Martelli et al. 2010; Taussig et al. 2010). Goardon et al analysed primary human $CD34^+$ AML samples (>5% of cells are $CD34^+$) and showed that GMPs ($CD34^+CD38^+CD110^+CD45RA^+$) and LMPPs ($CD34^+CD38^-CD90^+CD45RA^+$) both possessed LSC activity and were capable of generating transplantable AML (Goardon et al. 2011). The study also showed the hierarchical organisation of $CD38^-CD45RA^+$ forming GMP-like cells with occurrence of higher frequency of LSCs than GMP-like cells (Goardon et al. 2011). Together, the studies demonstrate that HSCs, LMPPs and/or GMPs can act as the cell of origin for LSCs in the majority of AML subtypes (Figure 1.10).

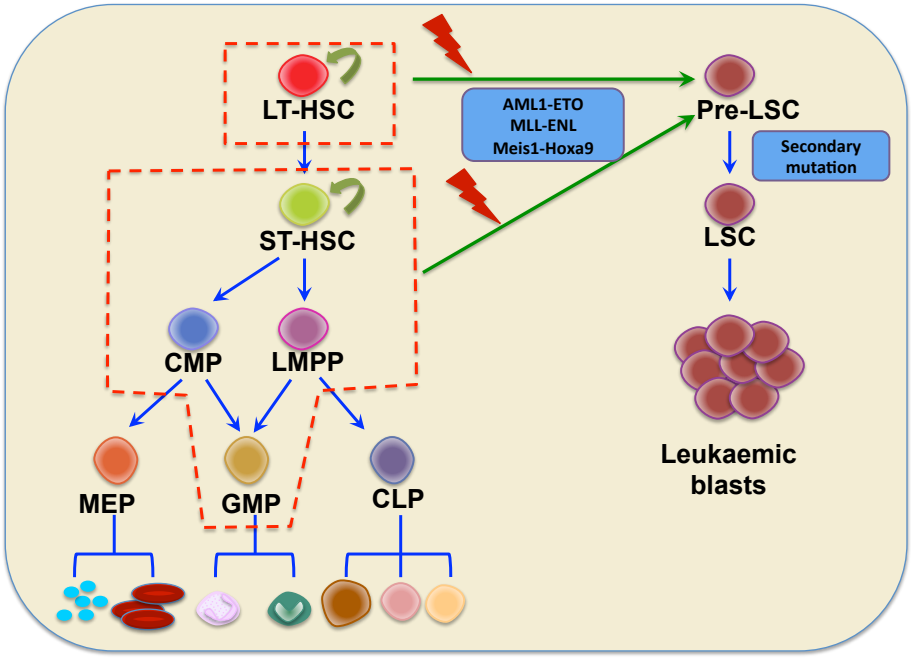
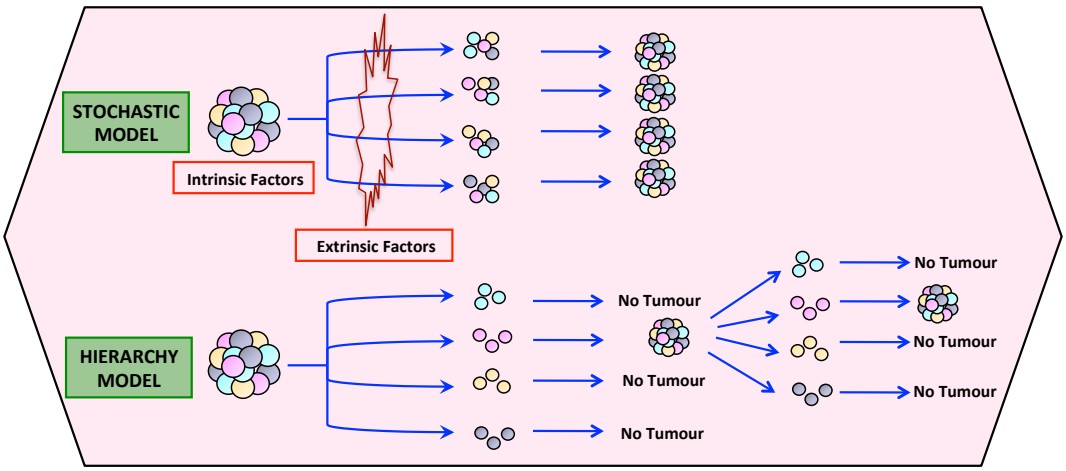



**Figure 1. 10: Ontogeny of leukemic stem cell.**

During normal haemopoiesis, LT-HSCs and ST-HSCs display self-renewal potential giving rise to primitive progenitors (LMPPs and CLPs) and more committed progenitors (GMPs, CLPs, MEPs) that differentiate into mature blood lineages. The cell of origin in AML is either a HSC or a downstream progenitor cell. Formation of fusion proteins (AML-ETO, MLL-ENL), co-expression of Meis1-Hoxa9 and mutations in LT-HSCs, or in the progenitor cells (represented by doted lines), result in the generation of Pre-LSC. Pre-LSCs acquire secondary mutations forming LSCs leading to leukaemic blasts. LT-HSC=Long-term haemopoietic stem cells; ST-HSC=Short-term HSC; LMPP=Lymphoid primed multipotent progenitor; CMP=Common myeloid progenitor; CLP=Common lymphoid progenitor; GMP=Granulocyte/macrophage progenitor; MEP=Megakaryocyte/erythroid progenitors; LSC=Leukaemic stem cells.

### 1.6.1.3 Models of leukaemogenesis

A heterogeneous population of cells that are phenotypically and functionally distinct are reported in leukaemia (Hope et al. 2004; Jan and Majeti 2013). Two different models are proposed to explain the heterogeneity of AML. According to the stochastic model, leukaemia consists of a homogenous population of immature cells that self-renew or proliferates in a stochastic manner influenced by cell intrinsic and extrinsic factors (Till et al. 1964; Korn et al. 1973; Reya et al. 2001). In contrast, the hierarchical model of leukaemia consists of a heterogeneous population of cells within which a small percentage of LSCs generate leukaemia and sustain the disease (Ravandi and Estrov 2006) (Figure 1.11).




Adapted and Modified from Dick J. 2008

**Figure 1. 11: Model of tumour heterogeneity**

Explaining the heterogeneity of tumours, two theories are proposed: Stochastic model and hierarchy model. According to the stochastic model, all tumour cells are capable of giving rise to tumours and their heterogeneity is influenced by cell intrinsic and extrinsic factors. In contrast, the hierarchy model proposes that tumour cells are biologically distinct with a subset of tumour-initiating cells that retain the tumourigenic potential while others are non-tumourigenic progeny.

### 1.6.1.4 Molecular genetics of AML

Genetic mutations in *NPM1,* FMS-like tyrosine kinase 3 (*Flt3)* and CCAAT/enhancer binding protein alpha (*C/EBP*$\alpha$) that are reported in cytogenetically normal AML have improved the WHO classification of AML and are applied as molecular markers in disease prognostication (Nakao et

al. 1996; Kiyoi et al. 1998; Thiede et al. 2002; Falini et al. 2005; Thiede et al. 2006). Bearing in mind the vast number of genetic mutations that are reported in AML, I have described some of the commonly occurring genetic and epigenetic mutations.

#### 1.6.1.4.1 Flt3

*FLT3* gene encodes a class III tyrosine kinase protein expressed in haemopoietic progenitor cells regulating differentiation, proliferation and survival (Kiyoi and Naoe 2002). *FLT3* internal tandem mutations (ITD) have been reported in 20%-27% of AML in younger adults and occur mostly in exon 14 and 15 affecting the juxtamembrane region (Nakao et al. 1996; Kiyoi et al. 1998; Thiede et al. 2002). *FLT3*-ITD mutations have been associated with significantly inferior outcomes and high risk of relapse (Whitman et al. 2001; Frohling et al. 2002; Thiede et al. 2002). Another point mutation in the *Flt3* at TKD (tyrosine kinase domain) resulted in the activation of *Flt3* receptor generating leukaemic blasts with uncontrolled proliferation (Mead et al. 2007). Patients with *FLT3*-ITD mutations harbouring ITD integration in the TKD-1 domain and others with co-occurrence of *WT1* (Wilm's tumour 1) mutation have a particularly poor prognosis (Frohling et al. 2002; Thiede et al. 2002; Virappane et al. 2008). Patients with *FLT3* mutations failed to respond to chemotherapy and allogeneic HSC transplant, and have a poor outcome due to the high risk of relapse (Dohner and Gaidzik 2011). However, molecular targeted therapy using FLT3 inhibitors with more selectivity against *FLT3* have delivered promising results (Zarrinkar et al. 2009).

#### 1.6.1.4.2 NPM1

*NPM1* gene mutations are common single gene mutations reported in AML and occur in one third of AML cases (Falini et al. 2005; Thiede et al. 2006). *NPM1* mutations mostly occur in exon 12 with a frame shift resulting in the disruption of nucleolar localisation and functions of *NPM1*, including activation of tumour suppressors p53 and ARF (Falini et al. 2005; Grisendi et al. 2006). *NPM1* mutations have been associated with mutations in *FLT3*-ITD, *IDH1/2* and *DNMT3*$\alpha$, but are rarely seen in association with *C/EBP*$\alpha$

mutations (Schlenk et al. 2008; Green et al. 2010). However, treatments for patients with NPM1 mutations have favourable outcomes with lower risk of relapse (Dohner et al. 2005; Schlenk et al. 2008).

#### 1.6.1.4.3 CEBPA

*C/EBPα* gene encodes C/EBPα, a TF that belongs to the basic region leucine zipper (bZIP) family and is involved in myelopoiesis (Radomska et al. 1998). With a frequency of 5%-10% of AML, C/EBPα mutations occur in the amino-terminal region generating a truncated isoform of C/EBPα (p30) and loss of the full-length protein (p42) (Pabst et al. 2001). Mutations reported in the carboxy-terminal of C/EBPα resulted in defective dimerisation and DNA binding (Pabst et al. 2001). Studies involving murine models showed that loss of p42 or heterozygous mutations affecting both amino- and carboxy-terminal regions generated AML with defective haemopoiesis (Kirstetter et al. 2008). Prognosis of AML patients harbouring a C/EBPα mutation, particularly in patients without FLT3-ITD, has been reported to be relatively better than patients with both C/EBPα and FLT3-ITD mutations (Wouters et al. 2009).

### 1.6.1.5 Epigenetic modifiers in AML

Recent studies using genome-wide sequencing of AML patient samples have discovered a series of recurrent mutations in genes, including DNA methyltransferase 3 alpha (*DNMT3α*), Isocitrate dehydrogenase1/2 (*IDH1/2*), *ten-eleven-translocation gene 2* (*TET2*) and *EZH2*, that are involved in epigenetic regulation (Parsons et al. 2008; Delhommeau et al. 2009; Mardis et al. 2009; Abdel-Wahab et al. 2010; Gross et al. 2010; Ley et al. 2010; Yan et al. 2011). Some of the recently discovered epigenetic alterations have been summarised in the following paragraphs.

#### 1.6.1.5.1 DNMT3α

DNA methyltransferases are coded by *DNMT1, DNMT3α and DNMT3β* and have been shown to catalyse the methylation of cytosine residue of CpG (Cytosine-phosphate-Guanine) dinucleotide in the DNA (Bestor 2000). Mutations in *DNMT3α* are recurrent in 20%-22% of patients with de novo AML

and occur often with IDH mutations (Ley et al. 2010; Yan et al. 2011). *DNMT3α* mutations are also reported often in secondary AML and MDS patients (Walter et al. 2011). The somatic mutations include nonsense and missense of open-reading frame (ORF) of *DNMT3α* with a recurrent mutation at amino acid R882 that is often reported in AML and MDS patients (Ley et al. 2010; Walter et al. 2011). Several studies have associated *DNMT3α* mutations in AML with worse survival outcomes (Ley et al. 2010; Walter et al. 2011; Yan et al. 2011).

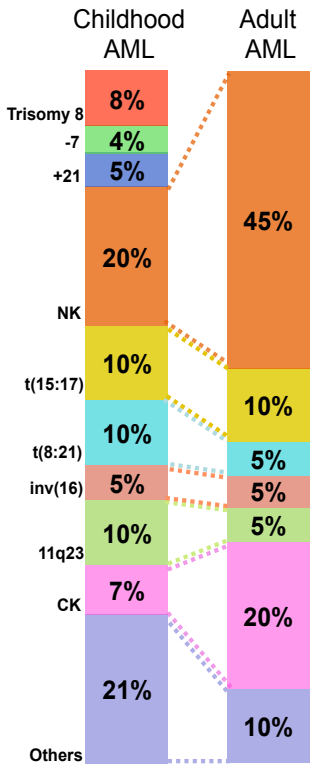
#### 1.6.1.5.2 IDH1/IDH2

IDH1/IDH2 is a cytosolic enzyme in the Krebs cycle and catalyses the decarboxylation of isocitrate to α-keto-gluterate (α-KG). Mutations in genes encoding *IDH1 and IDH2* are reported in genomic sequencing of AML (Parsons et al. 2008; Mardis et al. 2009; Gross et al. 2010). Heterozygous IDH1/2 mutations occur due to missense in the ORF region and are reported in 12%-22% of AML (Boissel et al. 2010; Gross et al. 2010). The mutated *IDH1* has been shown to catalyse α-KG to 2-hydroxygluterate (2-HG) (Boissel et al. 2010; Gross et al. 2010). The abnormal levels of 2-HG were reported in AML cells and sera from patients with IDH mutations (Boissel et al. 2010; Gross et al. 2010). The hypermethylation of various promoter sites that are specific to myeloid differentiation and leukaemogenesis has been associated with IDH mutations (Figueroa et al. 2010). Further evidence reported that the altered levels of 2-HG/α-KG in *IDH1/2* mutant cells disrupted the expression of jumonji histone lysine demethylases (JHDM), which regulates the expression of Mdm2 and p53 (Xu et al. 2011). Hence, epigenetic mutations, such as *IDH1/2,* are being extensively studied to understand the mechanisms regulating AML generation.

#### 1.6.1.5.3 TET2

*TET2* mutations were reported in 24% of patients with secondary AML (Delhommeau et al. 2009; Abdel-Wahab et al. 2010). DNA analysis showed that *TET2* mutations frequently occurred following transformation of myeloproliferative neoplasms (MPN) to AML (Delhommeau et al. 2009; Abdel-Wahab et al. 2010). The majority of *TET2* mutations were found in

the precursor cells of AML patients with mutations in $CD34^+$ progenitor cells (Langemeijer et al. 2009). Furthermore, mutations in *TET2* resulted in the defective demethylation of DNA and impaired conversion of 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC) (Ko et al. 2010). However, a rare co-occurrence of *TET2* mutation and *IDH1/2* mutation suggested the existence of a common mechanism that guides the leukaemogenic potential (Metzeler et al. 2011; Xu et al. 2011).




Adapted from Zeisig et al 2012

**Figure 1. 12: Frequency of cytogenetic aberrations in AML.**

Comparison of frequency of occurrence of cytogenetic aberrations in childhood and adult AML, NK – normal karyotype and CK – complex karyotypes.

### 1.6.1.6 AML-associated translocations: the paradigm of fusion proteins

AML associated with non-random translocations often results in the generation of a chimeric gene that encodes a novel fusion protein (Scandura et al. 2002; Cozzio et al. 2003; So et al. 2003; Somervaille and Cleary 2006). The fusion proteins retain the DNA-binding motifs and guide the interaction of the co-repressor complex with the active transcription locus, thus

altering the expression of the target genes that are essential for myeloid development and causing leukaemic transformation (Scandura et al. 2002). However, other abnormalities or 'second hits' appear to be necessary for full leukaemic development (Scandura et al. 2002). There are 749 recurrent aberrations reported in AML. Bearing in mind the enormity of oncogenic fusions identified in AML, I will be restricting the discussion to oncogenes (Meis1 and Hoxa9) and fusion proteins (AML1-ETO and MLL-fusions) studied in this thesis.

#### 1.6.1.6.1 AML1-ETO; t(8;21)

##### 1.6.1.6.1.1 Cytogenetic and clinical characteristics of AML1-ETO translocations

The t(8;21) translocation contributes to approximately 10% of AML cases (Martens and Stunnenberg 2010) that are associated strongly with childhood leukaemia and are classified as FAB M2 subtype of leukaemia with maturation (Mrozek et al. 2001). The chimeric oncofusion is produced by aberrant translocation of the *AML1* gene on chromosome 21q22 with the *ETO (Eight Twenty One)* gene on chromosome 8q22, generating a fusion protein AML1-ETO (Peterson and Zhang 2004). The AML1-ETO protein contains the N-terminal of AML1 with DNA binding and core binding factor ß (CBFß)-binding runt homology domain (RHD), while the C-terminal is replaced by almost the entire ETO protein (Wotton et al. 1994; Mao et al. 1999).

AML1 is a DNA-binding TF and plays a vital role in haemopoiesis (de Bruijn and Speck 2004). AML1 is a component of the CBF complex with a central RHD domain that binds to DNA and interacts with various TFs, such as CBFß, Ets proteins, smads and C/EBP$\alpha$ (Wotton et al. 1994; Mao et al. 1999; Scandura et al. 2002). AML1 interacts with CBP/p300 and activates transcription of IL-3, M-CSFR (macrophage colony-stimulating factor receptor), T- and B-cell receptors, as well as functions as a co-repressor of CD4, Sin3 and N-CoR, along with various other nuclear proteins that play a key role in chromatin remodelling (Wotton et al. 1994; Wang et al. 1998; Mao et al. 1999; Scandura et al. 2002). ETO is a member of the nuclear protein family (Feinstein et al. 1995) and functions as a transcriptional

repressor by interacting with *promyelocytic leukaemia zinc finger (PLZF)* and *Gfi-1* that regulate haemopoiesis (Melnick et al. 2000; Davis et al. 2003). Further, ETO has been shown to be involved in the recruitment of histone deacetylase (HDAC) proteins in the multi-protein complexes, such as N-CoR, SMRT or Sin3 (Jakubowiak et al. 2000).

#### 1.6.1.6.1.2 AML1-ETO fusion protein

AML1-ETO fusion protein dominantly perturbs the normal function of AML1 as the truncated AML1 of AML1-ETO lacks the transcriptional-activating domain and CBP/p300 binding domain, hence inhibiting AML1-dependent transactivation (Okuda et al. 1998; Davis et al. 2003). It has been suggested that AML1-ETO fusion protein functions as a dominant negative regulator of wild type (WT) AML1 and as a transcriptional repressor, by recruiting co-repressor NCoR/SMRT/HDAC complexes to DNA, through its ETO portion (Frank et al. 1995; Meyers et al. 1995; Uchida et al. 1997). AML1-ETO fusion protein functions as an activator of transcription by influencing the expression of genes controlled by AML1 protein. AML1-ETO fusion protein activates AML1 target genes, such as *jagged1* (a notch-ligand that maintains proliferation of stem cells), *ß-Catenin* and *Plakoglobin* (involved in Wnt-signalling) and anti-apoptotic gene *BCL-2* (Klampfer et al. 1996; Alcalay et al. 2003; Muller-Tidow et al. 2004). Previous studies demonstrated that AML1-ETO protein was a suppressor of cell differentiation and inhibited key TFs involved in haemopoietic differentiation, like PU.1, C/EBP$\alpha$ and GATA-1 (Petrovick et al. 1998; Mao et al. 1999). Also, AML1-ETO has been shown to physically interact and silence the E-protein family, particularly, HEB/TCF12, hence targeting DNA through E-box motifs (Zhang et al. 2004; Gardini et al. 2008). AML1-ETO has also been involved in the formation of pre-leukaemic cells with primitive cell-characteristics (expression of c-Kit) and defective differentiation potential (Beghini et al. 2000). Hence, AML1-ETO is a multifunctional protein affecting various cell programs, such as differentiation, proliferation, apoptosis and self-renewal. However, additional mutations along with the formation of AML1-ETO are essential for the development of AML (Scandura et al. 2002).

#### 1.6.1.6.2 MLL-ENL, t(11;19)(q23;p13.3)

##### 1.6.1.6.2.1 Cytogenetic and clinical characteristics of MLL translocations

MLL has been associated with a chromosomal rearrangement at 11q23 and has been reported in both adult and paediatric AML and ALL patients (Djabali et al. 1992; Tkachuk et al. 1992). MLL translocations result in binding of N-terminal of MLL with DNA and its methytransferase domain with the C-terminal portion of its translocation partner (Yano et al. 1997). MLL translocation accounts for 10% of human acute leukaemia and has poor prognosis (Eguchi et al. 2005). The MLL has more than 60 translocation partner genes and has been involved in more than 100 recurrent translocations (Meyer et al. 2009). The most frequent MLL rearrangements include AF4, AF9, ENL, ELL and AF10. MLL-ENL fusion protein with t(11;19) translocation is frequently reported in infants and results in a medial survival of less than 1 year (Meyer et al. 2009; Muntean and Hess 2012).

MLL protein is a member of the trithorax group family of proteins that is evolutionarily conserved (Ringrose and Paro 2004). It is a positive regulator of Hox gene expression through histone H3 lysine 4(H3K4)-methytransferase activity (Ringrose and Paro 2004). PHD (plant homeodomain) zinc-fingers located centrally in the MLL protein mediate homodimerisation and it's binding to other nuclear proteins (Chang et al. 2010b; Milne et al. 2010). The C-terminal of MLL protein, called the SET domain, is involved in homodimerisation of the MLL protein, which is necessary for the interaction with mammalian ATP-dependent chromatin remodelling SWI/SNF complex (Milne et al. 2002; Nakamura et al. 2002).

##### 1.6.1.6.2.2 MLL-ENL fusion protein

The MLL-ENL fusion protein is capable of initiating an aberrant self-renewal program in the targeted HSPCs, leading to transformation of both HSCs and myeloid progenitors, like CMPs and GMPs, to form LSCs (Horton et al. 2013; Krivtsov et al. 2013). The MLL-ENL fusion protein contains the N-terminal transactivating domain retained in the MLL gene portion and the C-terminal is replaced by the fusion partner i.e. ENL (Muntean et al. 2008; Yokoyama et al. 2010). Hence, both PHD zinc fingers and SET domain are lost from the

fusion protein. A previous publication showed that the conserved transcriptional activation domain of ENL was both necessary and sufficient to immortalise murine myeloid cells *in vitro* (Zeisig et al. 2005). ENL is a nuclear protein and evidence suggests that ENL, as a fusion partner, forms a higher-order super elongation complex (SEP), which is recruited to MLL-target loci for transcriptional activation and elongation in MLL-ENL transformed cells (Lin et al. 2010). Further, studies demonstrated that DOT1L, a histone methyltransferase, is involved in MLL-ENL mediated oncogeneic transformation (Chang et al. 2010a; Jo et al. 2011). Evidence gathered from previous studies showed that the MLL-ENL fusion protein acts as a transcriptional regulator and induces aberrant expression of downstream targets of MLL, such as *Hoxa7, Hoxa9, EPHA7, Meis1* and *Pbx* (Nakamura et al. 1996; Armstrong et al. 2002; Muntean and Hess 2012). Also, MLL-ENL has been shown to interact with c-Myc and to block cell differentiation (Schreiner et al. 2001). However, further research is essential to understand the precise mechanism for this aberrant transcriptional activity.

#### 1.6.1.6.3 Meis1 and Hoxa9 induced AML

Hox family members represent TF with DNA binding domains consisting of 60 amino acids, termed homeodomains (Boncinelli et al. 1989; Pearson et al. 2005). Hox genes are important for embryonic development and are key regulators of haemopoiesis (Pearson et al. 2005). Among Hox genes, Hoxa9 is highly expressed in haemopoietic progenitors and is linked with various forms of leukaemia (Argiropoulos and Humphries 2007). Elevated expression of *Hoxa9* and *Meis1* gene expression is often observed in AML patients and *Hoxa9* is a highly correlated gene for poor prognosis in AML (Golub et al. 1999; Lawrence et al. 1999; Afonja et al. 2000). Studies involving retroviral gene transfer models of AML showed that enforced overexpression of *Hox* genes, like *Hoxa9,* enhanced proliferation, causing leukaemia with long latency (Kroon et al. 1998). However, co-expression of *Meis1* with *Hoxa9* accelerated the onset of AML suggesting an inevitable role of Hox pathways in leukaemic transformation (Kroon et al. 1998; Thorsteinsdottir et al. 2001).

*Meis1*, a proto-oncogene, was identified as a common viral integration site in myeloid leukaemic cells of BXH-2 mice (Nakamura et al. 1996). Several studies have demonstrated that overexpression of *Meis1,* together with *Hox* gene or *NUP98-Hox* fusion gene, induced rapid onset of AML in murine BM transplantable models (Shen et al. 1997; Kroon et al. 1998; Lawrence et al. 1999; Afonja et al. 2000; Kroon et al. 2001; Thorsteinsdottir et al. 2001; Pineault et al. 2002). Hoxa9 protein binds with TALE (3-amino-acid-loop-extension) protein family members, like Meis and Pbx (Pre-B cell leukaemia homeobox), to form heterodimers that define the binding affinity and specificity of Hoxa9 (Shen et al. 1997). The molecular mechanism of interaction between *Meis1* and *Hox* genes in leukaemic transformation is not fully understood. However, studies in Hoxa9 and NUP98-Hox models of AML demonstrated that Meis1 upregulated *Flt3*, suggesting *Flt3* as a possible downstream target of *Hoxa9* and *Meis1* (Wang et al. 2005; Palmqvist et al. 2006; Wang et al. 2006). This is further supported by the elevated *Flt3* levels reported in AML samples that correlate with high levels of *Hoxa9* and *Meis1* expression (Ozeki et al. 2004; Quentmeier et al. 2004). Nevertheless, characterisation of Meis1-Hoxa9 regulated pathways controlling AML development is essential.

#### 1.6.1.7 Retroviral transduction/ transplantation model

Retroviral transduction of AML oncogenes or oncogenic fusions into murine primitive haemopoietic cells successfully generated the pathological AML (Lavau et al. 1997; Kroon et al. 1998; Zeisig et al. 2003). The retroviral transduction and transformation assay (RTTA) involves oncogenes and oncogenic fusions that are cloned into a retroviral expression vector, MSCV and transduced into primitive murine BM cells *in vitro* cultured in cytokines (IL-3, IL-6 and) substituted media (Lavau et al. 1997; Kroon et al. 1998; Lavau et al. 2000). Transduced cells are selected by reporter genes and transplanted into lethally irradiated recipients and are tracked for AML development (Lavau et al. 1997; Kroon et al. 1998; Lavau et al. 2000).

RTTA mouse models have provided an insight into the clonality of AML and reinforced the need for more than one mutation to cause AML (Kroon et al. 2001; Kelly et al. 2002a; Grisolano et al. 2003). RTTA of Flt3-ITD or

NUP98/HoxA9 resulted in myeloproliferative disorder (Yuan et al. 2001; Kelly et al. 2002b). However, genetic mutations in receptor tyrosine kinase such as Flt3 and TEL/PDGFβR coupled with transduction of oncogenic fusions (including PML-RARα or AML1-ETO) led to AML development in transplanted recipients (Kroon et al. 2001; Kelly et al. 2002a; Grisolano et al. 2003). The retroviral gene transfer system also shed light on the cell of origin of AML (Westervelt and Ley 1999). This model allowed investigators to test the oncogenic transformation potential of haemopoietic cells, including HSCs and cells along their differentiation hierarchy (Kroon et al. 2001; Huntly et al. 2004). RTTA studies of NUP98/HoxA9 generated oligoclonal AML in secondary recipients, suggesting HSCs as the target cells hit by the oncogenic fusions (Kroon et al. 2001). Retroviral introduction of oncogenes or oncogenic fusions is a great tool to explore the molecular pathways underlying AML (Wong et al. 2007; Yeung et al. 2010; Smith et al. 2011). Also, the transduction/transplantation could be taken forward to human cells and immunocompromised NOD/SCID mice that will provide a more relevant model for human AML and also serves as a novel model for preclinical therapeutic studies such as administration of small molecule tyrosine kinase inhibitors (Stover et al. 2005).

Despite the merits of RTTA model, drawbacks include the influence of genetic background of the mice on the outcome of type and latency of leukaemia (Zhang et al. 1998; Kelly et al. 2002a). Additionally, retroviral transduction resulted in the dysregulated transgene expression controlled by the long terminal repeat (LTR) promoter (Westervelt et al. 2003; Ren 2004). Hence, the irregulated expressions of oncogenes or oncogenic fusions are toxic or are associated with increased disease penetration (Westervelt et al. 2003; Ren 2004). Also, the lack of control over proviral integration site in the genome leads to insertional mutagenesis and MSCV retroviral vector by itself is associated with leukaemogenesis (Kohn et al. 2003; Du et al. 2005).

## 1.6.2 Hypothesis:

The main goal of my PhD is to test the hypothesis that hypoxia-signalling pathways regulate HSC fate decisions and under certain pathological conditions facilitate leukaemogenesis. I focused on the following objectives:

a. To study the role of *Hif-*$\alpha$ in normal haemopoiesis and HSC functions.

b. To explore and validate the role of the Cited2-Mcl-1 pathway in leukaemogenesis.

# Chapter 2

# Materials and methods

# 2.1 Mouse genetics

## 2.1.1 Mouse husbandry

All the experiments were approved by the Home Office under the project licence 60/4076 and personal licence (60/12879). Mice were housed in the dedicated facility that meets the "Code of practice for the housing and care of animals used in scientific procedures". Vigilant measures were followed to comply the 3Rs principles. Special care was taken to provide pathogen free environment. Mice that were 6-8 weeks old were used for breeding. Breeders were housed as pairs or trios. Pups were weaned at 3-4 weeks of age. At weaning, the pups were ear notched to give each one a unique identity and the ear-notches were collected for genotyping.

## 2.1.2 Generation of CKO transgenic mice

Hif-1$\alpha^{fl/fl}$ and Hif-2$\alpha^{fl/fl}$ mice were provided by Professor Sir Peter Ratcliffe on a collaborative basis. CKO for *Hif-1$\alpha$* were generated using the Cre-loxP technology (Gu et al. 1994) by Ryan et al (Ryan et al. 2000). A conditional allele of *Hif-1$\alpha$* was generated by introducing a loxP site at intron 1 and a loxP flanked neo-cassette at intron 2. The target vector was introduced by electroporation into F1-derived R1 embryonic stem cells. The homologous recombination of the target vector resulted in modified *Hif-1$\alpha$* allele with a loxP site 59 and loxP-flanked neo-cassette 39,both at exon 2. Transient expression of cre recombinase in the targeted ES cells, excised neomycin resistant gene. Clones containing loxP-flanked exon 2 and deleted neo-cassette, as confirmed by PCR were injected into the blastocysts of recipient C57BL/6. This generated Hif-1$\alpha^{fl/fl}$ chimeric mice with normal levels of *HIF-1$\alpha$* expression (Figure 2.1). Crossing Hif-1$\alpha^{fl/fl}$ mice with cre recombinase mice resulted in excision of exon 2, producing modified *Hif-1$\alpha$* mRNA transcript with non-functional protein. Hif-1$\alpha^{fl/fl}$ mice imported from Prof. Ratcliffe's lab were then crossed to C57BL/6 mice carrying cre under the Mx-1 promoter (HIf-1$\alpha^{+/+}$ Mx-1 Cre) (Table 2.1). This generated *Hif-1$\alpha$* CKO mice in which the deletion of *Hif-1$\alpha$* is induced by administration of immuno-stimulant pIpC.

*Hif-2α*$^{fl/fl}$ mice were generated by Gruber et al (Gruber et al. 2007). In order to generate conditional allele for *Hif-2α*, loxP sites were introduced at 0.5 Kbp upstream and downstream (with PGK-neo cassette) of exon 2. The construct with loxP sites was transfected into the 129X1/SvJ derived RW-4 embryonic stem cells. ES cell clones with correct 3-loxP allele were injected to the recipient blastocysts for germ line transfer of targeted allele. The offspring with heterozygous 3-loxP allele was crossed to E2A-cre to remove loxP flanked PGK neomycin cassette (Figure 2.1). Mice with exon 2-flanked loxP sites were crossed with C57BL/6 WT mice to separate E2A-Cre. Cre-mediated recombination lead to the loss of loxP-flanked exon2 and produced mutant *Hif-2α* transcript. The Hif-2α$^{fl/fl}$ mice obtained from Prof. Ratcliffe's lab were crossed to C57BL/6 mice carrying cre under the Mx-1 promoter. This generates *Hif-2α* CKO mice in which the deletion of *Hif-2α* is induced by administration of immuno-stimulant pIpC(Table 2.1).

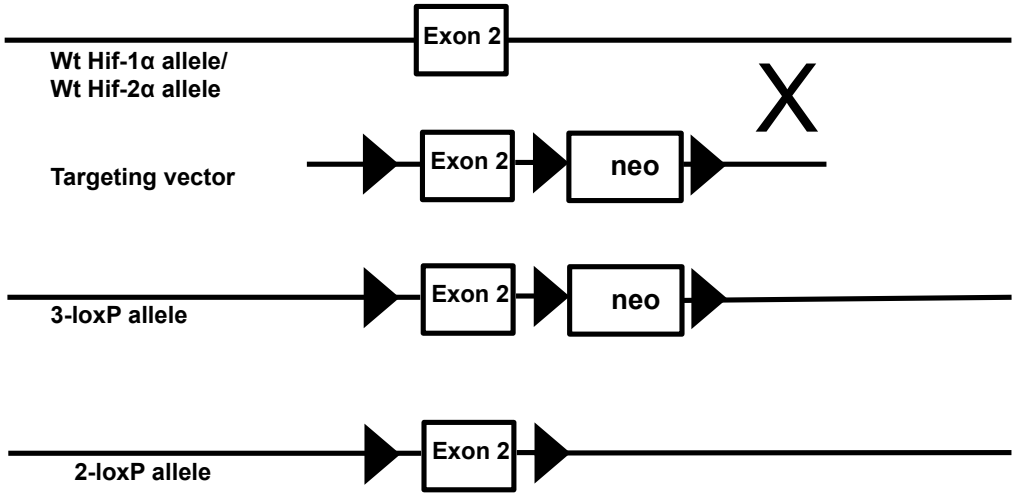



**Figure 2. 1: Targeting strategy for generation of Hif-loxP alleles.**

Structure of WT allele of *Hif-1α* or Hif-2α, targeting vector, 3-loxP allele and conditional 2-allele for *Hif-1α* or *Hif-2α* is generated. LoxP sites are introduced at intron 1 and 2 with frt-PGK::NeoR-frt (FNF) cassette sites at intron 2 flanked by loxP site. ▶ – loxP sites.

Cited2$^{fl/fl}$ transgenic mice were generated by MacDonald et al (MacDonald et al. 2008). The mice were given to us by Professor Shoumo Bhattacharya (University of Oxford) on a collaborative basis. The targeting vector was constructed by introducing sites in the intron 1 followed by frt-PGK::NeoR-frt (FNF) cassette downstream of exon 2 and second site as well as splice-acceptor-internal-ribosome-entry-site-lacZ cassette (IRES-Lacz) and a thymidine kinase (TK) cassette was introduced. The targeting vector

linearised by NotI and electroporated into 129Sv derived ES cells. The ES cells were selected for right target by PCR and injected in to blastocysts of CD57BL/6J and crossed to C57BL/6J females (Figure 2.2). Heterozygous mice generated were crossed to FLPeR mice to remove the FNF selection cassette and generated Cited2$^{+/fl}$ mice. These mice were intercrossed to obtain Cited2$^{fl/fl}$ mice. The Cited2$^{fl/fl}$ mice from Prof. Bhattacharya were crossed to C57BL/6 mice carrying cre under the Mx1 promoter or Vav promoter to generate CKO mice (Table 2.1).

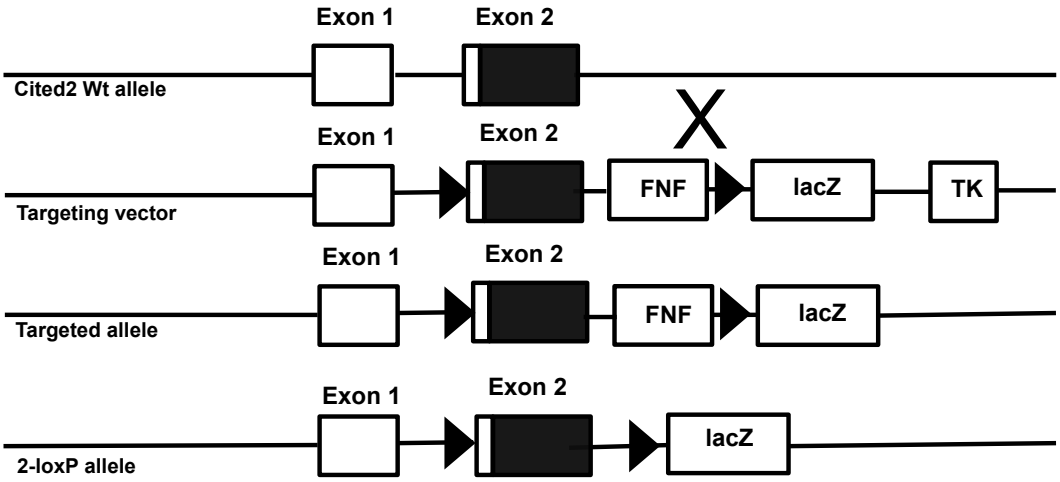



**Figure 2. 2: Targeting stratergy for the generation of *Cited2* 2-loxP allele.**

Structure of WT allele of *Cited2*, targeting vector, targeted allele and conditional loxP allele for Cited2 is shown. LoxP sites are introduced at introns and frt-PGK::Neo-frt (FNF) cassette downstream of exon2, followed by a second loxP site, a splice-acceptor-internal ribosome entry site-lacZ cassete, and a thymidine kinase (TK) cassette. ▶ –loxP site and block square-ORF.

Mcl-1 transgenic mice were generated by Campbell et al that express a FLAG-tagged mouse Mcl-1 cDNA in a vector bearing transcriptional regulatory sequences from the vav gene (Campbell et al. 2010b). Transgenic vector with murine Mcl-1 cDNA was introduced with to N-terminal FLAG epitope and inserted into Vav transgenic vector (Ogilvy et al. 1998). The transgene was excised by digesting with HindIII and into C57/BL6 mice by pronuclear injection as described above. Successful expression of transgene was confirmed by PCR using specific primers to the SV40 pA region (Campbell et al. 2010a) (Figure 2.3).

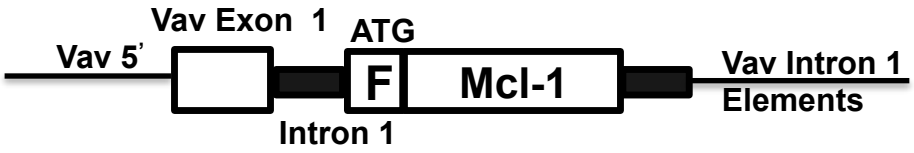



**Figure 2. 3 Schematic representation of vector overexpressing murine Mcl-1.**

Transgenic mice were generated that express a FLAG-tagged mouse Mcl-1 cDNA in a vector bearing transcriptional regulatory sequences from the vav gene.

| Desired genotype | Crosses |
|---|---|
| Hif-1 $\alpha^{+/+}$: Hif-2 $\alpha^{fl/fl}$<br>Hif-1 $\alpha^{+/+}$: Hif-2 $\alpha^{fl/fl}$ Mx1 Cre | Hif-1 $\alpha^{+/+}$: Hif-2 $\alpha^{+/fl}$ X Hif-1 $\alpha^{+/+}$: Hif-2 $\alpha^{+/fl}$<br>Hif-1 $\alpha^{+/+}$: Hif-2 $\alpha^{fl/fl}$ X Hif-1 $\alpha^{+/+}$: Hif-2 $\alpha^{fl/fl}$<br>Hif-1 $\alpha^{+/+}$: Hif-2 $\alpha^{fl/fl}$ Mx1Cre X Hif-1 $\alpha^{+/+}$: Hif-2 $\alpha^{fl/fl}$ |
| Hif-1 $\alpha^{+/fl}$: Hif-2 $\alpha^{fl/fl}$<br>Hif-1 $\alpha^{+/fl}$: Hif-2 $\alpha^{fl/fl}$ Mx1 Cre | Hif-1 $\alpha^{+/+}$: Hif-2 $\alpha^{+/fl}$ X Hif-1 $\alpha^{+/fl}$: Hif-2 $\alpha^{+/fl}$<br>Hif-1 $\alpha^{+/fl}$: Hif-2 $\alpha^{fl/fl}$ X Hif-1 $\alpha^{+/fl}$: Hif-2 $\alpha^{fl/fl}$<br>Hif-1 $\alpha^{+/fl}$: Hif-2 $\alpha^{fl/fl}$ Mx1Cre X Hif-1 $\alpha^{+/fl}$: Hif-2 $\alpha^{fl/fl}$ |
| Hif-1 $\alpha^{fl/fl}$: Hif-2 $\alpha^{fl/fl}$<br>Hif-1 $\alpha^{fl/fl}$: Hif-2 $\alpha^{fl/fl}$ Mx1 Cre | Hif-1 $\alpha^{+/fl}$: Hif-2 $\alpha^{+/fl}$ X Hif-1 $\alpha^{+/fl}$: Hif-2 $\alpha^{+/fl}$<br>Hif-1 $\alpha^{fl/fl}$: Hif-2 $\alpha^{fl/fl}$ X Hif-1 $\alpha^{fl/fl}$: Hif-2 $\alpha^{fl/fl}$<br>Hif-1 $\alpha^{fl/fl}$: Hif-2 $\alpha^{fl/fl}$ Mx1Cre X Hif-1 $\alpha^{fl/fl}$: Hif-2 $\alpha^{fl/fl}$ |
| Cited2$^{fl/fl}$<br>Cited2$^{fl/fl}$ Vav-iCre | Cited2$^{+/fl}$: Cited2$^{+/fl}$<br>Cited2$^{fl/fl}$: Cited2$^{fl/fl}$<br>Cited2$^{fl/fl}$ Vav-iCre: Cited2$^{fl/fl}$ |
| Cited2$^{fl/fl}$ Vav-iCre<br>Cited2$^{fl/fl}$ Vav-iCre Mcl-1 | Cited2$^{fl/fl}$ Vav-iCre: Cited2$^{fl/fl}$<br>Cited2$^{fl/fl}$ Vav-iCre: Cited2$^{fl/fl}$ Mcl-1 |

**Table 2. 1: Strategy used to generate CKO mouse models for *Hif-1α, Hif-2α* and *Cited2*.**

Transgenic mice with codon improved cre (*iCre*), with reduced epigenetic silencing in mammals) (Shimshek et al. 2002) transgene expression regulated by the haemopoietic specific Vav promoter element were generated by de Boer et al (de Boer et al. 2003a). The endogenous murine *Vav* gene is expressed in all the haemopoietic cells and *Vav* promoter element drives the expression of transgene efficiently and exclusively in all haemopoietic system (Ogilvy et al. 1998). Tissue restricted expression of iCre driven by Vav promoter was confirmed in all the haemopoietic system including spleen, thymus, lymph nodes with an exception in the testis (de Boer et al. 2003a) (Figure 2.4).

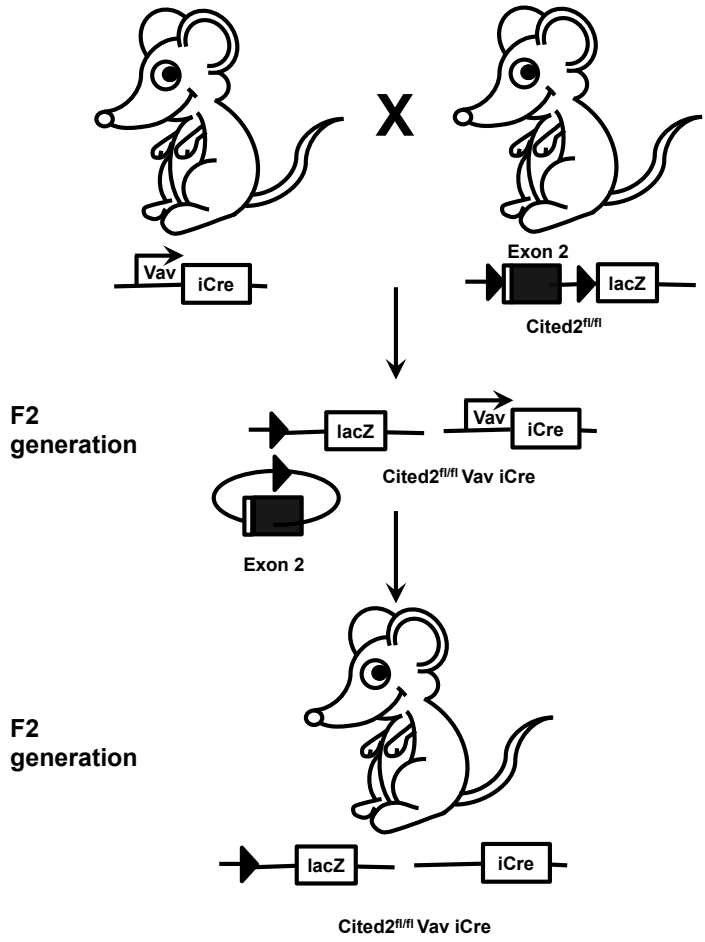



**Figure 2. 4: Model of Vav iCre mediated gene deletion.**

Conditional deletion of *Cited2* floxed allele in haemopoietic tissues is mediated by expression of iCre driven by the Vav promoter. Once recombined, the excision of floxed *Cited2* exon 2 results in expression of lacZ cassette driven by *Cited2* promoter confirming the gene deletion. – loxP site, block square – ORF. ▶

Mx1-Cre inducible gene deletion system in mice provides an advantage of gene deletion at given time during ontogeny (Kuhn et al. 1995). The expression of Cre recombinase is regulated by promoter element of *Mx1* gene. Endogenous *Mx1* gene is silenced in the healthy mice and expressed as a defense to viral infection (Hug et al. 1988). The expression of Mx1 promoter can be transiently activated to high levels by administration of interferon $\alpha$ (INF-$\alpha$), interferon ß (INF-ß) or an IFN inducer like pIpC, a synthetic double-stranded RNA (Kuhn et al. 1995). pIpC is a analog of ds-RNA that mimic the molecular pattern associated with viral infection (GE Healthcare, Cat No: 27-4732-01) . pIpC is recognised by Toll-like receptor 3 (TEL3) resulting in production of type-I IFN-ß, inflammatory cytokines and chemokines (including IL-6, TNF-$\alpha$, Cxcl-10) and activate NF-kB TF

(Alexopoulou et al. 2001; Matsumoto et al. 2002). The mice harboring a floxed target gene and Mx1-Cre transgene when treated with pIpC results in the activation of Mx1 promoter, drives the Cre expression that recognises the loxP sites and by homologous recombination generates inactivating mutation of the target gene (Kuhn et al. 1995) (Figure 2.5).

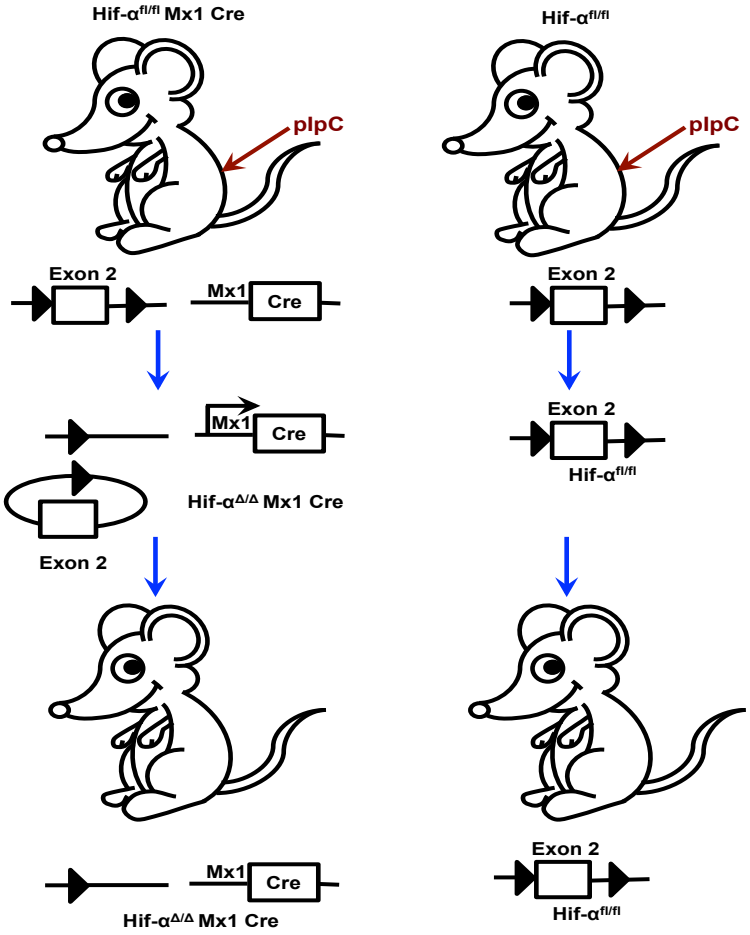



**Figure 2. 5: Model of Mx1 Cre mediated gene deletion.**

Conditional deletion of Hif-α floxed allele is mediated by expression of Cre driven by the Mx1 promoter. pIpC administration activates the Mx1 promoter leading to Cre expression. The Cre mediated recombination at the loxP site of exon 2 results in the excision of floxed Hif-α allele.

Supplier details of all the reagents used in this thesis is summarised with the catalogue numbers in appendix Supplementary table.

## 2.1.3 Preparation and administration of pIpC

pIpC is a synthetic double stranded RNA. It induces interferon response in the mice activating the Mx1 promoter. Thus inducing the deletion of floxed allele in the Mx1 driven cre mice (Kuhn et al. 1995). To prepare 1mg/ml of pIpC solution, 100ml of sterile PBS was added to 265mg of lyophilised pIpC and dissolved at 50$^{o}$C for 2-3 hours. 165ml of pre-warmed PBS was added to pIpC solution to make up the volume to 265ml. The solution was placed at 50$^{o}$C for additional 1hour until pIpC was fully dissolved. The solution was allowed to cool overnight at room temperature and 1ml aliquots of 1mg/ml concentration were prepared and stored at -20$^{o}$C.

Mice with 8-12 weeks of age received 6 doses of pIpC (300mg/dose) via intra-peritoneal (IP) injections every alternate day. Injected mice were monitored for the health status. The mice were sacrificed at day 11 after the last injection. Deletion of the floxed gene was confirmed by PCR of genomic DNA. Transplanted recipients with floxed allele of *Hif-1α* and/or *Hif-2α* received 5 doses of pIpC (250mg) via IP injections following 8 weeks after transplant every alternate day.

## 2.1.4 Genotyping

### 2.1.4.1 Extraction of genomic DNA

Genomic DNA was extracted from ear notch samples and haemopoietic cells using the ISOLATE II Genomic DNA Kit. The tissue samples or cell pellets were lysed in lysis buffer and proteinase K solution at 56°C, for 4 hours. Ethanol was then added to provide optimal DNA binding condition. The lysate was passed through spin column with silica membrane enabling binding of DNA. Remnant salts, cellular components and other impurities were removed in two wash steps by centrifugation. Genomic DNA was eluted from the silica membrane using preheated ddH20. DNA concentration was measured by nanodrop spectrometer (Nanodrop ND1000 Spectrophotometer; Labtech International Ltd, East Sussex, UK) by recording the absorbance at wavelength 260nm. The purity of DNA was assessed by the ratio of absorbance at 260nm and 280nm. DNA with A260/A280 ratio of 1.7-1.9 was considered pure.

### 2.1.4.2 Polymerase chain reaction (PCR)

The genomic DNA extracted was used to determine the genotypes of the chimeric mice. Primers for the PCR reactions are summarised in Table 2.2. Each PCR reaction with final volume of 25μl contained 2ng – 200ng of DNA template, 10μM of each of the primers, 2mM of MgCl2, 1X coloured buffer solution, 0.2mM of each of the dNTPs and 1 unit of Mango Taq (Bioline) (Table 2.3-2.7). The PCR product was loaded on to the Agarose gel directly and analysed by electrophoresis.

| Gene | | Primer | Band length |
|---|---|---|---|
| ***Cited2*** | WT | 5'-AAA GGC GCT AAG GAT AGA CAC-3'<br>5'-ATA CTG AGG TCC CTG GCA C-3' | 300bp |
| | Floxed | 5'-ATA ATA ATA ACC GGG CAG G-3'<br>5'-ATA CTG AGG TCC CTG GCA C-3' | 280bp |
| ***Hif-1*** $\alpha$ | WT | 5'-GCA GTT AAG AGC ACT AGT TG-3'<br>5'-GGA GCT ATC TCT CTA GAC C-3' | 200bp |
| | Floxed | 5'-TTGGGGATGAAAACATCTGC-3'<br>5'-GGA GCT ATC TCT CTA GAC C-3' | 2-loxP-250bp<br>1-loxP-280bp |
| ***Hif-2*** $\alpha$ | WT | 5'-CAG GCA GTA TGC CTG GCT AAT TCC AGT T-3'<br>5'-CTT CTT CCA TCA TCT GGG ATC TGG GAC T-3' | 430bp |
| | Floxed | 5'-CAG GCA GTA TGC CTG GCT AAT TCC AGT T-3'<br>5'-GCT AAC ACT GTA CTG TCT GAA AGA GTA GC-3' | 2-loxP– 460bp<br>1-loxP– 360bp |
| **Vav-iCre** | | 5'-CCG AGG GGC CAA GTG AGA GG-3'<br>5'-GGA GGG CAG GCA GGT TTT GGT G-3' | 380bp |
| **Mx1-Cre** | | 5'-CGT TTT CTG AGC ATA CCT GGA-3'<br>5'-ATT CTC CCA CCG TCA GTA CG-3' | 400bp |
| **Mcl-1** | | 5'-GGACTACAAAGACGATGACG-3'<br>5'-GGTATAGGTCGTCCTCTTCC-3' | 500bp |

| | Temperature | Time |
|---|---|---|
| 1 | 95ºC | 5 min |
| 2 | 95ºC | 30 sec |
| 3 | 67.5ºC | 30 sec<br>(-0.7ºC / cycle) |
| 4 | 72ºC | 40 sec |
| | From step 2 repeat 12 cycles | |
| 5 | 95ºC | 30 sec |
| 6 | 60ºC | 30 sec |
| 7 | 72ºC | 40 sec |
| | From step 5 repeat 22 cycles | |
| 8 | 72ºC | 5 min |
| 9 | 10ºC | Forever |

**Table 2. 3: Touchdown PCR programme used for genotyping *Cited2* mutant mice**

| Step | Temperature | Time |
|---|---|---|
| 1 | 94ºC | 5 min |
| 2 | 94ºC | 30 sec |
| 3 | 57ºC | 45 sec |
| 4 | 72ºC | 60 sec |
| | From step 2, repeat 35 cycles | |
| 5 | 72ºC | 5 min |
| 6 | 10ºC | Forever |

**Table 2. 4: PCR programme used for genotyping *Hif-1α* and *Hif-2α* mutant mice**

| Step | Temperature | Time |
|---|---|---|
| 1 | 94ºC | 15 min |
| 2 | 95ºC | 40 sec |
| 3 | 50ºC | 30 sec |
| 4 | 72ºC | 60 sec |
| | From step 2, repeat 33 cycles | |
| 5 | 72ºC | 10 min |
| 6 | 14ºC | Forever |

**Table 2. 5: PCR programme used for genotyping *Mcl-1* mutant mice.**

| Step | Temperature | Time |
|---|---|---|
| 1 | 95ºC | 5 min |
| 2 | 94ºC | 40 sec |
| 3 | 58C | 40 sec |
| 4 | 72ºC | 30 sec |
| | From step 2, repeat 30 cycles | |
| 5 | 72ºC | 5 min |
| 6 | 10ºC | Forever |

**Table 2. 6: PCR programme used for genotyping Vav-iCre mice.**

| Step | Temperature | Time |
|---|---|---|
| 1 | 95ºC | 5 mn |
| 2 | 95ºC | 30 sec |
| 3 | 67ºC | 45 sec |
| 4 | 72ºC | 1 min |
| | From step 2, repeat 39 cycles | |
| 5 | 72ºC | 5 min |
| 6 | 10ºC | Forever |

**Table 2. 7: PCR programme used for genotyping Mx1-Cre mice**

#### 2.1.4.3 Gel electrophoresis

Agarose gel of required concentration was prepared by dissolving agarose in 1X TAE buffer (40 mM Tris acetate, pH approx. 8.3, containing 1 mM EDTA). Sybr safe, a substitute for ethidium bromide was added to the gel at 0.0001X concentration. The gel was run at 100V in 1X TAE buffer and the band length was measured against a standard DNA ladder (Bioloine easy ladder I).

## 2.2 Molecular and cell biology

### 2.2.1 Cultural conditions of cell lines and primary murine cells

Platinum E cells are the retroviral packaging cell lines derived from 293T cells. These were kindly gifted by Dr. Peter Laslo (Leeds Univeristy). The cells have EF1alpha promoter region and high expression of gag, pol and ecotropic env structural proteins. IRES drive the expression of selectable markers puromycin and blasticidin resistance. The cells are cultured in

Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2.0 mM L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin. The efficiency of cells to produce stable retrovirus was maintained by selection against puromycin (1μg/ml) and blasticidin (10μg/ml), every 2 months. The cells were split when they were 70%-80% confluent (Morita et al. 2000).

HEK 293 cells are transformed human embryonic kidney cell with sheared adenovirus 5 DNA (Graham et al. 1977). The cells lines were used to produce lentivirus. Cells were cultured in DMEM, supplemented with 10% heat-inactivated FBS, 2.0 mM L-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin.

NIH 3T3 cells are immortalised murine embryonic fibroblasts. They were generated from outbred NIH Swiss mice using 3T3 protocol (Jainchill et al. 1969). NIH 3T3 cells were cultured in DMEM, supplemented with 10% heat-inactivated FBS, 2.0 mM L-glutamine, 100units/ml penicillin, and 100units/ml streptomycin. These cells were used for retroviral and lentiviral titration experiments.

Adherent cells were passaged when they were 70% -80% confluent. Cells were washed with PBS, trypsinised and cell pellet was re-suspend in complete DMEM medium. Cell viability was calculated by trypan blue staining and haemocytometer.

To freeze harvested cells (cell lines and primary murine cells), cell pellet was re-suspended in freezing solution (FBS and 10% DMSO) and aliquoted to pre-chilled 2ml cryovials. Each vial contained about 0.5E6-3E6 cells. These cryovials were transferred to Mr.Frosty (allows slow cooling at the rate of -10°C/min) and placed overnight at -80°C. The cells were then moved to liquid nitrogen for long-term preservation. In order to culture cells from liquid nitrogen, cells in the cryovials were thawed rapidly at 37°C and were swiftly re-suspended in 10ml of FBS, pre-warmed at 37°C. The centrifuge at 450xg for 5min and cells were plated at respective densities based on the cell viability.

HSPCs were grown in Iscove's Modified Dulbecco's Media (IMDM) supplemented with cytokines including 40ng/ml SCF, 20ng/ml Interlukine-3 (IL-3), 20ng/ml Interluekine-6 (IL-6) along with 10% heat-inactivated FBS (Invitrogen), 2.0 mM L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin. Cells were plated at density of 0.3E6-1E6 cells/ml.

## 2.2.2 Retroviral/lentiviral gene transfer and expression

### 2.2.2.1 Design and production of retroviral/lentiviral constructs

The retroviral vector with oncogenic fusions pMSCVneo MLL-ENL, pMSCVneo AML1-ETO, pMSCVpuro Meis1a pMSCVneo Hoxa9 and Control vector pMSCVneo, pMSCVpuro were kindly gifted by Dr. Tim Somervaille (Manchester University) and Prof. Eric So (King's College London).

Murine Stem Cell Virus (pMSCV) vector was employed for the consistent high-level expression of gene of interest in haemopoetic system (Hawley et al. 1996). A 6kb fragment encoding MLL-ENL fusion transcript was inserted using the EcoRI site downstream of proviral LTR region of pMSCV vector. LTR region drives the transcription of the inserted cDNA. The murine PGK-1 internal promoter encodes the puromycin resistant gene and enables the selection of infected haemopoetic cells by making them resistant to antibiotics (Lavau C et al 1997) (Figure 2.6).

cDNA encoding Meis1a was cloned into the EcoRI and HpaI site of MSCVPGK-PAC vector. The PGK promotor controls the expression of puromycin resistance cassette. The LTR region regulates the expression of Meis1a gene expression. The complete length of murine Hoxa9 fragment was inserted at BamHI-XhoI site in the pMSCV vector. The LTR region drives the expression of Hoxa9 gene while the PGK promoter encodes the neomycin resistance gene. The cells infected with both Meis1a and Hoxa9 were selected by double resistance against puromycin and neomycin (Kroon et al. 1998) (Figure 2.6).

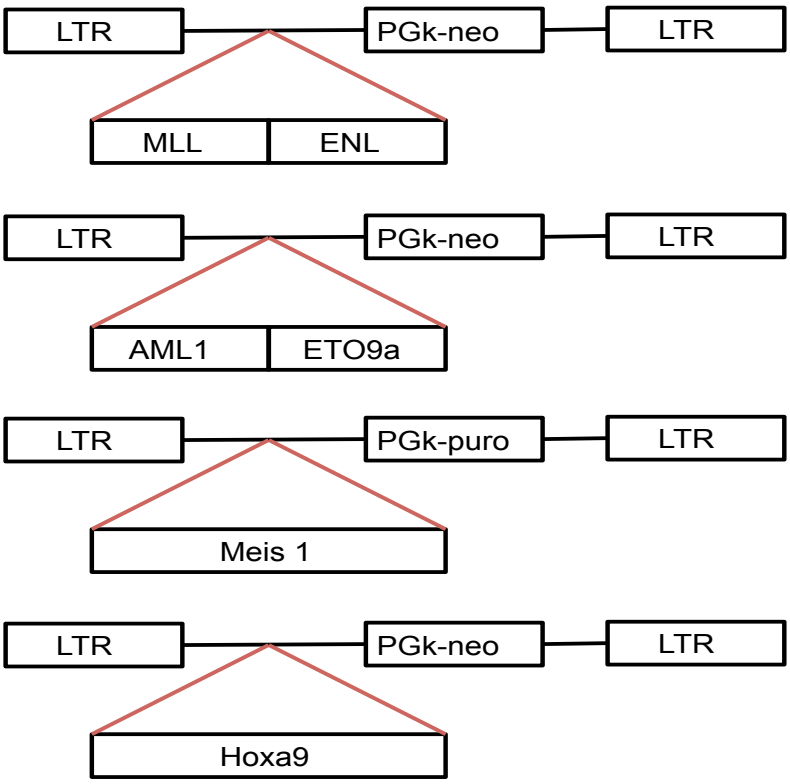



**Figure 2. 6: Schematic representation of the retroviral vectors employed for transformation of murine HSPCs.**

A CSI lentiviral vector (obtained from Professor Tariq Enver, UCL) was used for the expression of *Cited2* and *Mcl-1* genes in the haemopoietic cells. The vector consists EF-1alpha promoter that drives the expression of gene of interest and an IRES region that regulates the expression of reporter GFP. Human CITED2 or MCL-1 cDNA was inserted at the BamHI site, downstream of the promoter region (Personal communication with Prof. Kamil Kranc). Cells incorporated with gene of interest were sorted based on the expression of GFP.

### 2.2.2.2 Transformation and preparation of plasmid DNA

The plasmids were transformed into a competent bacterial cell. 2μl of plasmid was mixed with 25μl of competence bacteria in an ependoff tube. After 15 min of incubation on ice, the tube was placed in water bath at 42°C for 30 seconds followed by 5 min of incubation on ice. The mix was streaked on the LB agar plate containing 100μg/ml of ampicillin and incubated overnight at 37°C. An individual colony from the LB agar plate was selected

and cultured in 250ml of LB broth with ampicillin (100ug/ml) for 14-16 hours at 37°C in the bacterial shaker. Large volume of plasmid DNA was prepared using maxi-prep kit (Qiagen) using manufacturer's instructions. DNA was eluted in pre-warmed ddH2O, quantified and quality tested by nanodrop as described in section 2.1.3.1.

### 2.2.2.3 Retrovirus/lentivirus production

Platinum-E cells and HEK 293 cells were used to transfect pMSCV constructs and lentivectors, respectively. 60mm tissue culture dishes were coated with 0.1% sterile gelatine for 20 minutes at room temperature. The cells were trypsinised with extra care to obtain single cell suspension. 2E6 cells suspended in 10ml of substituted DMEM were plated into each 60mm dish and incubated at 37°C, 5% $CO_2$ for 24 hours. For transfection, 10μg of plasmid DNA and 3μg of VSV-G packaging plasmid (encodes for viral envelop protein) was diluted in sterile $H_2O$ to make up the volume to 420ml in sterile Bijou tubes. For lentiviral transfection, additional packaging plasmid p8.91 that encodes for viral capsid (gal, pol, REE sequence) was used. 60μl of 2M CaCl2 was added to the above mix, gentle finger flicked and incubated at room temperature for 10 min. 500μl of 2X HBS solution (NaCl, KCL, Glucose, HEPES, Na2HPO4, pH 7.1) in the bijou tube was bubbled using 1ml pipette while simultaneously adding the plasmid and CaCl2 solution drop wise into the 2X HBS solution. The transfection mix was incubated at room temperature for 20 minutes; added drop wise into the packaging cells and returned back to the incubator.

After 12-16 hours of incubation, the cells were fed with fresh DMEM without any substitution and cultured for 24-48 hours. The virus supernatant was collected at periods of 24 and 48 hours, filtered using 0.45μm filters and snap frozen as 1ml aliquots using dry ice. These aliquots were stored at -80°C for future use.

NIH3T3 cells were used to test the infection efficiency. 3T3 cells were plated at 0.1-0.3E5 cells/ml in 6 well plates in complete DMEM and incubated for 6-8hours. Once the cells were adherent, DMEM was replaced by infection cocktail (virus supernatant and 4ug/ml polybrene). After 12-16

hours of infection, the cells were washed and substituted with fresh media. The infection efficiency for retrovirus was tested by culturing the infected cells and uninfected cells in substituted DMEM along with antibiotics puromycin (1.5mg/ml) and neomycin (1mg/ml) for 48 hours. The infection efficiency was recorded by comparing the percentage of live cells in infected and uninfected cells. To test the lentivirus infection efficiency, infected cells were examined for GFP expression by flow cytometry.

#### 2.2.2.4 Isolation and transduction protocol of murine HSPCs

Cells were extracted from the BM as described in section 1.42. The cells were pelleted by centrifugation (450g for 5minutes) and re-suspended in RBC lysis buffer ($NH_4Cl$) for 1 minute at room temperature. The cells were washed in sterile wash buffer (PBS with 2% FBS). Up to 100E6 cells were re-suspended in 100μl of wash buffer and 2.5μl of CD117 (c-Kit) magnetic beads. The cells were incubated in dark rotor at 4°C for 20 minutes, washed and re-suspend in 3 ml of wash buffer. A MS column was placed in the MACS separation unit attached to the MACS multi-stand. To equilibrate the MS column, 3 ml of wash buffer was passed through the column via gravity. The cells were added through the 0.45μm filter into the MS column and allowed to flow through. To ensure the clear separation of c-Kit positive cells, the MS column was washed 3 times using a wash buffer (PBS/2% FBS) via gravity. The flow through from the MS column was collected to compare the purity of isolated of CD117 cells. 5ml of wash buffer was added to the MS column and immediately removed from the MACS separation unit. Using the plunger, the cells attached to the column via magnetic beads were flushed into the 15ml falcon tube. This step was repeated to ensure all the cells were extracted from the column. The c-Kit positive cells (HSPCs) were counted and checked for purity of c-Kit positive cells by staining with c-Kit antibody and flow cytometry.

For each infection, 0.5E6 HSPCs were plated in 500μl of IMDM with 40ng SCF, 20ng IL-3, 20ng IL-6, 10%FBS, 2mM L-glutamine, 100U/ml of penicillin and streptomycin (supplemented IMDM) and incubated overnight. Cells along with the media were transferred into 50ml falcon tubes with 1ml of virus supernatant and 4μg/ml of polybrene. The falcon tubes were placed in

centrifuge pre-warmed to 32°C and spun for 2 hours at 800g. After spinoculation, the cells were re-suspended in 500ul of fresh supplemented IMDM and returned to the incubator for 6-8 hours. For second run of infection, 500ul of virus with polybrene (4mg/ml final concentration) were added drop wise into the cells and returned to the incubator for overnight infection. Spinoculation procedure was repeated as mentioned before. Retrovirus transduced cells were cultured in supplemented IMDM for 24-36 hours, later transferred to antibiotic medium for selection of puromycin and/or neomycin resistance. Lentivirus transduced cells were cultured for 24 hours and checked for infection efficiency by expression of GFP using flow cytometry.

### 2.2.3 Serial re-plating transformation assay

HSPCs, retrovirally transduced with proto-oncogenes are selected for antibiotic resistance for 36 hours. The viable cells positively selected for antibiotic resistance were counted by tryphan blue staining. Methylcellulose (M3231, Stem cell technologies) was thawed at 37$^{o}$C and supplemented with 20ml IMDM, 20 ng/ml SCF, 10 ng/ml, IL-3, 10 ng/ml IL-6, 10 ng/ml GM-CSF, 100Units/ml penicillin, and 100mg/ml streptomycin. Contents of methylcellulose were mixed by vigorous shaking and aliquoted into 15 ml falcons as 2.25ml aliquots, stored at -20$^{o}$C. HSPCs are re-suspended in IMDM and appropriate antibiotics [puromycin (1.5µg/ml) and/or neomycin (1mg/ml)] making up the final volume to 250µl. Cell suspension was immediately added to the pre-warmed methylcellulose aliquot and vortex for even mixing of cells. 1ml of methylcellulose with 10E3 cells was plated into each well of 6 well plates using 18g blunt needle attached to 3ml syringe. The plates were humidified and incubated for 6 days at 37$^{o}$C, 5% $CO_2$.

At day 6, colonies were counted and the morphology of the colonies was examined. To re-plate CFCs, methylcellulose wells were washed using IMDM with 2% FBS using 10ml syringe. The process was repeated to extract all the colonies from the well into the 50ml falcon. Cells were centrifuged a 450xg for 10minutes and the step was repeated with fresh IMDM (2% FBS) to get rid of any remnant methylcellulose. Cell pellets were re-suspended in 1 ml of

IMDM and counted for viable cells using tryphan blue. Cells were plated onto methylcellulose at density of 10E3 cells/well as mentioned above with appropriate antibiotics and incubated for 6 days at 37°C, 5% $CO_2$. An aliquot of cells were used for immunostaining and the rest was frozen in multiple aliquots. The process is repeated until CFC-3 to produce pre-LSCs (Figure 2.7).

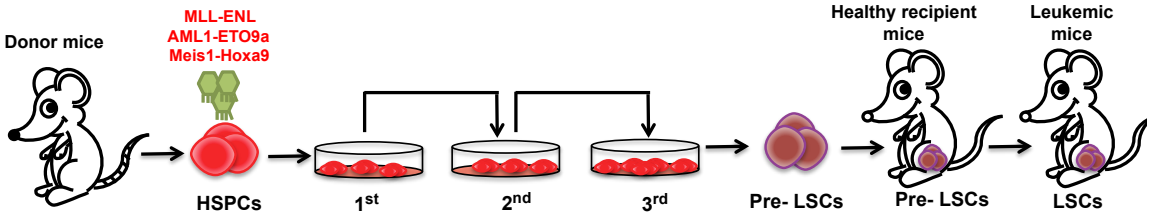



**Figure 2. 7: Schematic representation of RTTA.**

BM cells from donor mice are enriched for haemopoietic stem and progenitor cells (HSPCs), transduced with retrovirus expressing fusion genes AML1-ETO9a, MLL-ENL or co-expression of Meis1 and Hoxa9 are serially re-plated to form pre-LSCs. At CFC-3, pre-LSCs are injected in to lethally irradiated mice that generate LSCs and transplantable AML.

## 2.3 Isolation and flow cytometry for haemopoietic cells from the BM, Spleen, PB and Thymus

Dissected tibias and femurs were crushed and the cells were collected in PBS (2% FBS) by filtering through 70μM filters. Spleen and thymi were dissected and crushed using in 2ml PBS (2% FBS) with 5ml pluger. Isolated cells were collected in PBS (2% FBS). Peripheral blood (PB) was incubated in RBS lysis buffer ($NH_4Cl$, Sigma) for 20 min at room temperature and washed with PBS/2% FBS.

BM, spleen and PB cells (10,000,000) were incubated in CD16/CD32 antibody in 100μl of cold PBS/2% FBS for 5 minutes on ice and were stained with conjugated antibody for immunophenotypic analysis by flow cytometry (FACS). For slam staining, cell surface markers with rat anti-lineage (CD2, CD3, CD5, CD4, CD8a, Gr-1, CD19, B220, Ter119) with biotin (BD biosciences), Streptavidin-PE or streptavidin-PB, c-Kit-APC/APC-Cy7, Sca-1-APC-Cy7/PECy7, CD150 APC/PE-Cy7, CD48-PE (BD Biosciences, Biolegend)

was used. For lineage staining, antibodies Gr-1-APC/Pe-Cy7, CD11b-PE/PE-Cy7 (myeloid cells), CD19-APC-Cy7, B220-PE (B-cells), CD4-APC/PE, Ter119-FITC (erythroid) and CD8a-APC/PE (T-cells) (Table 2.8) were used. Cells were incubated with antibody cocktail in cold PBS/2% FBS for 20 minutes in dark on ice. Washed with PBS/2% FBS and re-suspended in 500µl of PBS/2% with viability dye DAPI. FACS analysis was performed using FACSCanto II flow cytometer (BD Biosciences) and the data acquired through BD FACSDiva (BD Biosciences) was analysed by Flowjo software (Tree Star Inc., USA).

| Conjugated fluorochrome | Antigen | Host | Clone | Concn | Supplier | Cat # |
|---|---|---|---|---|---|---|
| Biotin | CD2 | Rat IgG2b, λ | RM2-5 | 1/800 | BioLegend | 100103 |
| Biotin | CD3 | Hamster IgG1, K | 145-2C11 | 1/800 | BD Biosciences | 553060 |
| PE | CD4 | rat IgG2a, κ | H129.19 | 1/200 | BioLegend | 130310 |
| Biotin | CD4 | rat IgG2a, κ | H129.19 | 1/1600 | BD Biosciences | 553649 |
| APC | CD4 (L3T4) | rat IgG2a, κ | RM4-5 | 1/200 | BD Biosciences | 553051 |
| Biotin | CD5 | rat IgG2a, κ | 53-7.3 | 1/800 | BD Biosciences | 553019 |
| PE | CD8a | rat IgG2a, κ | 53-6.7 | 1/200 | BioLegend | 100708 |
| Biotin | CD8a | rat IgG2a, κ | 53-6.7 | 1/800 | BD Biosciences | 553029 |
| APC | CD8a | rat IgG2a, κ | 53-6.7 | 1/200 | BioLegend | 100712 |
| PE-Cy7 | CD11b | rat IgG2b, κ | M1/70 | 1/1500 | BioLegend | 101215 |
| APC | CD11b | rat IgG2b, κ | M1/70 | 1/1500 | BioLegend | 101211 |
| Biotin | CD19 | rat IgG2a, κ | 1D3 | 1/200 | BD Biosciences | 553784 |
| APC-Cy7 | CD19 | rat IgG2a, κ | 6D5 | 1/200 | BioLegend | 115530 |
| APC | CD31 | rat IgG2a, κ | MEC13.3 | 1/200 | BioLegend | 102510 |
| FITC | CD45.1 | mouse IgG2a, k | A20 | 1/200 | BioLegend | 110706 |
| Pacific Blue | CD45.2 | mouse IgG2a, κ | 104 | 1/200 | BioLegend | 109820 |
| Biotin | CD45R / B220 | rat IgG2a, κ | RA3-6B2 | 1/200 | BD Biosciences | 553086 |
| APC | CD45R / B220 | rat IgG2a, κ | RA3-6B2 | 1/2000 | BioLegend | 103212 |
| PE | CD48 | Hamster IgG | HM48-1 | 1/400 | BioLegend | 103406 |
| APC | CD48 | Hamster IgG | HM48-1 | 1/400 | BioLegend | 103411 |
| AF 488 | CD48 | Hamster IgG | HM48-1 | 1/400 | BioLegend | 103414 |
| APC-Cy7 | CD117 / c-Kit | rat IgG2b, κ | 2B8 | 1/200 | BioLegend | 105826 |
| APC | CD117 / c-Kit | rat IgG2b, κ | 2B8 | 1/200 | BioLegend | 105812 |
| PE-Cy7 | CD150 / SLAM | rat IgG2a, κ | TC15-12F12.2 | 1/200 | BioLegend | 115914 |
| APC | CD150 / SLAM | rat IgG2a, κ | TC15-12F12.2 | 1/200 | BioLegend | 115910 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Biotin | Gr-1 / Ly-6G/C | rat IgG2b, κ | RB6-8C5 | 1/200 | BD Biosciences | 553125 |
| APC | Gr-1 / Ly-6G/C | rat IgG2b, κ | RB6-8C5 | 1/1500 | BioLegend | 108412 |
| FITC | Sca-1 / Ly-6A/E | rat IgG2a, κ | E13-161.7 | 1/200 | BioLegend | 122506 |
| APC-Cy7 | Sca-1 / Ly-6A/E | rat IgG2a, κ | E13-161.7 | 1/200 | BioLegend | 122506 |
| PE-Cy7 | Sca-1 / Ly6-A/E | rat IgG2a, κ | E13-161.7 | 1/200 | BioLegend | 122514 |
| FITC | Ter119 | rat IgG2b, κ | TER-119 | 1/100 | BioLegend | 116206 |
| Biotin | Ter119 | rat IgG2b, κ | TER-119 | 1/50 | BD Biosciences | 553672 |
| PE | Streptavidin | | | 1/200 | BD Biosciences | 554061 |
| Pacific Blue | Streptavidin | | | 1/100 | Molecular probes invitrogen | S11222 |
| PE | Annexin V | | | 1/50 | BD Biosciences | 556422 |

**Table 2. 8: List of antibodies used for flow cytometric analysis.**

## 2.4 Isolation and flow cytometry for HSC niche cells from the BM

Briefly crushed bones (tibia and femur) were washed with PBS/2% FBS to remove haemopoietic cells. The bones fragments were digested in 3mg/ml collagenase VIII (Sigma) dissolved in HBSS/2% FBS for 2 hours at 37$^{o}$C by constant agitation at 11rpm. The released cells were washed with PBS/2% FBS and filtered using 70μM filters. Cells were stained with flourochrome-conjugated antibodies (CD45, Ter119, CD31, CD166 (ALCAM), Sca-1) and were analysed by FACS as mentioned above.

## 2.5 Detection of apoptosis using Annexin V

The 1,000,000 cells from the BM was re-suspended in 100μl PBS/2 % FBS containing 5μl of Annexin V APC and incubated for 20 minutes at RT in dark. 500μl of PBS/2% FBS was added to stop the reaction. DAPI, viability dye was added prior to acquisition and immediately analysed using FACS as described in the section 2.3.

## 2.6 Analysis of LacZ expression using FDG (Fluorescein di[β-D-galactopyranoside)

FACS based detection of LacZ+ cells by using fluorogenic substrate of galactosidase, FDG. Glactosidase based FDG cleavage releases flourescene product FITC that can be detected by FACS (Guo and Wu 2008). Cells were re-suspended in 50μl of PBS/2% FBS and warmed for 10 min at 37$^{o}$C in water bath. 50 ul of pre-warmed (37$^{o}$C) FDG was added with rapid mixing and incubated in 37$^{o}$C water bath for 1 min. To stop the reaction, 1 ml of cold PBS/2% FBS (frozen pipettes tips) was added and centrifuged in pre-cooled 4$^{o}$C micro-centrifuge 3200 rpm for 4 min. cells were re-suspended in cold 0.5 ml of PBS/2% FBS and transferred into cold FACS tubes and analysed for FITC using flow cytometer.

## 2.7 Cell sorting

Cells were isolated from the BM and PB as mentioned in section 2.3. Isolated BM cells were enriched for c-Kit$^{+}$ cells using magnetic beads and

MACS column as described in section 2.2.2.4. The cells were re-suspended in 100µl of cold PBS/2% FBS with antibodies and incubated for 200 minutes on ice in dark. The cells were washed with PBS/2% FBS and re-suspended in 500µl of PBS/2% FBS filtering through 70µM filters. The sorting was performed using BD FACSAria (BD Biosciences).

## 2.8 *In vivo* serial transplantation

Competitive BM transplantation assay was performed using B6.SJL mice with CD45.1 isoform as recipients. Mice were lethally irradiated (9Gy) using cobalt source irradiator. They were maintained in sterile conditions within IVC (individually ventilated cages) cages and treated with antibiotic (Baytril-0.15mg/ml) for 2 weeks after irradiation. The health status was monitored throughout the experiment period and scored according to project license 60/4076 CD45.2+ donor BM cells were isolated as mention above (section 2.3) and were mixed with 500,000 CD45.1+ competitor BM cells in a 1:1 ratio and injected intravenously (IV) into lethally irradiated recipients at 200µl volume/mice. For secondary and tertiary transplantation, BM cells were sorted for LSK as mentioned in the section 2.6 and together with 200,000 CD45.1+ support BM cells, 2000 LSKs were injected into the lethally irradiated recipients.

## 2.9 Gene expression assay

### 2.9.1 Extraction of RNA

RNA was extracted and purified using RNeasy micro Kit (Qiagen) according to manufacturers protocol. Equal number of cells was sorted directly in to lysis buffer (RNeasy micro kit) using FACSAria as described in section 2.6. RNA was extracted in 15µl of RNase-free water.

### 2.9.2 Quantitative PCR- total RNA

cDNA was prepared from total RNA using high capacity cDNA reverse transcription kit (Applied Biosystems) according to manufacturers protocol. 13µl of total RNA extracted from equal number of cells was used as template as the RNA obtained was below the quantification level due to

sample restrictions. Quantitative reverse transcription PCR (RT-PCR) was performed using universal probe library assay (UPL) for *Hif-1α* and *Hif-2α* and housekeeping gene (TATA-binding protein –TBP) (Table 2.9). Reactions were run on a LightCycler 480 System. 4.8µl of 10 times diluted cDNA was used per 10µl reaction of Primers (100µM), LAN-based probe (locked-nucleic acid) and Light-cycler 480 probes master (2X) programmed for 40 cycles (Table 2.10). All experiments were performed in technical triplicate. Differences in input cDNA were normalised using TBP expression and the mRNA expression levels were determined using comparative $C_T$ method (Schmittgen and Livak 2008).

| Genes | Primers |
|---|---|
| TBP | 5'-GGCGGTTTGGCTAGGTTT-3'<br>5'-GGGTTATCTTCACACACCATGA-3' |
| *Hif-1 α* | 5'-TTACGTGTGAGAAAACTTCTGGAT-3'<br>5'-GCCATCTAGGGCTTTCAGATAA-3' |
| *Hif-2 α* | 5'-TGACAGCTGACAAGGAGAAAAA-3'<br>5'-CAACTCATAGAAGACCTCCGTCTC-3' |

**Table 2. 9: Primers used for quantitative RT-PCR**

| Step | Temperature | Time |
|---|---|---|
| 1 | 95°C | 10 min |
| 2 | 95°C | 10 sec |
| 3 | 60°C | 30 sec |
| | Repeat from step 2 for 40 cycles | |
| 4 | 72°C | 1 sec |
| 5 | 40°C | 30 sec |

**Table 2. 10: PCR programme for quantitative analysis of *Hif-1α* and *Hif-2α* using LightCycler 480 system**

# Chapter 3

# The role of *Hif-1α* and *Hif-2α* in cell-autonomous HSC functions

## 3.1 Introduction

Previous studies have suggested that HSCs are localised in the special microenvironment of the BM, called the haemopoietic niche (Schofield 1978; Calvi et al. 2003; Zhang et al. 2003; Kiel et al. 2005; Scadden 2006; Morrison and Spradling 2008; Wang and Wagers 2011). BM is believed to be hypoxic compared to other tissues and HSCs reside in the most hypoxic region of the BM (Cipolleschi et al. 1993; Levesque et al. 2007; Parmar et al. 2007; Kubota et al. 2008; Eliasson and Jonsson 2010; Eliasson et al. 2010; Simsek et al. 2010; Takubo et al. 2010). The two isoforms of HIFs, HIF-1$\alpha$ and HIF-2$\alpha$ are key elements of cellular and systemic hypoxia responses and regulate gene expression to adapt to a hypoxic environment (Semenza 1999; Coleman and Ratcliffe 2007; Gruber et al. 2007; Kaelin and Ratcliffe 2008). Several studies have reported the hypoxic nature of HSCs (Danet et al. 2003; Hermitte et al. 2006; Shima et al. 2009; Shima et al. 2010; Takubo et al. 2010). Analysis of BM HSCs in mice with the hypoxia probe Pimo showed that HSCs retained the dye highly demonstrating that they were hypoxic (Danet et al. 2003; Takubo et al. 2010). Transplantation experiments revealed that human HSCs acquired hypoxic status when transplanted into irradiated mice and entered the quiescent phase (Shima et al. 2010). Human HSCs that were cultured ex vivo under hypoxia retained the reconstitution potential when transplanted into immunodeficient mice (Danet et al. 2003; Hermitte et al. 2006). Human HSCs and primitive progenitors when cultured under hypoxia *in vitro*, stabilised Hif-1$\alpha$ protein, retained the primitive phenotype and induced cell cycle quiescence (Danet et al. 2003; Hermitte et al. 2006; Shima et al. 2009). Therefore, the hypoxic nature of HSCs and the HSC niches is conserved across species.

Hifs were proven to be critical during embryogenesis and early development, as deletion of *Hif-1$\alpha$* and *Hif-2$\alpha$* in mice was embryonic lethal (Maltepe et al. 1997; Iyer et al. 1998; Ryan et al. 1998; Tian et al. 1998; Adelman et al. 1999; Adelman et al. 2000; Peng et al. 2000; Compernolle et al. 2002; Scortegagna et al. 2003a; Ramirez-Bergeron et al. 2006; Gruber et al. 2007). *Hif-1$\alpha$* null embryos showed defective neural tubes, cardiovascular defects, including hyperplasia and reduced viable

mesenchymal cells in the cranial region with enhanced hypoxia (Iyer et al. 1998; Ryan et al. 1998). Deletion of *Hif-1ß* subunit in mouse embryos resulted in decreased *Vegf* expression, leading to haemopoietic defects with decreased numbers of haemopoietic progenitors and mature cells due to increased apoptosis (Adelman et al. 1999; Ramirez-Bergeron et al. 2006). In adult mice, Takubo et al used a CKO approach using Mx1-Cre transgene and showed that acute deletion of *Hif-1α* resulted in loss of the cell cycle quiescence of HSCs and progressive loss of long-term repopulating capability of HSCs in chemotherapeutic 5-FU treated mice following serial transplantation (Takubo et al. 2010). Deletion of *Hif-1α* resulted in elevated expression of p16Ink4a and p19Arf, products of Ink4a that regulate cellular senescence (Takubo et al. 2010). Transplantation assay showed that the decrease in HSCs and primitive progenitor cells (LSKs) was regulated by *Hif-1α* in an Ink4 dependent manner and suppression of *p16Ink4a* and *p19Arf* in *Hif-1α*-deficient HSCs restored their repopulation potential (Takubo et al. 2010). During aging, *Hif-1α* deletion resulted in loss of the hypoxic nature of HSCs, decreased HSC number and resulted in extra medullary haemopoiesis (Takubo et al. 2010). It is noteworthy that HSC lacking *Hif-1α* maintained normal haemopoiesis under steady state and successfully reconstituted primary recipients when transplanted into lethally irradiated recipients (Takubo et al. 2010). Loss of repopulation potential of HSCs lacking *Hif-1α* was observed in secondary transplantation (Takubo et al. 2010). Stabilisation of Hif-1α protein by deletion of one or both alleles of *Vhl* enhanced the quiescent state in HSCs. However, over stabilisation of *Hif-1α* by deletion of both alleles of *Vhl* in HSCs led to loss of their reconstitution ability (Takubo et al. 2010). Additionally, it was shown that conditional deletion of *Hif-1α* resulted in dysregulated mitochondrial respiration (Simsek et al. 2010). Meis1 regulates the transcription of *Hif-1α* in LT-HSCs thereby contributing to HSC metabolism and enhanced glycolytic respiration in HSCs (Simsek et al. 2010). In conclusion, genetic studies, mostly from Prof Suda's laboratory, indicated an important role for *Hif-1α* in HSC maintenance under stressful conditions.

Investigations using murine models have stressed the importance of *Hif-2α* during embryogenesis (Tian et al. 1998; Peng et al. 2000; Compernolle et al.

2002; Scortegagna et al. 2003a; Gruber et al. 2007). Mouse embryos lacking *Hif-2α* exhibited variable phenotypes, including embryonic lethality (Peng et al. 2000), mid-gestation lethality (Tian et al. 1998) and perinatal death (Compernolle et al. 2002). Scrotegagna et al. generated congenic mixed strain F1 hybrids (Estill and Garcia 2000) with germ-line knockout of *Hif-2α* by interbreeding C57BL/6 and 129S6/SvEvTac (sub-strain of 129 strain called "steel") background *Hif-2α* heterozygotes (Simpson et al. 1997; Scortegagna et al. 2003a). These unique mixed strain background mice lacking *Hif-2α* survived until adulthood, developed pancytopenia and multiple organ pathologies (in the eyes, heart, liver, testis and BM) involving mitochondrial dysfunction, sensitive to high oxidative stress (Scortegagna et al. 2003a). To further investigate the role of *Hif-2α* in adult mice, Gruber et al took a CKO approach using the ubiquitously expressed Cre transgene (Ubc-CreERT2), where *Hif-2α* deletion in Hif-2$\alpha^{fl/fl}$ Ubc-CreERT2 was induced by tamoxifen in widespread tissues, including brain, intestine, liver, heart, kidney, BM, spleen, thymus (Gruber et al. 2007; Ruzankina et al. 2007). Postnatal deletion of *Hif-2α* generated anaemia with reduced RBC, haematocrit and haemoglobin values (Gruber et al. 2007). Reduced erythroid colony forming potential of haemopoietic progenitors and reduced circulating Epo in the serum at steady state and under stress conditions were also reported (Gruber et al. 2007). Previous studies indicated that the downstream target genes of *Hif-1α* and *Hif-2α,* such as *Cited2*, *Vegfα* and *Foxo3a*, were essential for HSC maintenance (Gerber et al., 2002; Kranc et al., 2009; Miyamoto et al., 2007; Rehn et al., 2011). Together these results suggested that *Hif-1α* and *Hif-2α* were required for normal haemopoiesis. However, the cell-autonomous requirement of *Hif-2α* in regulating haemopoiesis is not known.

*Hif-1α* and *Hif-2α* share similar domain structure and undergo the same proteolytic degradation (Wang et al. 1995). The expression of *Hif-1α* is ubiquitous while *Hif-2α* expression is restricted to certain cell types, such as vascular endothelial cells, hepatocytes, kidney fibroblasts, glial cells, pancreas, epithelial cells lining intestinal lumen, neural crest cell derivatives, and lung type II pneumocytes and in HSCs and haemopoietic progenitor cells (Tian et al. 1997; Jain et al. 1998; Wiesener et al. 2003).

While *Hif-1α* is essential for maintenance of LSCs (Zhang et al. 2012), *Hif-2α* expression is associated with various cancers, such as breast cancer, squamous cell carcinoma, lung cancer and Vhl associated renal clear cell carcinoma (RCC) and hemangiomas (Maxwell et al. 1999; Harris 2002; Covello et al. 2005; Hu et al. 2006). In addition, specific expression of *Hif-2α* and not *Hif-1α* in RCC patient samples has been reported (Maxwell et al. 1999). *Hif-1α* and *Hif-2α* share overlapping as well as opposing roles in different cell types. In RCC cells, *VEGF, GLUT-1* and *IL-6* were regulated by both *Hif-1α* and *Hif-2α* (Hu et al. 2003), but in mouse ESCs *Vegf* was regulated by *Hif-1α* alone (Iyer et al. 1998; Ryan et al. 1998; Hu et al. 2003). However, *Hif-1α* played a key role in glycolysis and controlled the expression of key genes, including *HK1, HK2, PGK1* and *LDHA* in RCC and mouse ES cells (Iyer et al. 1998; Ryan et al. 1998). *Hif-2α,* on the other hand had unique target genes like *Epo* in kidney (Scortegagna et al. 2003a; Gruber et al. 2007; Rankin et al. 2007) and liver (Kapitsinou et al. 2010), *CCND1* (Raval et al. 2005) in RCC cells and *Oct4* in mouse ES cells (Covello et al. 2006). Therefore, *Hif-1α* and *Hif-2α* have similar and distinct functions in different cell types and their functions are cell context-specific. However, the interplay between *Hif-1α* and *Hif-2α* in the HSCs remains unknown.

## 3.2 Outline of experiments described in Chapter 3

Very little is known about the role of *Hif-2α* in adult haemopoiesis. Specific functions of *Hif-2α* and its role within HSCs remain unexplored. Furthermore, the genetic requirement for and functional overlap between both *Hif-1α* and *Hif-2α* in the HSC functions remain to be investigated. Therefore, in this Chapter, I have focused on investigating the cell-autonomous roles of *Hif-1α* and *Hif-2α* in HSC survival and function. I conducted transplantation experiments where BM cells from mice carrying a floxed allele of *Hif-2α* or *Hif-1α* and *Hif-2α* with inducible Mx1-Cre and Control mice were transplanted to syngeneic recipients. Upon inducible gene deletion, the floxed alleles were deleted specifically in the donor derived haemopoietic cells and the haemopoietic organs were analysed. There was no difference in the number of HSCs or primitive progenitors

lacking *Hif-1α* and *Hif-2α* or *Hif-2α* alone, suggesting that *Hif-2α* is dispensable for adult haemopoiesis and additional deletion of *Hif-1α* did not affect HSC functions. I also aged the transplanted mice harbouring the haemopoietic system lacking *Hif-2α* alone or both *Hif-1α* and *Hif-2α* and showed that *Hif-2α* or both *Hif-1α* and *Hif-2α* were dispensable for stress induced haemopoiesis. Furthermore, I conducted serial transplantation experiments that determined the self-renewal properties of HSCs lacking *Hif-2α* or *Hif-1α* and *Hif-2α*. HSCs of all genotypes retained self-renewal ability equivalent to Control HSCs and repopulated the donor mice through successive transplantations. Taken together these experiments imply that *Hif-2α* is not essential for cell-autonomous maintenance of HSC functions under steady state and stressful conditions of serial transplantation and aging. It is also evident that HSCs lacking *Hif-2α* together with *Hif-1α* efficiently sustain normal haemopoiesis (Guitart, Subramani et al, Blood, 2013).

## 3.3 Result

### 3.3.1 The lack of requirement of *Hif-2α* in cell-autonomous HSC maintenance

To study the requirement of *Hif-2α* specifically within the haemopoietic system, I employed a competitive BM transplantation assay. I generated Hif-2α CKO mice, by crossing Hif-2α$^{fl/fl}$ mice to Hif-2α$^{+/+}$ Mx1-Cre mice. This cross subsequently generated Hif-2α$^{fl/fl}$ Mx1-Cre (in which floxed *Hif-2α* allele can be deleted upon administration of pIpC) and Hif-2α$^{fl/fl}$ without Mx1-Cre (hereafter referred to as Control). I transplanted 500,000 CD45.2$^{+}$ BM cells taken from untreated Hif-2α$^{fl/fl}$ Mx1-Cre mice and Hif-2α$^{fl/fl}$ Control mice, along with 500,000 CD45.1$^{+}$ support BM cells into lethally irradiated congenic CD45.1$^{+}$ recipients (Figure 3.1A). Having confirmed the engraftment of donor cells (Figure 3.1B) 8 weeks after transplantation the recipient mice were injected with 5-6 doses of pIpC (250ug per injection) on alternate days. Two weeks after the last pIpC injection, deletion of the floxed *Hif-2α* alleles in donor derived CD45.2$^{+}$ sorted cells from PB was confirmed by PCR (Figure 3.1C). Mice were sacrificed at 2 weeks and 4

weeks after last pIpC injection and haemopoietic organs were analysed for contribution of donor-derived cells. In the recipients, there was equal reconstitution of donor-derived CD45.2$^+$ cells in PB, BM and spleen from pIpC treated Hif-2$\alpha^{fl/fl}$ Mx1-Cre (hereafter referred to as Hif-2$\alpha^{\Delta/\Delta}$) and Control mice (Figure 3.2A). There was equal contribution of Hif-2$\alpha^{\Delta/\Delta}$ -derived and Control-derived CD45.2$^+$ Lin$^-$, LK (Lin$^-$ c-Kit$^+$), LSK (Lin$^-$Sca-1$^+$c-Kit$^+$), LMMPs (LSK CD150$^-$CD48$^+$ and LSK CD150$^+$CD48$^+$), HSC (LSK CD150$^+$CD48$^-$) and ST-HSC (LSK CD150$^-$CD48$^-$) cells in the BM of recipient mice (Figure 3.2B-H and 3.3). In mice sacrificed 2 weeks after the last pIpC injection the frequency of CD45.2$^+$ derived myeloid (Gr-1$^+$ and Mac-1$^+$) cells and erythroid (Ter119$^+$) cells in the PB, BM and spleens was similar in both groups of recipients (Fig 3.4A, 3.4B and 3.4C). The same frequencies of CD45.2$^+$ T-cells (CD4$^+$, CD8$^+$) in the PB, spleen and thymus (CD4$^+$and CD8$^+$) (Figure 3.4) were observed. However, there was an increase in the frequency of CD45.2$^+$ derived B-cells (CD19$^+$B220$^+$ cells) in the spleens of Hif-2$\alpha^{\Delta/\Delta}$ recipients compared to Controls (Figure 3.4C). The contribution of donor-derived cells to multilineage haemopoiesis in mice sacrificed 4 weeks after last pIpC injection showed a decrease in CD45.2$^+$ myeloid cells in PB, BM and spleens of Hif-2$\alpha^{\Delta/\Delta}$ recipients and an increase in B-cells in the BM of Hif-2$\alpha^{\Delta/\Delta}$ recipients compared to Control recipients (Figure 3.5). The above results showed that HSCs lacking Hif-2$\alpha$ efficiently repopulated primary recipient mice and therefore *Hif-2$\alpha$* was not absolutely essential for cell-autonomous HSC functions. The decrease in myeloid cells in the PB, BM and spleens and an increase in B-cells in the BM of Hif-2$\alpha^{\Delta/\Delta}$ recipients observed in primary transplant recipients suggested that *Hif-2$\alpha$* might play a role in the differentiation of myeloid and B-cells. However, more research is required to fully understand the importance of *Hif-2$\alpha$* in myeloid and lymphoid differentiation.

### 3.3.2 The requirement for *Hif-1$\alpha$* and *Hif-2$\alpha$* in cell-autonomous HSC maintenance

*Hif-1$\alpha$* is expressed at higher levels in HSCs compared to progenitor cells (Takubo et al. 2010). Loss of *Hif-1$\alpha$* has been shown to lead to reduced numbers of HSCs and loss of HSC quiescence under stressful conditions, such

as aging, serial transplantation and 5-FU treatment (Takubo et al. 2010). *Meis1*, a transcriptional regulator of *Hif-1α* and *Hif-2α*, was shown to be necessary for HSC quiescence and HSCs lacking *Hif-1α* expression failed to repopulate BM upon secondary transplantation (Simsek et al. 2010; Kocabas et al. 2012). The expression of *Hif-2α* in HSCs was higher than in the downstream progenitor cells (Takubo et al. 2010). However, the relationship between *Hif-1α* and *Hif-2α* and the ability of *Hif-2α* to compensate for the loss of *Hif-1α* in cell-autonomous HSC maintenance remains to be investigated. To dissect the role of *Hif-1α* and *Hif-2α* in cell-autonomous HSC maintenance and to uncover possible functional compensatory mechanism between *Hif-1α* and *Hif-2α*, I performed competitive BM transplantation experiments. 500,000 $CD45.2^+$ BM cells from untreated $Hif\text{-}1\alpha^{fl/fl}$ $Hif\text{-}2\alpha^{fl/fl}$ Mx1-Cre mice (double knockout, DKO) and Control mice were transplanted into lethally irradiated $CD45.1^+$ recipients along with 500,000 $CD45.1^+$ support BM (Figure 3.6A). Mice were treated with 5-6 doses of pIpC (250ug/injection) on alternate days and analysed after 2 weeks and 4 weeks of the last pIpC treatment. Deletion of floxed alleles in the sorted $CD45.2^+$ haemopoietic cells was confirmed by PCR (Figure 3.6B). Analysis of BM after 2 weeks and 4 weeks post pIpC injection showed no difference in the reconstitution of donor-derived HSCs, LMPPs, LSKs, LK or $Lin^-$ cells in DKO and Control recipients (Figure 3.7 and 3.8). The frequencies of donor-derived mature blood lineages of DKO recipients were equal to Control recipients (Figure 3.9A-3.9D and 3.10A-3.10D). These experiments showed that deletion of *Hif-1α* and *Hif-2α* within HSCs had no effect on short-term and long-term reconstitution of primary recipients. Taken together these data indicate that HSCs lacking both *Hif-1α* and *Hif-2α* efficiently repopulate primary recipients and sustain multilineage haemopoiesis.

### 3.3.3 The requirement for *Hif-1α* and *Hif-2α* in the maintenance of HSC self-renewal capacity in serial transplantation

Takubo et al reported that HSCs lacking *Hif-1α* displayed reduced self-renewal potential and progressive HSC loss when transplanted into lethally irradiated secondary recipients (Takubo et al. 2010). In primary recipients,

cells lacking *Hif-1α* exhibited successful haemopoietic reconstitution, but displayed reduced LSKs and failed to repopulate secondary recipients (Takubo et al. 2010). Serial transplantation is the most stringent measure of self-renewal potential of HSCs (Siminovitch et al. 1964; Harrison 1972; Harrison et al. 1978; Harrison and Astle 1982; Iscove and Nawa 1997; Sudo et al. 2000; Kondo et al. 2003; Ito et al. 2006; Janzen et al. 2006; Purton and Scadden 2007; Liu et al. 2009; Takubo et al. 2010; Kocabas et al. 2012). HSCs have self-renewal potential and efficiently sustain multilineage haemopoiesis during serial transplantation (Rosendaal et al. 1979; Lemischka et al. 1986; Purton et al. 2006). HSC population from the donor mice is transplanted into sequential irradiated recipients and their ability to repopulate recipients and sustain haemopoiesis is determined. I conducted serial transplantation experiments to test the self-renewal properties and long-term repopulation potential of HSCs deficient of *Hif-2α* or both *Hif-1α* and *Hif-2α*. After 4 weeks of last pIpC treatment (14 weeks post transplant) of primary recipients, 3000 CD45.2$^{+}$ LSK cells were sorted from primary recipients of Hif-2$\alpha^{\Delta/\Delta}$, DKO and Control cells. LSK cells were then transplanted into lethally irradiated secondary recipients along with 200,000 CD45.1$^{+}$ support BM (Figure 3.11A). Analysis of PB, BM and spleens showed equal reconstitution of donor-derived CD45.2$^{+}$ cells of Hif-2$\alpha^{\Delta/\Delta}$, DKO and Control recipients (Figure 3.11B and 3.11C). Analysis of Hif-2$\alpha^{\Delta/\Delta}$ and DKO with respective Control recipients at 24 weeks and 28 weeks after transplantation showed comparable frequencies of donor-derived HSCs, primitive progenitors (Figure 3.12 and 3.13) and equal reconstitution of mature blood lineages irrespective of the genotype (Figure 3.14). Further, I transplanted 3000 donor-derived LSKs from secondary recipients of Hif-2$\alpha^{\Delta/\Delta}$, DKO and Control into lethally irradiated tertiary recipients (Figure 3.15A). The analysis of PB, BM and spleens of the recipients at 16 weeks post transplantation indicated that Hif-2$\alpha^{\Delta/\Delta}$, DKO and Control donor-derived HSCs equally efficiently reconstituted the recipient mice (Figure 3.15B and 3.15C). There were no significant differences in HSCs or primitive progenitors (Figure 3.16 and 3.17), which successfully formed all the mature lineages irrespective of the genotype (Figure 3.18). However, the cells from DKO recipients seemed to have an advantage in reconstitution potential in comparison with Control recipients, but this did not reach statistical

significance (Figure 3.15C and 3.17). This might be due to the low engraftment of cells derived from Control mice in contrast to cells from DKO mice. Nevertheless, DKO HSCs efficiently repopulated lethally irradiated secondary and tertiary recipients. These experiments suggested that HSCs lacking *Hif-2α* alone or both *Hif-1α* and *Hif-2α* maintained the reconstitution potential during serial transplantation and Hif-α–dependent signalling is dispensable for cell-autonomous HSC function.

### 3.3.4 The requirement for *Hif-1α* and *Hif-2α* in normal haemopoiesis in aged mice

Several studies involving murine models have investigated the age-related modulations of HSC functions (de Haan et al. 1997; Sudo et al. 2000; Rossi et al. 2005; Chambers et al. 2007). Studies in aged mice reported an increase in the number of HSCs accompanied by decreased self-renewal potential and myeloid-biased defective differentiation (de Haan et al. 1997; Sudo et al. 2000; Rossi et al. 2005; Chambers et al. 2007). HSCs display defective homing ability and increased stress induced apoptosis with age (Morrison et al. 1996; Liang et al. 2005). Expression of cyclin-dependent inhibitor, *p16Ink4a* was increased with age in HSCs from mice as well as in human $CD34^+$ cells and deletion of p16In4a reinstated the self-renewal and apoptotic defects (Janzen et al. 2006; de Jonge et al. 2009). Notably, according to Takubo et al, aged *Hif-1α* CKO mice exhibited decreased HSC number compared to Control (Takubo et al. 2010). Defective transplantation phenotype of *Hif-1α*-deficient HSCs was Ink4a-dependent as suppression of *p16Ink4a* and *p19Arf* restored the transplantation defects (Takubo et al. 2010). Hence, it was interesting to see the effect of aging on HSCs lacking both *Hif-1α and Hif-2α*. To examine the effect of deletion of *Hif-2α* or *Hif-1α* and *Hif-2α* in aged mice, Hif-$2\alpha^{\Delta/\Delta}$, DKO and Control primary recipients were aged for 37 weeks after transplantation and the Hif-$2\alpha^{\Delta/\Delta}$ secondary recipients were aged for 32 weeks post transplantation while DKO secondary recipients were aged for 35 weeks. Details of recipient's age, pIpC treatment and analysis are summarised in the table3.1. Aged mice were sacrificed and analysed for donor-derived cell contribution using flow cytometry. The analysis of PB, BM and spleens of aged Hif-$2\alpha^{\Delta/\Delta}$ and DKO

primary recipients at week 27 after last pIpC injection showed an equal contribution of donor-derived HSCs, primitive progenitors (Figure 3.19) and multilineage re-constitution compared to Control recipients (Figure 3.20). Secondary recipients transplanted with Hif-2$\alpha^{\Delta/\Delta}$ and DKO cells analysed at week 24 and week 27 after last pIpC displayed no significant difference between donor-derived HSCs, LMPPs, LSKs or $Lin^-$ cells from Hif-2$\alpha^{\Delta/\Delta}$, DKO and Control recipients (Figure 3.21). There were equal frequencies of donor-derived mature blood lineages in the PB, BM and Spleen (Figure 3.22). These experiments further showed that HSCs lacking both *Hif-1$\alpha$ and Hif-2$\alpha$* retained reconstitution capacity during serial transplantation and maintenance of multilineage haemopoiesis during the course of aging.

| | | | | Analysis | |
|---|---|---|---|---|---|
| | **Donor** | **Cell type** | **pIpC administration** | **Weeks after last pIpC injection** | **Weeks after transplantation** |
| **Primary Transplant** | Hif-2α$^{fl/fl}$ Mx1-Cre, Hif-1α$^{fl/fl}$:Hif-2α$^{fl/fl}$ Mx1-Cre and Control | 500000 BM cells | 8 weeks after transplant | 2 weeks | 12 weeks |
| | | | | 4 weeks | 14 weeks |
| | | | | 27 weeks | 37 weeks |
| **Secondary Transplant** | Primary recipient (Hif-2α$^{\Delta/\Delta}$ and Control) | 3000 LSK | - | - | 24 weeks |
| | | | - | - | 32 weeks |
| | Primary recipient (DKO and Control) | 3000 LSK | - | - | 28 weeks |
| | | | - | - | 35 weeks |
| **Tertiary Transplant** | Secondary recipient (Hif-2α$^{\Delta/\Delta}$ and Control) | 3000 LSK | - | - | 16 weeks |
| | Secondary recipient (DKO and Control) | 3000 LSK | - | - | 16 weeks |

**Table 3. 1: Details of time frame between transplantation, pIpC treatment and analyses of transplantation recipients.**

# 3.4 Discussion

## 3.4.1 *Hif-2α* is dispensable for cell-autonomous HSC maintenance in stressful conditions of transplantation

Hypoxia is believed to be important in regulating HSC fate decisions of quiescence, self-renewal, apoptosis and differentiation (Miyamoto et al. 2007; Kranc et al. 2009; Simsek et al. 2010; Takubo et al. 2010; Suda et al. 2011). *Hif-1α*–deficient HSCs displayed functional defects with progressive loss of self-renewal potential and increased apoptosis (Takubo et al. 2010; Kocabas et al. 2012). Scortegagna and collegues studied congenic mixed breed Hif-2α knockout mice (C57BL/6 Hif-2α heterozygote:129S6/SvEvTac Hif-2α heterozygote) with global deletion of *Hif-2α* (Scortegagna et al. 2003a). These Hif-2α null mice showed increased perinatal lethality and a small number of Hif-2α null mice survived till adulthood, developed multiple organ pathologies, impaired mitochondrial metabolism, elevated oxidative stress and pancytopenia (Scortegagna et al. 2003a). Conditional postnatal ablation of *Hif-2α* in mice using ubiquitously expressed Ubc-Cre activated by tamoxifen treatment developed anaemia and defective erythropoiesis (Gruber et al. 2007). Acute deletion of *Hif-2α* in mice resulted in decreased RBC number, haematocrit and haemoglobin levels with reduced *Epo* production (Gruber et al. 2007). Lack of *Hif-2α* expression led to a decrease in erythroid progenitors in the BM, while in the spleen deletion of *Hif-2α* led to increase in erythroid colony forming potential activating potential compensatory mechanism (Gruber et al. 2007). However, specific role of *Hif-2α* in the regulation of cell-autonomous HSC functions remained to be investigated.

In this study, I have analysed the cell-autonomous effects of *Hif-2α* deficiency on HSC functions. The results in this Chapter demonstrate that HSCs lacking *Hif-2α* sustained normal haemopoiesis under stressful conditions of transplantation. Serial transplantation experiments showed that *Hif-2α*-deficient HSCs repopulated the secondary and tertiary recipients and maintained multilineage haemopoiesis. In the study by Takubo et al, the authors conditionally deleted *Hif-1α* in mice using Mx1-Cre

(Takubo et al. 2010). PIpC treated Hif-1$\alpha^{fl/fl}$ Mx1-Cre mice showed that the frequency of LSKs, HSCs, differentiation capacity and their short-term reconstitution potential were identical to the Controls. Primary recipients transplanted with Hif-1$\alpha^{fl/fl}$ Mx1-Cre displayed efficient haemopoietic reconstitution upon *Hif-1*$\alpha$ deletion. However, a decrease in number of LSK cells and a progressive loss of quiescent HSCs was observed in cells lacking *Hif-1*$\alpha$ (Takubo et al. 2010). *Hif-1*$\alpha$ deficiency ultimately resulted in the loss of long-term reconstitution capacity of HSCs in the PB and BM upon transplantation to secondary recipients (Takubo et al. 2010).

My data suggest that unlike *Hif-1*$\alpha$ (Takubo et al. 2010), *Hif-2*$\alpha$ is not essential for maintenance of self-renewal properties of HSCs. The results in this Chapter imply that regardless of the fact that HSC and progenitors inherently express high levels of *Hif-2*$\alpha$ (Takubo et al. 2010), HSCs in which *Hif-2*$\alpha$ was deleted were capable of regulating multilineage haemopoiesis, demonstrating conclusively that *Hif-2*$\alpha$ was dispensable for maintenance of HSC functions. Analysis of aged primary and secondary recipients with Hif-2$\alpha^{\Delta/\Delta}$ and Control HSCs showed equal reconstitution of HSCs, primitive progenitors and mature blood lineages. These observations are in agreement with results from Prof. Kranc's laboratory showing that conditional deletion of *Hif-2*$\alpha$ specifically in the haemopoietic system using Vav-iCre (efficiently deletes the floxed gene during early haemopoietic development in the embryos) did not perturb normal haemopoiesis. HSCs lacking *Hif-2*$\alpha$ were capable of normal multilineage haemopoiesis under steady state conditions and upon serial transplantation (Guitart, Subramani et al, Blood, 2013). In conclusion, my data taken together with other observations from our laboratory indicate that *Hif-2*$\alpha$ is dispensable for cell-autonomous HSC maintenance in stressful conditions of transplantation and aging.

### 3.4.2 Additional deletion of *Hif-1*$\alpha$ has no impact on self-renewal capacity of *Hif-2*$\alpha$-deficient HSCs

To examine the interplay between *Hif-1*$\alpha$ and *Hif-2*$\alpha$ and to address the hypothesis that additional deletion of *Hif-2*$\alpha$ exacerbates the phenotypes observed in *Hif-1*$\alpha$-deficient mice (Takubo et al. 2010), I conducted serial

transplantation assays using BM cells lacking *Hif-1α*/*Hif-2α* and Control BM cells. HSCs from DKO mice exhibited equal engraftment and repopulation potential as the Control HSCs. DKO HSCs sustained normal multilineage haemopoiesis in the primary recipient mice. Furthermore, surprisingly, HSCs lacking *Hif-1α* and *Hif-2α* sorted from primary recipients were capable of repopulating the secondary and tertiary recipients. While, according to the published data (Takubo et al. 2010), *Hif-1α*-deficient HSCs can repopulate primary, but not secondary recipients, HSCs lacking both *Hif-1α* and *Hif-2α* were able to repopulate primary and secondary recipients and sustained long-term multilineage haemopoiesis. These results are further supported by data from Prof. Kranc's laboratory that show primary recipients lacking *Hif-1α* and *Hif-2α* mediated by Vav-iCre had equal reconstitution in PB and BM with similar number of LSK and HSCs compared to Control (Unpublished data, Prof. Kranc lab). Mice lacking *Hif-1α* under stressful conditions of aging showed reduced numbers of HSCs and extra-medullary haemopoiesis in the spleen (Takubo et al. 2010). Data from the experiments in this thesis showed that aged primary and secondary recipients of recipients of DKO and Hif-2$\alpha^{\Delta/\Delta}$ HSCs had normal donor-derived multilineage haemopoiesis compared to respective Controls. Although, *Hif-1α* was shown to be critical for maintenance of HSC functions under various stress conditions, such as aging, 5-FU treatment and serial transplantation, *Hif-1α*-deficient HSCs were able to sustain steady state haemopoiesis (Takubo et al. 2010). Additionally, a recent publication reported a prominent increase in *Hif-2α* mRNA levels in *Hif-1α*-deficient HSCs (Kocabas et al. 2012). I have shown that HSCs lacking *Hif-1α*, with additional deletion of *Hif-2α*, sustained self-renewal properties and long-term reconstitution. Hence, it is possible that the HSC phenotype described in the *Hif-1α*-deficient mice (Takubo et al. 2010) was caused by overexpression of *Hif-2α* and that in our system co-deletion of *Hif-2α* rectified the defective HSC phenotype in a cell-autonomous manner. However, further studies need to be conducted to understand the underlying mechanism. Collectively, these results imply that *Hif-2α* is not required for cell-autonomous HSC maintenance and additional deletion of *Hif-2α* along with *Hif-1α* in HSCs does not enhance the phenotype reported in *Hif-1α*-deficient HSCs.

Previous studies have shown that hypoxia signalling pathways might play an important role in development and in the regulation of differentiated cells, including, functions of myeloid, B-cells and T-cells (Walmsley et al. 2005; Cramer et al. 2003; Kojima et al. 2002; Walmsley et al. 2005; Kojima et al. 2010). In this Chapter, analysis of primary recipients for mature blood lineages revealed a decrease in donor-derived myeloid cells in PB, BM and spleen of Hif-2$\alpha^{\Delta/\Delta}$ recipients and an increase in B-cells in the BM and spleen of Hif-2$\alpha^{\Delta/\Delta}$ recipients compared to Control. A previous publication showed that deletion of *Hif-1*$\alpha$ specifically in the myeloid lineages resulted in increased apoptosis and reduced neutrophils (Walmsley et al. 2005). Also another study illustrated that deletion of *Hif-1*$\alpha$ in myeloid cells led to a drastic reduction in cellular ATP pool, resulting in impaired survival and functional defects in myeloid cell infiltration, mobilisation and aggregation (Cramer et al. 2003). *Hif-1*$\alpha$–deficient mice exhibit impaired maturation of B-cells (Kojima et al. 2002; Walmsley et al. 2005; Kojima et al. 2010). Taken together these studies along with the results in this Chapter imply that *Hif-1*$\alpha$ and *Hif-2*$\alpha$ play a role in the maintenance of myeloid cell proliferation and functions. It might be possible that *Hif-1*$\alpha$ and *Hif-2*$\alpha$ play antagonistic roles in the maintenance of B-cells. However, more detailed study to understand the contributions of *Hif-1*$\alpha$ and *Hif-2*$\alpha$ in the development and maintenance of myeloid and lymphoid cell functions is necessary to explain the differences in myeloid lineages observed in this study.

Hypoxia has been shown to regulate angiogenesis and other organs apart from BM are hypoxic, including thymus, retina, liver and kidney (Moss 1968; Vanderkooi et al. 1991; Linsenmeier and Braun 1992; Hale et al. 2002; Biju et al. 2004). Thymocytes from mice showed decreased apoptosis during *in vitro* hypoxic culture (Hale et al. 2002). Analysis of thymi from the Hif-2$\alpha^{\Delta/\Delta}$ mice and DKO showed no difference in the number of $CD4^+$, $CD8^+$ or $CD4^+CD8^+$ cells compared to Controls. Contrastingly, studies of murine models with deletion of *pVhl* in the thymocytes resulting in enhanced stabilisation of the Hif-1$\alpha$ protein have shown reduced $CD4^+CD8^+$ thymocytes due to increased apoptosis (Biju et al. 2004). Further, inactivation of *Hif-1*$\alpha$ in *pVhl* deficient thymocytes restored their number and reduced apoptosis,

both *in vivo* and *in vitro* (Biju et al. 2004). Taking these results into account, though increased expression of *Hif-1α* via *pVhl* affected thymocyte development, deletion of *Hif-1α* and *Hif-2α* did not affect thymocyte numbers. Hence the defective phenotype displayed in thymus (Biju et al. 2004) might be due to toxic effects of Hif-1α accumulation. However, further studies focusing on the functions of thymi need to be determined to understand the variable phenotype in the thymus with defective *Hif-1α* and *Hif-2α* expression.

Several publications have demonstrated that Hif target genes, such as *Vegf, Foxo3a, Cited2* and *Cripto* were essential for cell-autonomous HSC maintenance (Chen et al. 2007; Miyamoto et al. 2007; Kranc et al. 2009; Miharada et al. 2011; Rehn et al. 2011). Deletion of *Cited2*, a downstream target of *Hif-1α* and *Hif-2α* (Schodel et al. 2011), led to loss of HSCs via apoptosis and resulted in multilineage BM failure (Chen et al. 2007; Kranc et al. 2009). *Foxo3a* gene transcription was active in hypoxic conditions and regulated the transcription of *Cited2* in both normoxia and hypoxia (Bakker et al. 2007; Miyamoto et al. 2007). In hypoxic conditions, *Foxo3a* knockdown studies in MEFs, NIH3T3 cells and breast cancer cells, resulted in decreased survival rates (Bakker et al. 2007). *Foxo3a* was shown to inhibit *Hif-1α*-induced apoptosis by inducing *Cited2* expression (Bakker et al. 2007). Stabilised Hif-1α is known to bind the HRE element in the Cripto promoter region (Miharada et al. 2011). Hif-$1\alpha^{fl/fl}$ Mx1-Cre mice displayed reduced number of HSCs and decreased expression of Cripto in the endosteal niche (Miharada et al. 2011). Hence, under hypoxia heat shock protein GRP78 and its ligand Cripto regulated *Hif-1α*-mediated HSC quiescence and maintenance in the BM niche (Miharada et al. 2011). *Vegfα* expression is regulated by Hif by binding to the HRE region under hypoxic conditions (Rehn et al. 2011). Deletion of the HRE element prevented Hif-1α binding and thereby prevented Vegfα activation (Rehn et al. 2011). This resulted in increased HSC number with impaired reconstitution ability and defective differentiation potential (Rehn et al. 2011). Although Hif target genes were shown to be important in regulating HSC functions, it is possible that other Hif-independent pathways might be additionally regulating the Hif target genes to Control HSC functions (Figure 3.23).

*Meis1* is a member of homeobox genes that was shown to be highly expressed in primitive HSCs (Krumlauf 1994; Wong et al. 2007). *Meis1* regulates transcription of *Hif-1α* and *Hif-2α in vitro* (Simsek et al. 2010). A recent publication reports that loss of *Meis1* in the HSCs led to down-regulation of *Hif-1α* and *Hif-2α* (Simsek et al. 2010). The authors suggested that this decreased expression of *Hifs* resulted in defective HSC metabolism, loss of HSCs via apoptosis and impaired HSC long-term reconstitution (Kocabas et al. 2012). Considering the evidence from my experiments indicating that deletion of *Hif-2α* or both *Hif-1α* and *Hif-2α* within the HSCs did not perturb HSC functions, the phenotype observed in *Meis1* deficient HSCs might be due to the influence of other HSC regulators (e.g. ROS modulators) controlled by *Meis1*. In conclusion, down-regulation of *Hif-1α* and *Hif-2α* alone are unlikely to explain the phenotype observed in *Meis1*-deficient HSCs.

While several studies have suggested that HSCs are localised in special hypoxic niches and contribute to the maintenance of HSC functions (Cipolleschi et al. 1993; Scadden 2006; Levesque et al. 2007; Parmar et al. 2007; Suda et al. 2011), a recent publication by Nombela-Arrieta suggested that the hypoxic profile of HSCs was independent of the hypoxic state of the microenvironment (Nombela-Arrieta et al. 2013). Studies in mice using imaging techniques showed that HSPCs that retained the hypoxia marker, Pimo, at high levels and expressed *Hif-1α* were distributed throughout the BM which argued that localisation of HSCs was not restricted to the hypoxic niche (Nombela-Arrieta et al. 2013). HSPCs in Go and G1 of cell cycle phase retained similar levels of pimo and showed no change in the *Hif-1α* expression (Nombela-Arrieta et al. 2013). Opposed to what is published in the literature, Nombela-Arrieta et al. showed that post-treatment with the cytotoxic agent 5-FU, highly proliferating HSPCs displayed an increase in *Hif-1α* expression and high levels of Pimo (Nombela-Arrieta et al. 2013). This study reported that stabilisation *Hif-1α* in HSPCs was a cell-intrinsic phenomenon and not influenced by the possible different oxygen tension within the BM (Nombela-Arrieta et al. 2013). These results suggested that cell-intrinsic expression of *Hif-1α* with in the HSCs governs the glycolytic metabolism (Simsek et al. 2010) thereby regulating HSC functions and

revealed a surprising discovery that HSCs do not require expression of Hifs in the BM microenvironment for their normal functioning (Nombela-Arrieta et al. 2013). They also showed that the hypoxic profile of quiescent HSCs with high expression of *Hif-1α* and high levels of pimo incorporation were retained by cycling and proliferating HSCs (Nombela-Arrieta et al. 2013). These findings provide novel insights into the molecular and cellular mechanisms governing HSC functions and their fate choices. This study provides an interesting approach to further dissect the complex Hif-regulated molecular pathways in HSCs.

## 3.5 Conclusion

The results presented in this Chapter indicate that *Hif-2α* is not essential for cell-autonomous maintenance of HSC functions under steady state and stressful conditions of serial transplantation and aging. HSCs lacking both *Hif-1α* and *Hif-2α* successfully engraft and sustain long-term serial transplantation. Additional deletion of *Hif-2α* does not exacerbate the phenotype published in mice lacking expression of *Hif-1α*. In conclusion, my data reveal unexpected findings indicating that regardless of the oxygen tension in anatomically different BM niches, HSCs functions, including self-renewal, long-term multilineage haemopoiesis and survival, do not critically require intrinsic *Hif-1α*- and *Hif-2α*-dependent signalling.

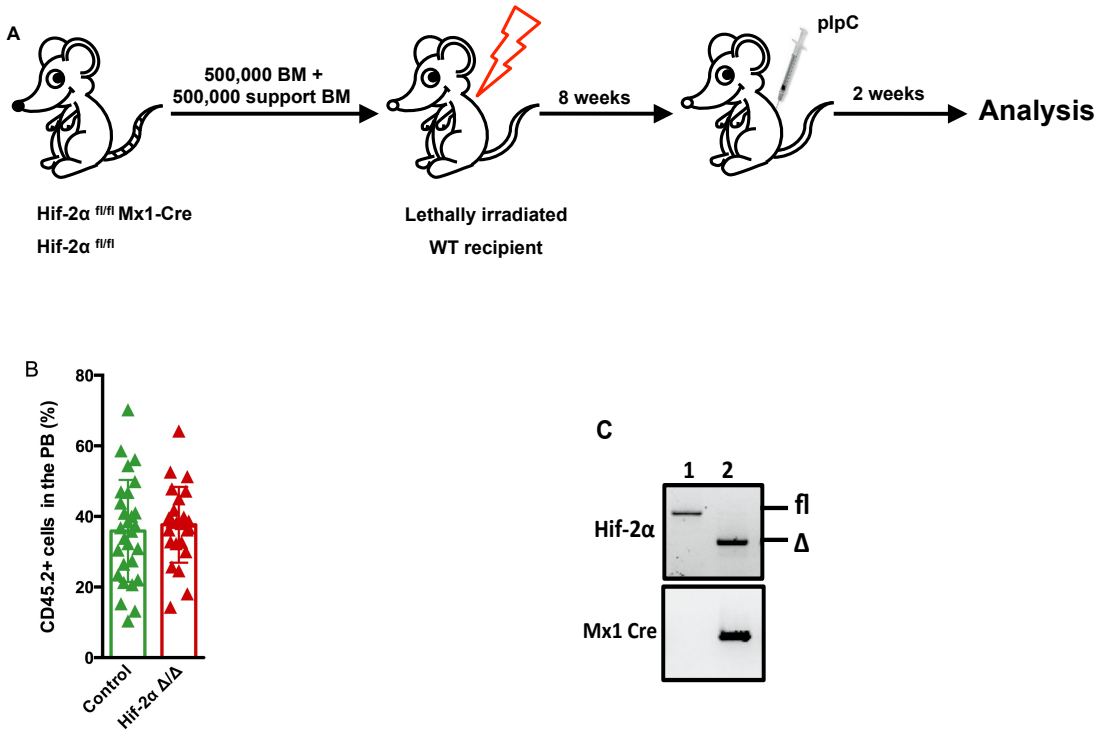



**Figure 3. 1: Primary transplantation of Hif-2α$^{fl/fl}$ and Control BM cells**

(A) Experimental design - 500,000 CD45.2$^{+}$ donor-derived BM cells from untreated Hif-2α$^{fl/fl}$ Mx1-Cre mice and Hif-2α$^{fl/fl}$ Control mice along with 500,000 CD45.1$^{+}$ support BM cells were injected into lethally irradiated B6.SJL (CD45.1) recipients, (B) Percentage of CD45.2$^{+}$ donor-derived cells at 8 weeks post-transplantation from PB of primary recipients transplanted with BM cells from untreated Hif-2α$^{fl/fl}$ Mx1-Cre mice (red, n=28) and Hif-2α$^{fl/fl}$ Control mice (green, n=25) showing successful engraftment of donor derived cells, (C) Gene deletion confirmed by genomic PCR: Deletion of *Hif-2α* in donor derived CD45.2$^{+}$ cells from the PB at 2 weeks after last pIpC injection. 1: Control cells, 2: Hif-2α$^{Δ/Δ}$ cells, PB- Peripheral blood.

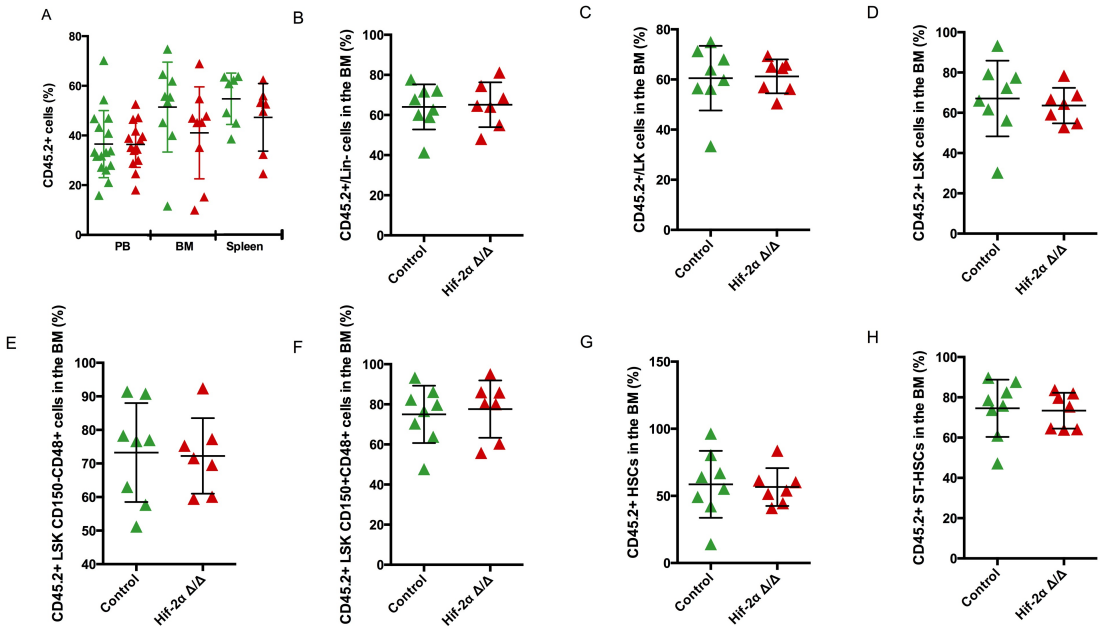



**Figure 3. 2: Analysis of chimerism in primary recipients transplanted with Hif-2α$^{fl/fl}$ Mx1-Cre and Control BM, 2 weeks after last pIpC injection**

(A) Percentage of CD45.2$^{+}$ donor-derived cells in PB, BM and spleen of primary recipients. Percentage of CD45.2$^{+}$ (B) Lin-, (C) LK, (D) LSK, (E) and (F) LMPPs, (G) HSCs and (H) ST-HSCs in the BM of primary recipients from Hif-2α$^{\Delta/\Delta}$ (red, n=7) and Control (green, n=8) cells. Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance. The data are obtained from two independent experiments.

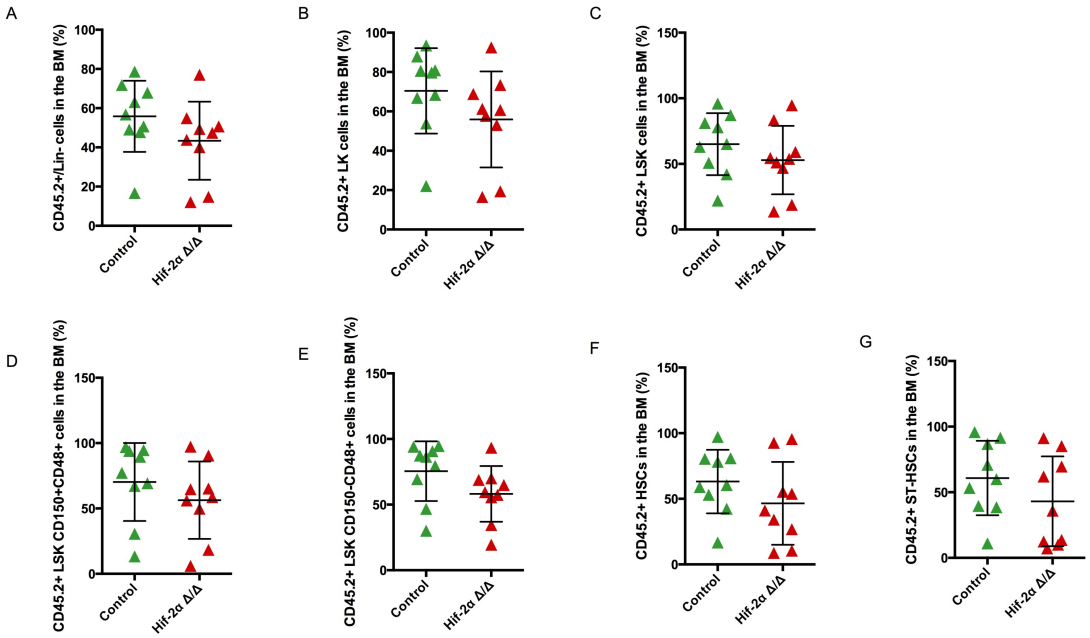



**Figure 3. 3: Analysis of primary recipients transplanted with Hif-2α$^{\Delta/\Delta}$ and Control BM 4 weeks after last plpC injection.**

Percentage of CD45.2$^{+}$ donor-derived cells (A) Lin-, (B) LK, (C) LSK, (D) and (E) LMPPs, (F) HSCs and (G) ST-HSCs in the BM of primary recipients from Hif-2α$^{\Delta/\Delta}$ (n=9) and Control (n=9) cells. Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

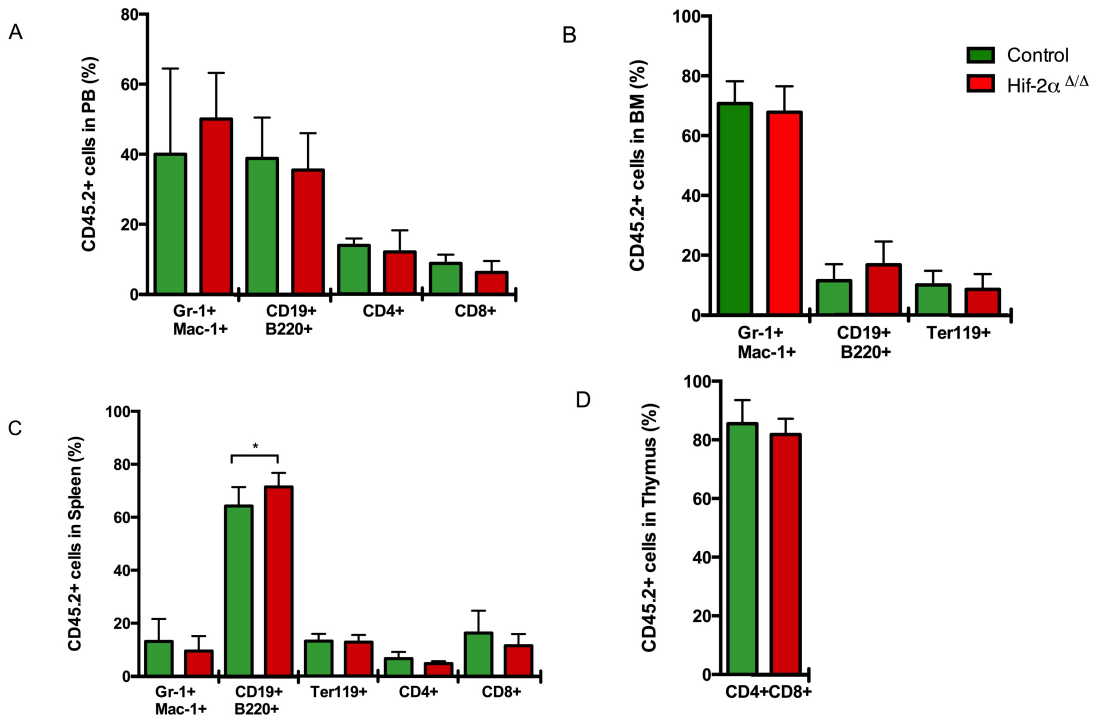



**Figure 3. 4: Percentage of donor– derived CD45.2$^{+}$ myeloid (Gr-1$^{+}$ Mac-1$^{+}$), B-cells (CD19$^{+}$ B220$^{+}$), erythroid (Ter119$^{+}$) and T-cells (CD4$^{+}$ CD8$^{+}$)**

(A) PB, (B) BM, (C) Spleen and (D) Thymus analysed 2 weeks after last pIpC injection. Recipients transplanted with Control cells (green, n=8) and Hif-2α$^{Δ/Δ}$ cells (Red, n=7). Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance, p value = ≤ 0.05 (*).

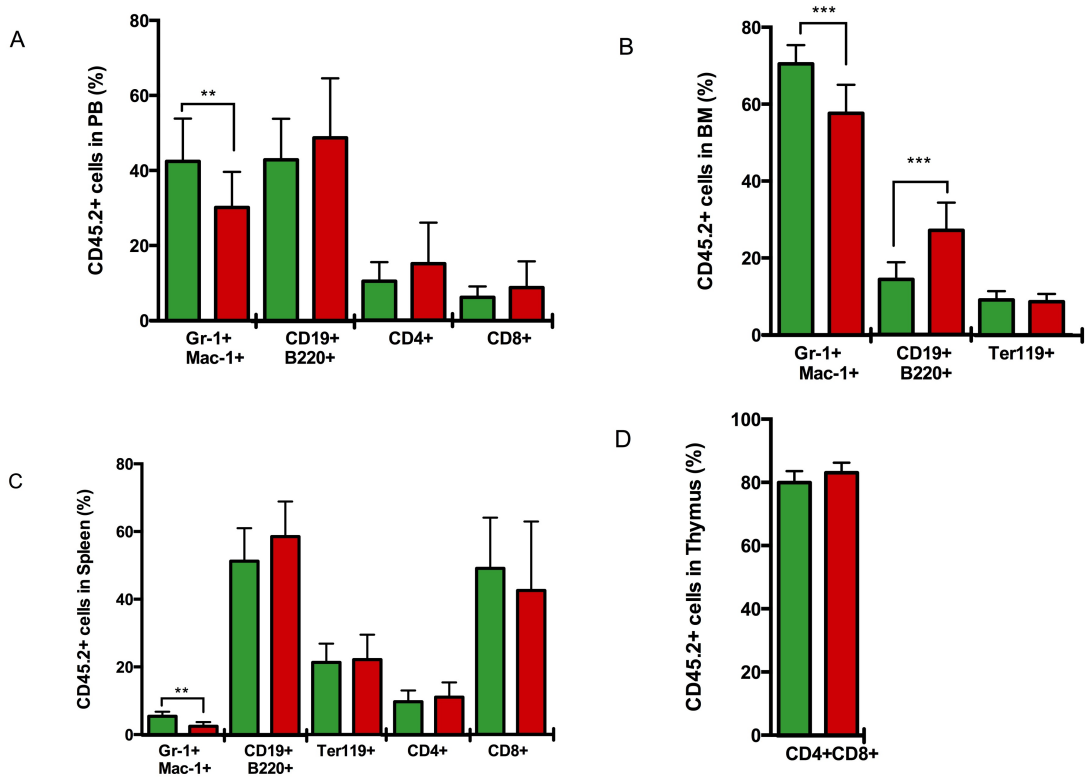



**Figure 3. 5: Percentage of donor –derived CD45.2$^+$ myeloid (Gr-1$^+$ Mac-1$^+$), B-cells (CD19$^+$ B220$^+$), erythroid (Ter119$^+$) and T-cells (CD4$^+$ CD8$^+$)**

(A) PB, (B) BM, (C) Spleen and (D) Thymus analysed 4 weeks after last pIpC injection. Recipients transplanted with Control cells (green, n=7) and Hif-2α$^{\Delta/\Delta}$ cells (red, n=7). Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance, p value ≤ 0.05 (*), p ≤ 0.005 (**) and p ≤ 0.0005 (***).

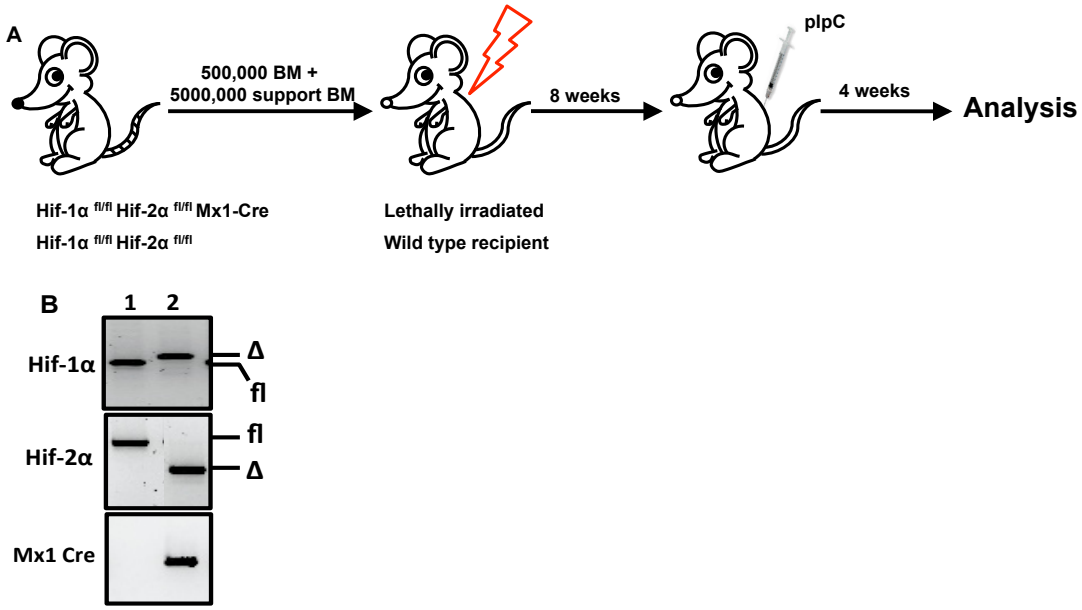



**Figure 3. 6: Primary transplantation of Hif-1α$^{fl/fl}$ Hif-2α$^{fl/fl}$ Mx1-Cre and Control BM cells**

(A) Experimental design - 500,000 donor-derived CD45.2$^{+}$ BM cells from untreated Hif-1α$^{fl/fl}$ Hif-2α$^{fl/fl}$ Mx1-Cre mice and Control mice together with 500,000 CD45.1$^{+}$ support BM were injected into lethally irradiated B6.SJL CD45.1 recipients. 8 weeks post transplantation, mice were injected with 5 doses of pIpC to induce gene deletion, (B) Gene deletion shown by genomic PCR: Deletion of *Hif-1α* and *Hif-2α* in donor derived CD45.2$^{+}$ cells from the PB at 2 weeks after last pIpC injection. 1: Control cells and 2: DKO cells.

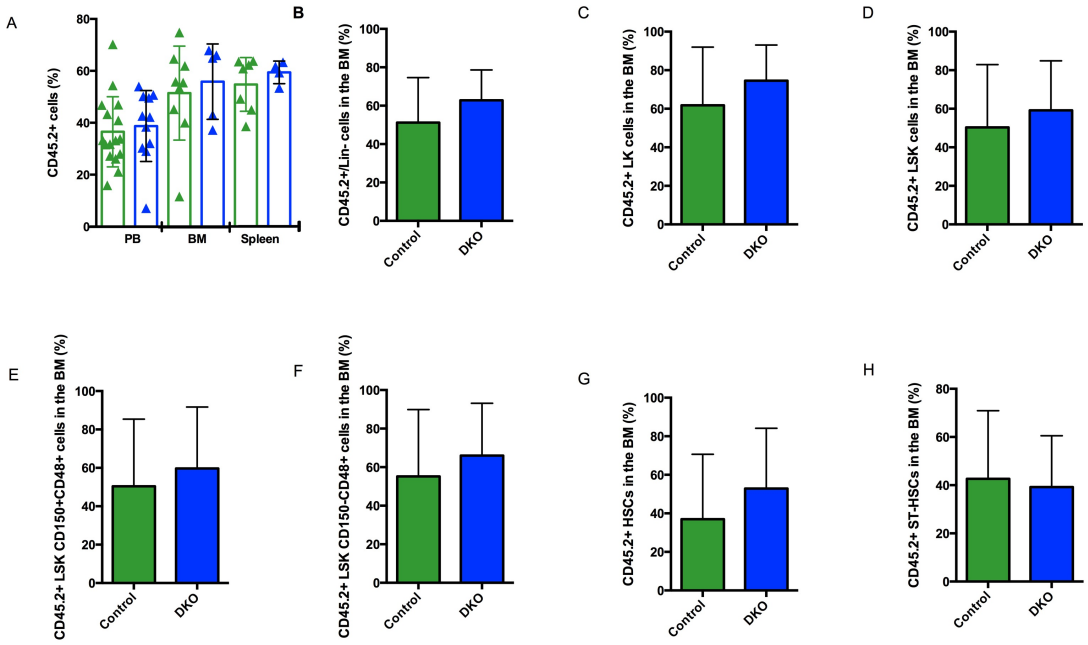



**Figure 3. 7: Analysis of primary recipients transplanted with Hif-1α$^{fl/fl}$ Hif-2α$^{fl/fl}$ Mx1-Cre and Control BM 2 weeks after last pIpC injection**

(A) Percentage of CD45.2$^{+}$ donor-derived cells in PB, BM and spleen of primary recipients. Percentage of CD45.2$^{+}$ in (B) Lin-, (C) LK, (D) LSK, (E) and (F) LMPPs, (G) HSCs and (H) ST-HSCs in the BM of primary recipients transplanted with Control cells (green, n=5) and DKO cells (blue, n=7). Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

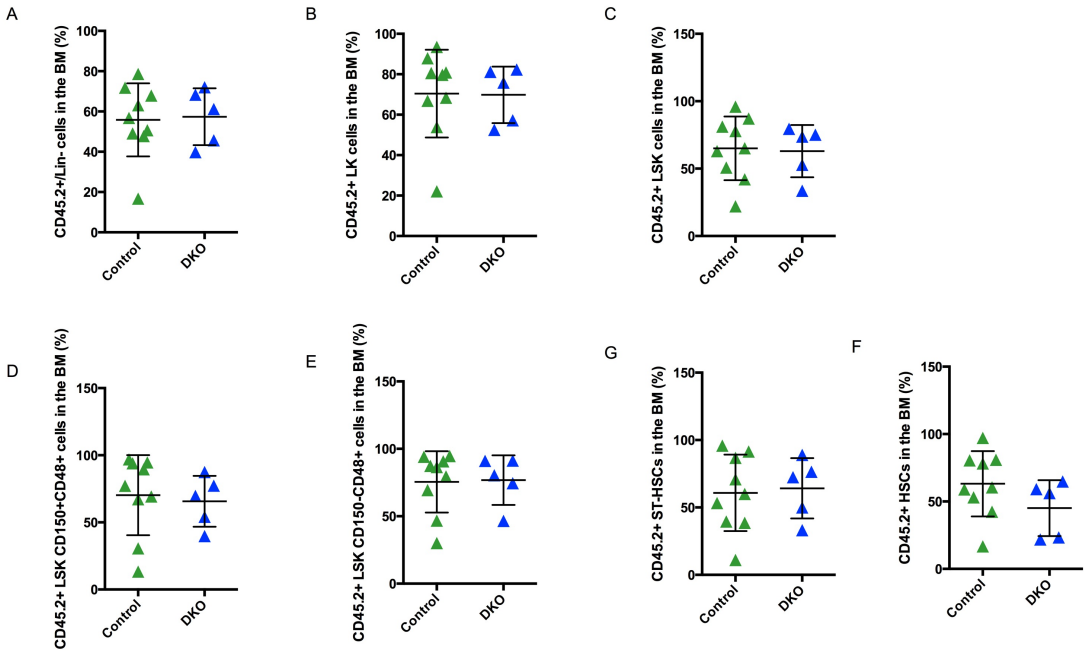



**Figure 3. 8: Analysis of primary recipients transplanted with Hif-1α$^{fl/fl}$ Hif-2α$^{fl/fl}$ Mx1-Cre and Control BM 4 weeks after last pIpC injection.**

Percentage of CD45.2$^{+}$ donor-derived CD45.2$^{+}$ (A) Lin-, (B) LK, (C) LSK, (D) and (E) LMPPs, (F) HSCs and (G) ST-HSCs in the BM of primary recipients transplanted with Control cells (green, n=9) and DKO cells (Blue, n=5). Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

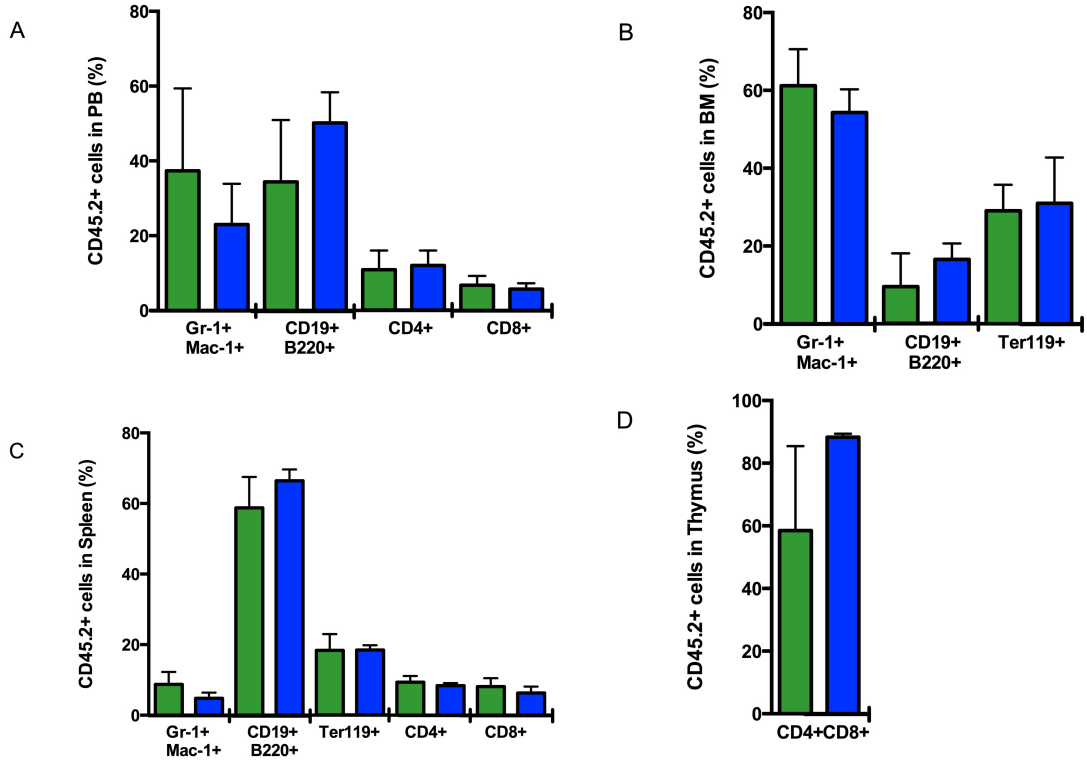



**Figure 3. 9: Percentage of donor –derived CD45.2$^+$ myeloid, B-cells, erythroid and T-cells**

(A) PB, (B) BM, (C) Spleen and (D) Thymus, analysed 2 weeks after last pIpC injection. Recipients transplanted with Control cells (green, n=5) and DKO cells (blue, n=8). Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

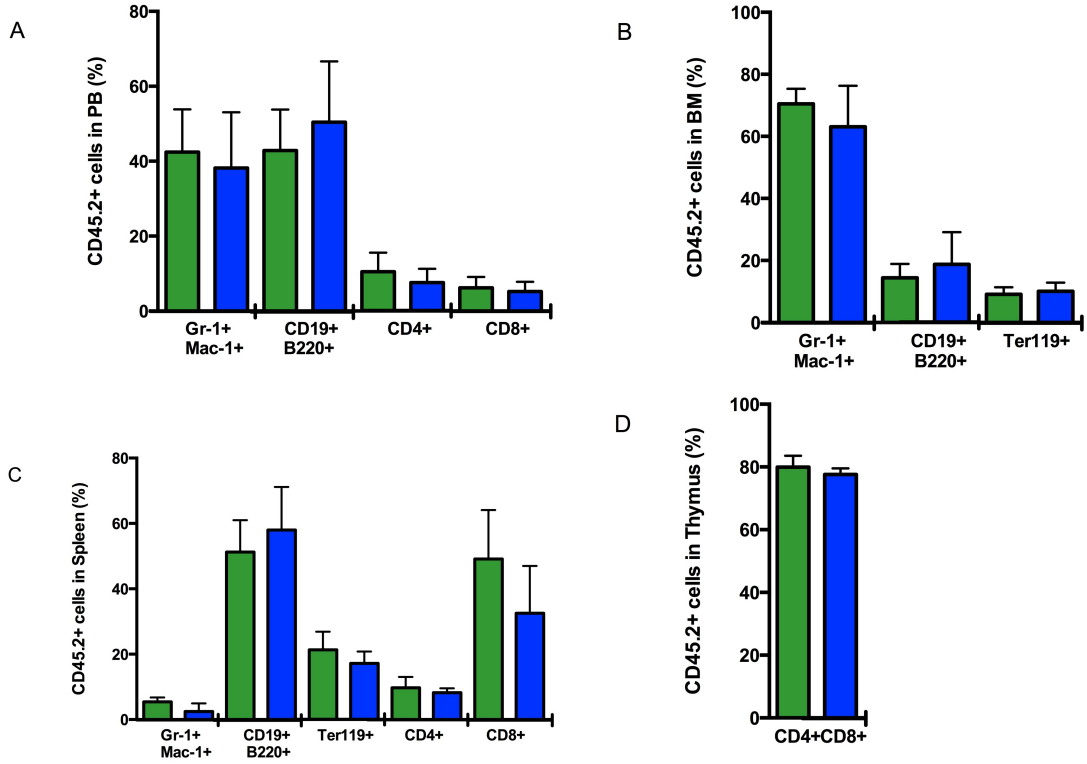



**Figure 3. 10: Percentage of donor –derived CD45.2+ myeloid, B-cells and T-cells** in (A) PB, together with erythroid cells in (B) BM, (C) Spleen and T-cells in (D) Thymus, in recipients transplanted with Control cells (green, n=9) and DKO cells (blue, n=5) analysed 4 weeks after last pIpC injection. Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

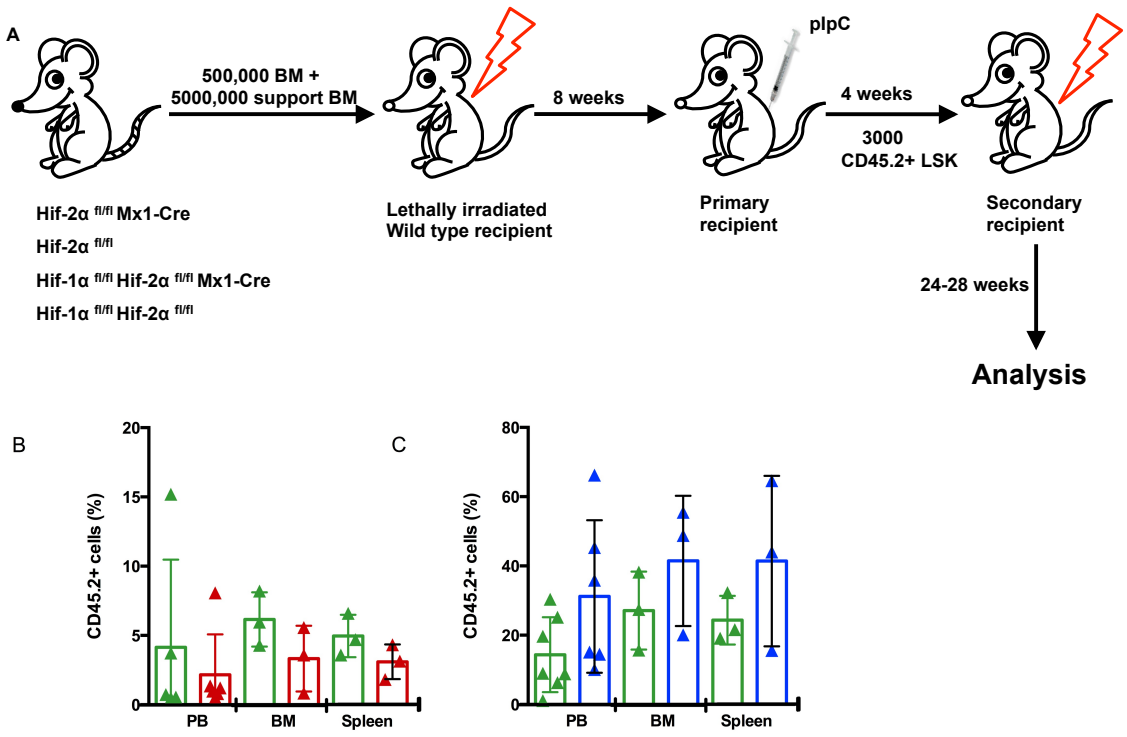



**Figure 3. 11: Secondary transplantation**

(A) Experimental design – 14 weeks post transplantation (4 weeks after last pIpC injection), 3000 donor-derived LSKs were sorted from primary recipients transplanted with Hif-2α$^{\Delta/\Delta}$, DKO and Control cells, Analysis of engraftment of donor-derived LSKs in the PB, BM and spleen of secondary recipients transplanted with (B) Hif-2α$^{\Delta/\Delta}$ (red) and (C) DKO (blue) with Control cells transplanted after 24 weeks and 28 weeks of transplant respectively. Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance (n=3).

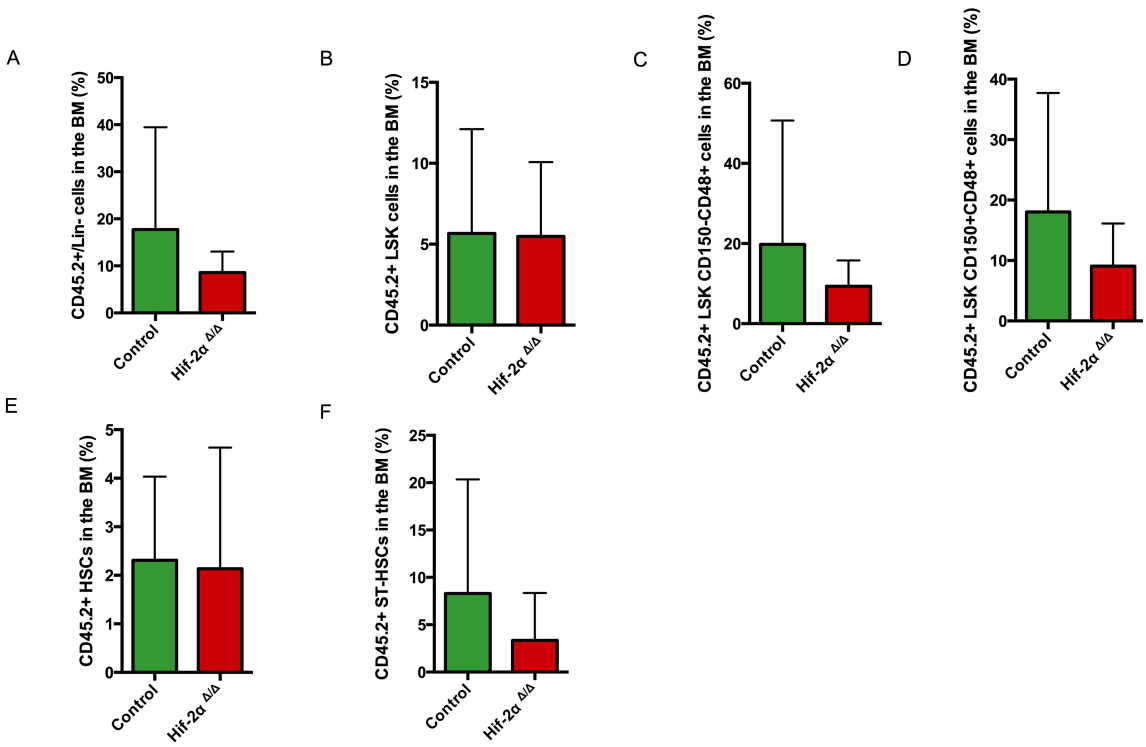



**Figure 3. 12: Analysis of BM from secondary recipients transplanted with Hif-2α $^{\Delta/\Delta}$ and Control LSKs at 24 weeks post transplantation.**

Percentage of donor-derived CD45.2$^{+}$ (A) Lin-, (B) LSK, (C) and (D) LMPPs, (E) HSCs and (F) ST-HSCs in secondary recipients transplanted with Hif-2α$^{\Delta/\Delta}$ (n=3) and Control cells (n=3). Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

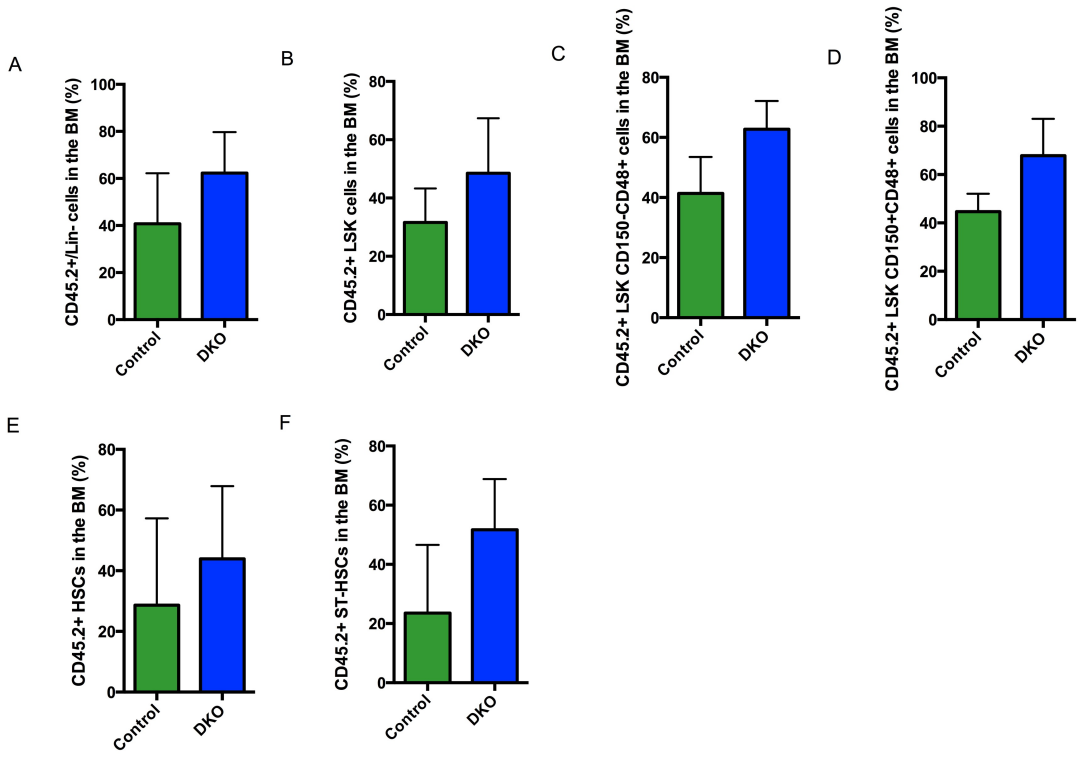



**Figure 3. 13: Analysis of BM from secondary recipients transplanted with DKO and Control LSKs at 28 weeks post transplantation.**

Percentage of donor-derived CD45.2$^{+}$ (A) Lin-, (B) LSK, (C-D) LMPPs, (E) HSCs and (F) ST-HSCs in secondary recipients transplanted with DKO (n=3) and Control cells (n=3). Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

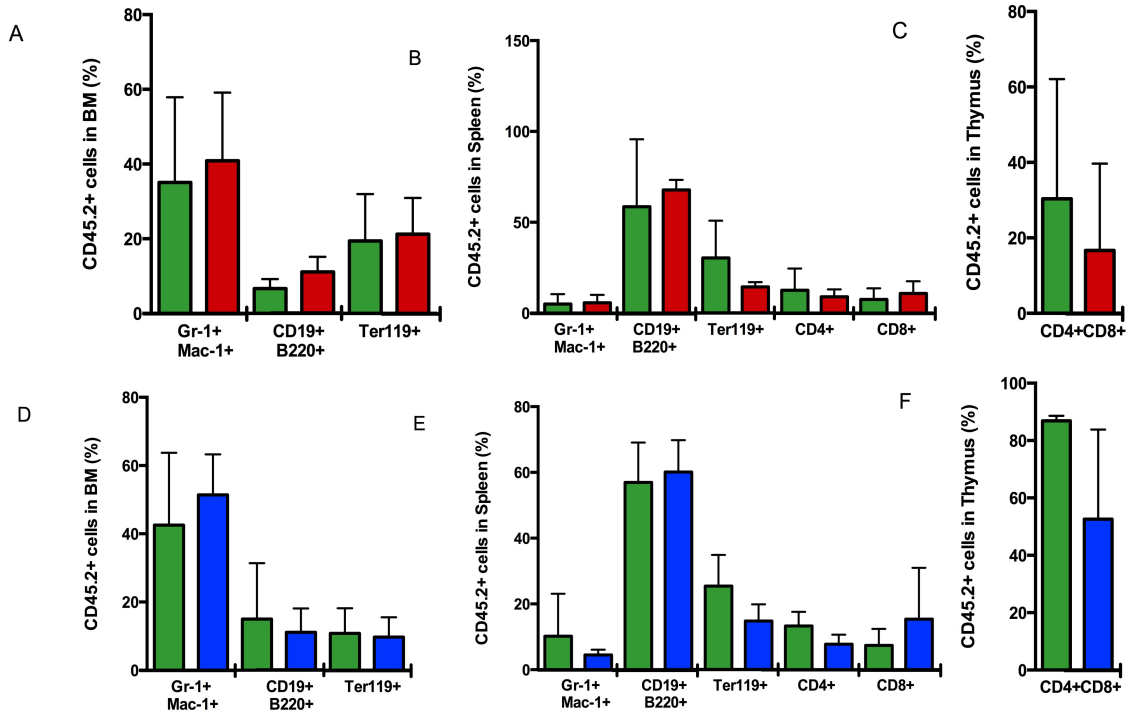



**Figure 3. 14: Analysis of contribution of donor-derived CD45.2$^{+}$ LSKs from primary recipients towards mature blood lineages in secondary recipients.**

Percentage of CD45.2$^{+}$ myeloid (Gr-1, Mac-1), B-cells (CD19, B220), erythroid (Ter119) in (A) BM along with T-cells (CD4 and CD8) in the (B) Spleen and (C) Thymus from Hif-2$\alpha^{\Delta/\Delta}$ (red) and (D) BM, (E) spleen and (F) thymus from DKO-derived (blue) cells with respective Controls (green), n=3. Results are represented as mean ± SD.

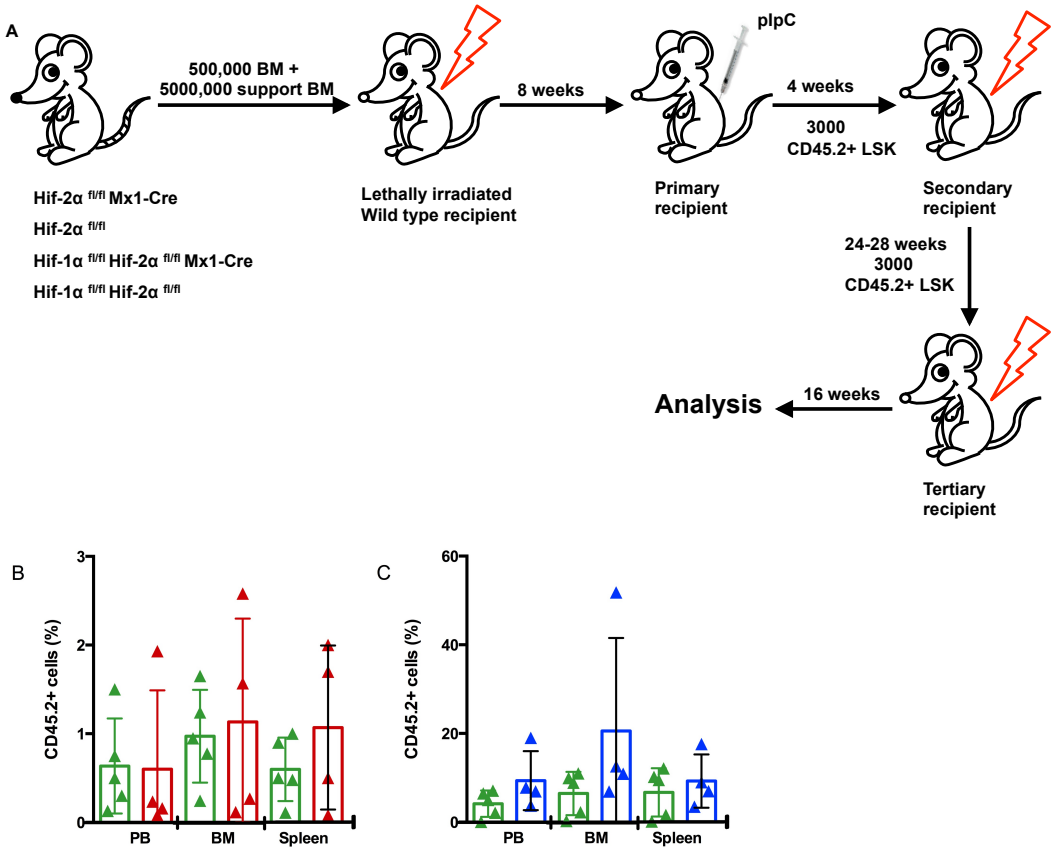



**Figure 3. 15: Tertiary transplantation**

(A) Experimental design, Engraftment of donor-derived LSKs in PB, BM and spleen of tertiary recipients transplanted with (B) Hif-2α$^{\Delta/\Delta}$ (red) (C) DKO (blue) LSKs from secondary recipients with respective Controls (green), n=3.

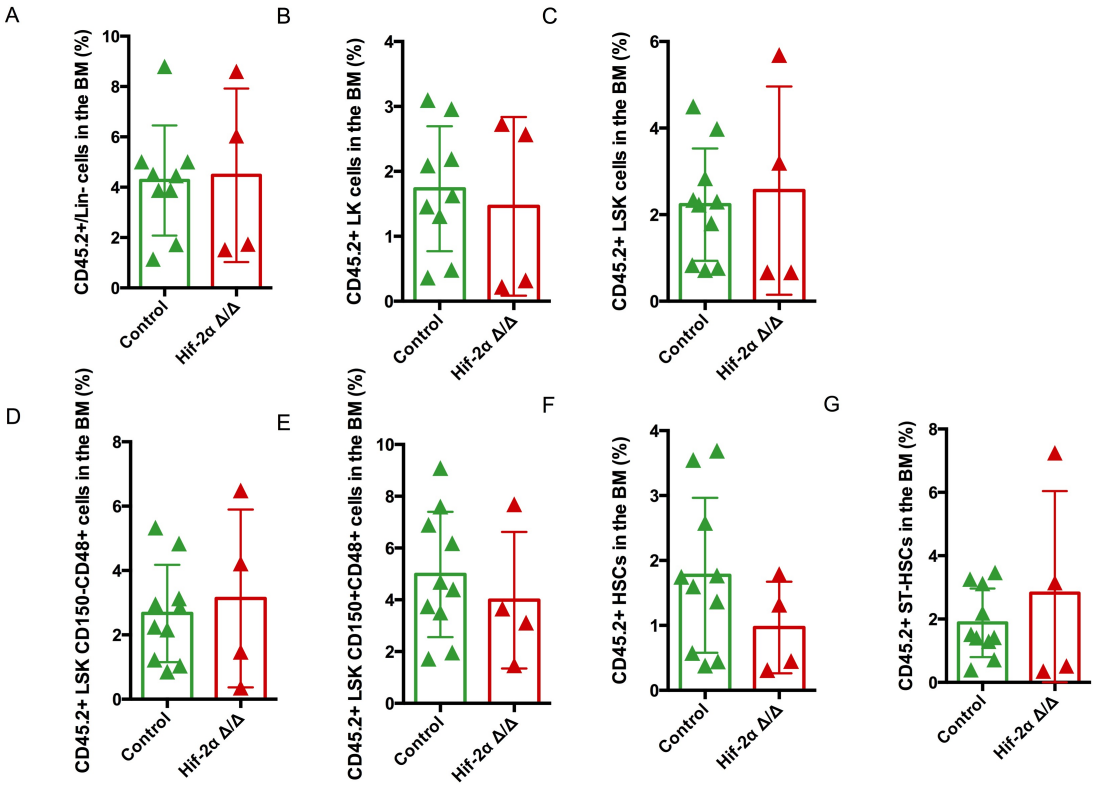



**Figure 3. 16: Analysis of BM from tertiary recipients for reconstitution potential of Hif-2α $^{\Delta/\Delta}$ LSKs with respective Control at 16 weeks post transplant.**

Percentage of CD45.2$^{+}$-derived (A) Lin-, (B) LK, (C) LSK, (D) and (E) LMPPs, (F) HSCs and (G) ST-HSCs in tertiary recipients transplanted with Hif-2α$^{\Delta/\Delta}$ (n=4) and Control cells (n=10). Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

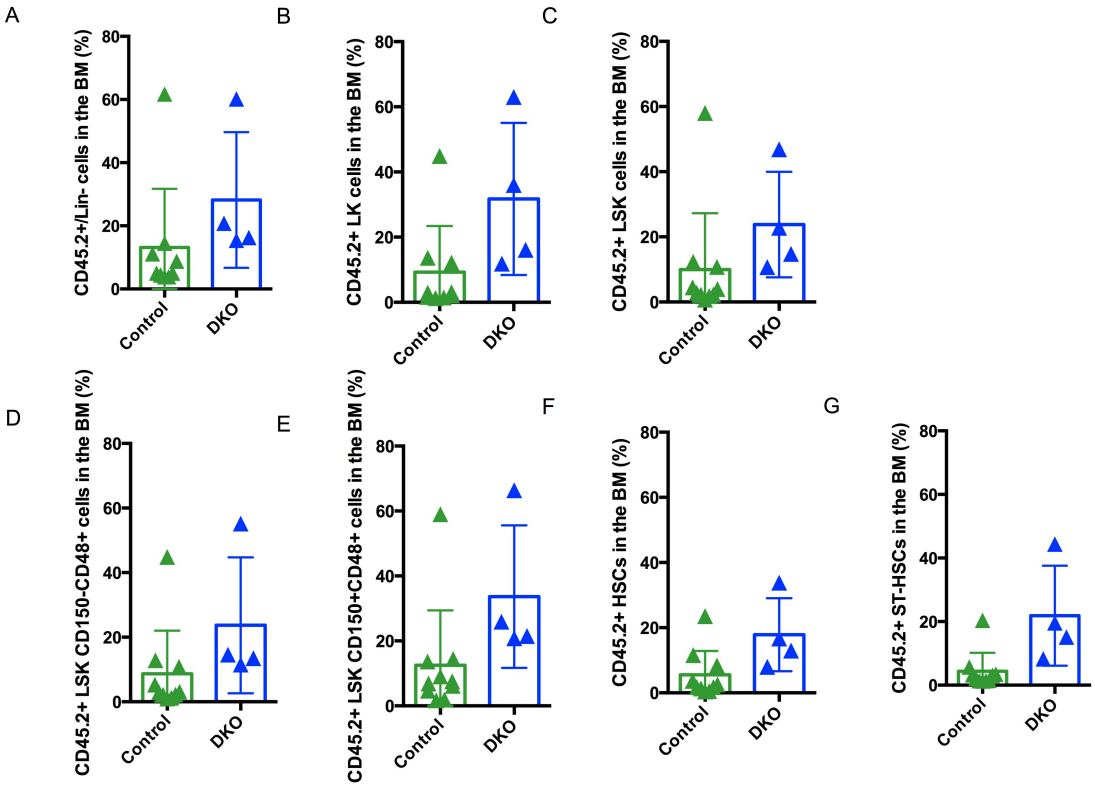



**Figure 3. 17: Analysis of BM from tertiary recipients for reconstitution potential of DKO LSKs with respective Control at 16 weeks post transplant.**

Percentage of CD45.2$^{+}$-derived (A) Lin-, (B) LK, (C) LSK, (D) and (E) LMPPs, (F) HSCs and (G) ST-HSCs in tertiary recipients transplanted with DKO (n=4) and Control cells (n=10). Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

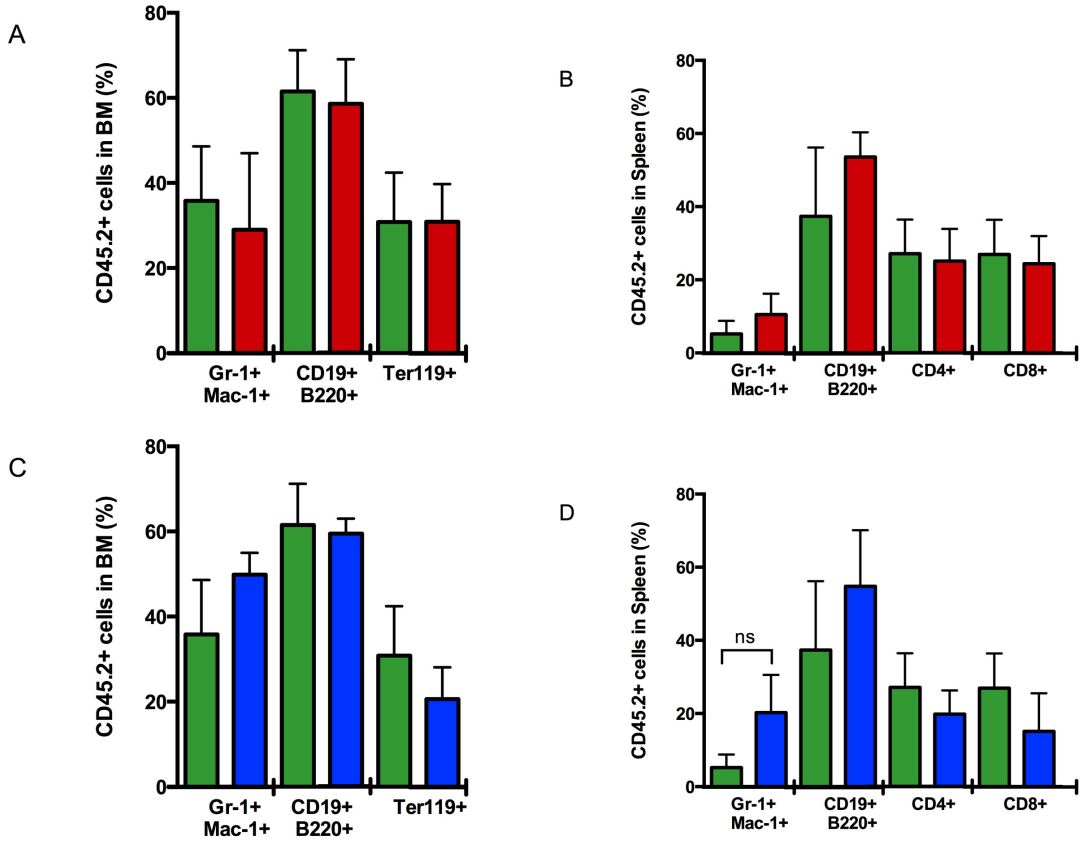



**Figure 3. 18: Analysis of PB, BM, spleen and thymus from tertiary recipients for Hif-2α $^{\Delta/\Delta}$ and DKO LSK-derived mature blood lineages at 16 weeks post transplant.**

Percentage of CD45.2$^{+}$-derived myeloid, B-cell, erythroid and T-cells in (A) BM and (B) spleen from Hif-2α $^{\Delta/\Delta}$ (red, n=4) and (C) BM, (D) spleen from DKO derived LSKs (blue, n=4) with Controls (green, n= 10). Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance, ns - not significant ($p \geq 0.05$).

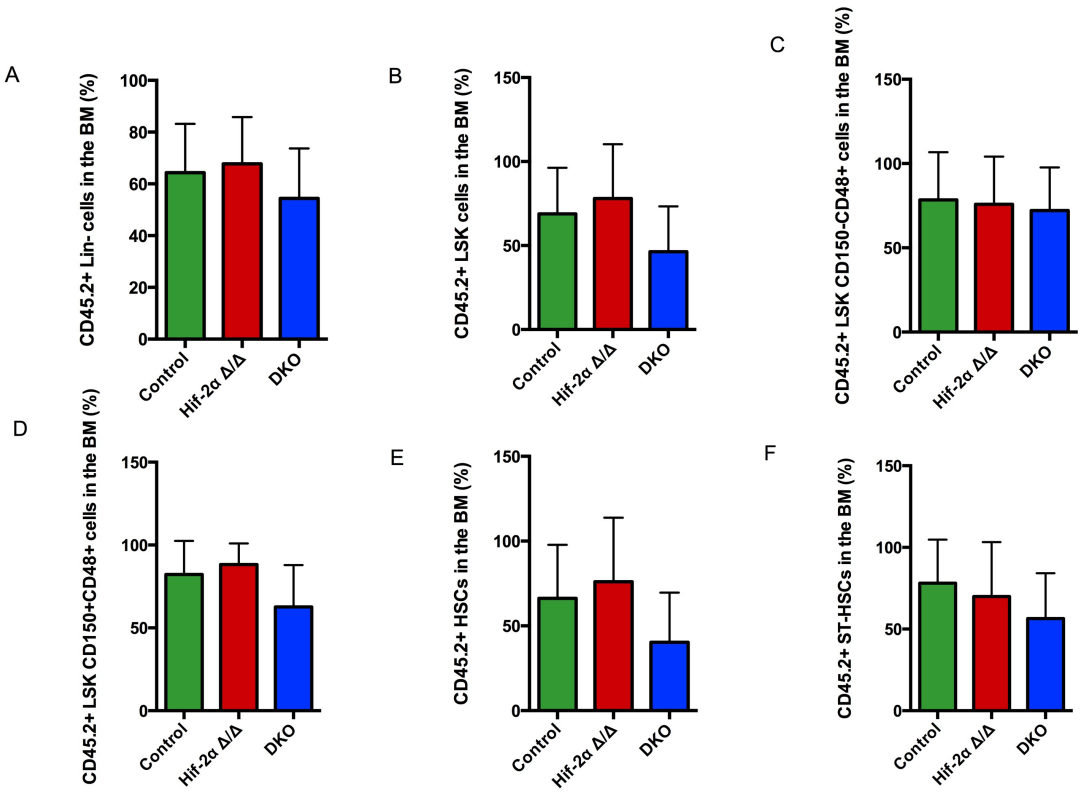



**Figure 3. 19: Analysis of aged primary recipients.**

Percentage of CD45.2-derived (A) Lin-, (B) LSKs, (C) and (D) LMPPs, (E) HSCs and (F) ST-HSCs in the BM of primary recipients transplanted with Hif-2$\alpha^{\Delta/\Delta}$ (red, n=5), DKO (blue, n=4) and Control (green, n=8) cells, analysed at 28 weeks after last pIpC injection. Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

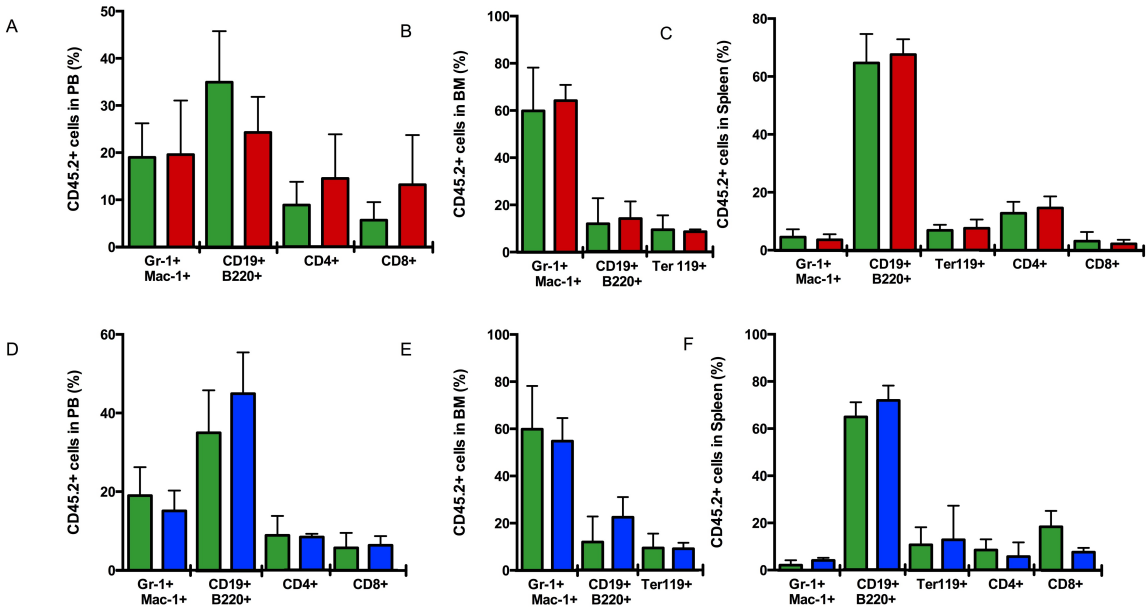



**Figure 3. 20: Analysis of mature blood lineages in aged primary recipients.**

Percentage of CD45.2-derived myeloid, B-cells, erythroid and T-cells in (A) PB, (B) BM, (C) spleen in primary recipients transplanted with Hif-2α$^{\Delta/\Delta}$ (red, n=5), (E) PB, (F) BM and (G) spleen in DKO (blue, n=4) recipients, analysed at 28 weeks after last pIpC injection. Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

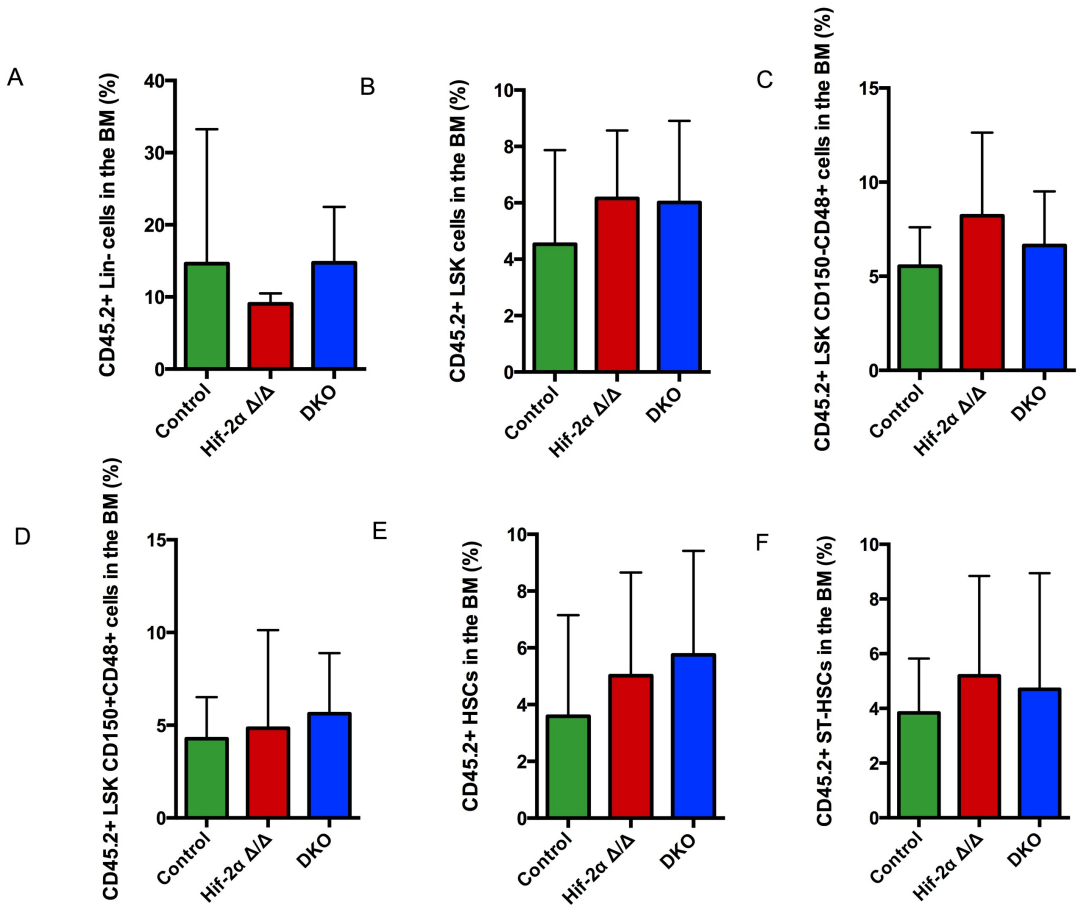



**Figure 3. 21: Analysis of aged secondary recipients.**

Percentage of CD45.2-derived (A) Lin-, (B) LSKs, (C) and (D) LMPPs, (E) HSCs and (F) ST-HSCs in the BM of secondary recipients transplanted with Hif-2α$^{\Delta/\Delta}$ (red, n=3), DKO (blue, n=3) and Control (green, n=5) cells, analysed at 24-27 weeks after transplantation. Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

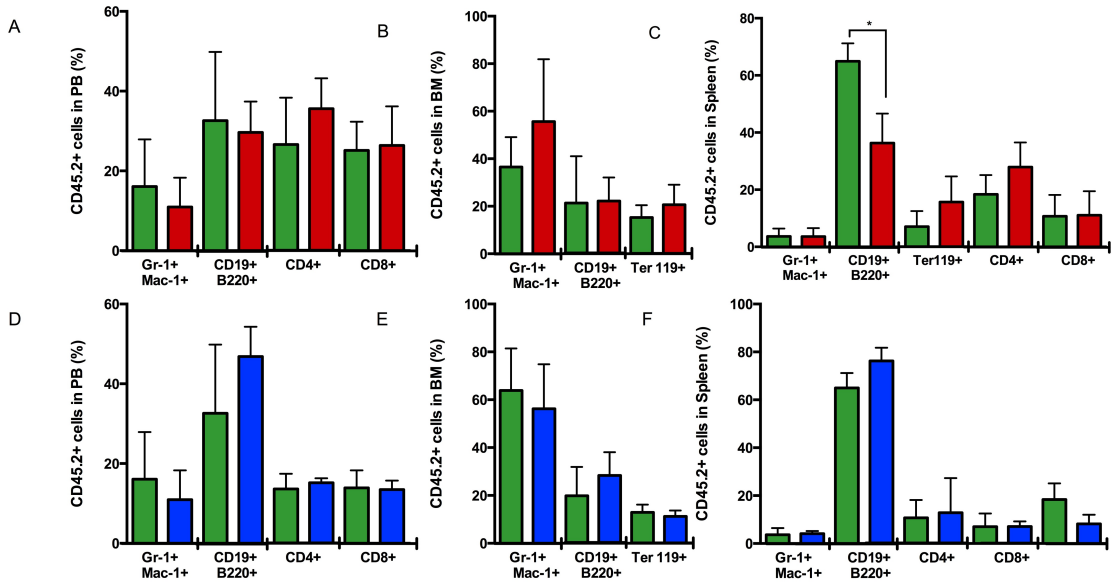



**Figure 3. 22: Analysis of mature blood lineages in aged secondary recipients.**

Percentage of CD45.2-derived myeloid, B-cells, erythroid and T-cells in (A) PB, (B) BM, (C) spleen in secondary recipients transplanted with Hif-2α$^{\Delta/\Delta}$ (red, n=3), (E) PB, (F) BM and (G) spleen in DKO (blue, n=3) recipients, analysed at 24-27 weeks after transplantation. Results are represented as mean ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

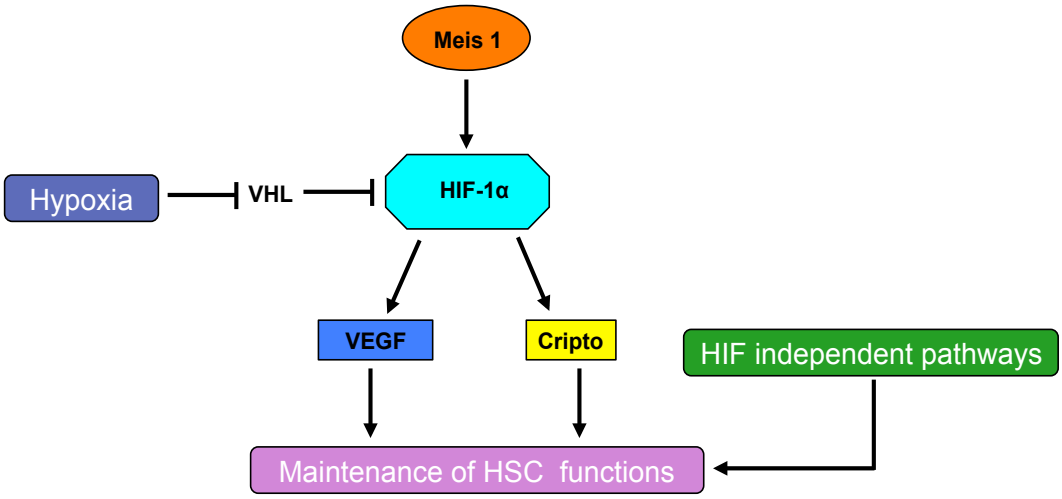



**Figure 3.23: Schematic representation of Hif-dependent and independent regulatory pathway for maintenance of HSC functions.**

# Chapter 4

# The non-cell-autonomous roles for *Hif-1α* and *Hif-2α* in HSC maintenance

# 4.1 Introduction

Adult haemopoiesis involves interaction between multipotent HSCs and their microenvironment (Schofield 1978; Scadden 2006; Jones and Wagers 2008; Suda et al. 2011). Various studies suggested that HSCs reside in the specialised BM microenvironment defined as the "HSC niche" that plays crucial roles in the maintenance of HSC properties and functions (Kiel et al. 2005; Sugiyama et al. 2006; Parmar et al. 2007; Sacchetti et al. 2007; Simsek et al. 2010; Takubo et al. 2010). Also, studies showed that HSCs are located in the endosteal zone of the BM characterised by a low partial pressure of oxygen (Draenert and Draenert 1980; Calvi et al. 2003; Zhang et al. 2003; Arai et al. 2004). Parmer et al, using Ho DNA dye demonstrated that HSCs were enriched in the region of BM, which exhibits very low perfusion of the dye (Parmar et al. 2007). Further studies showed that HSCs were positive for Pimo and displayed low metabolic activity (Parmar et al. 2007; Simsek et al. 2010; Takubo et al. 2010). These findings implied that the hypoxic profile of HSCs was determined by their position in the BM niche. In contrast, a recent study using quantitative imaging of femoral BM revealed a widespread distribution of HSCs in the BM, with no special preference for localisation (Nombela-Arrieta et al. 2013). The results also showed that hypoxic profile of HSC, with high expression of *Hif-1*$\alpha$ was dependent on intercellular glycolytic metabolism and not on the oxygen gradient or proximity to vasculature in the BM microenvironment (Nombela-Arrieta et al. 2013). This result was supported by *in vitro* BM assay in which hypoxic culture of BM cells enhanced colony formation and transplantation capacity (Cipolleschi et al., 1993; Goodell et al 1993; Danet et al., 2003; Ivanovic et al., 2004). In human $CD34^+$ cells, hypoxia was shown to induce quiescence under culture conditions (Hermitte et al. 2006; Shima et al. 2010). Also, studies of human HSCs reported stabilisation of Hif-1$\alpha$ in the circulating HSCs (Piccoli et al. 2007). Taking into account the discrepancies in previous studies describing HSC localisation in the BM niche and the role of hypoxia in regulating HSC fate, more studies dissecting the complex interplay between HSC, hypoxia and the BM niche and their influences in regulating HSC functions are essential.

Various studies have shown that the niche contributes to the maintenance of various HSC functions, including self-renewal, quiescence and differentiation (Blanpain et al. 2004; Parmar et al. 2007; Kubota et al. 2008). The cellular components of the HSC niche contributing towards regulation of these fates are debated. However, studies have shown that both the endosteal and vascular niches represent major components of the HSC niche. While some publications have supported the idea that endosteal niches are enriched with HSCs and are in close proximity to OBs during steady state and also after BM transplantation (Gong 1978; Zhang et al. 2003; Ellis et al. 2011), other publications have suggested that HSCs localise adjacent to the BM sinusoidal endothelial cells, which support the perivascular and endothelial niches harbouring HSCs (Duncan et al. 2005; Ding et al. 2012; Kiel et al. 2005).

Pervious publications have provided evidence that factors regulating endosteal niche cells influenced the HSC number and function (Calvi et al. 2003; Suda et al. 2011). Ex vivo real-time imaging of trabecular BM from irradiated mice showed that transplanted HSCs were in close proximity to the endosteum (Xie et al. 2009). Experimental evidence from the 3D live-imaging of calvarium BM of mice showed that after transplantation HSC-enriched cells gradually made their way into the osteoblastic region of the BM away from the vasculature (Lo Celso et al. 2009). This study revealed that HSCs in the murine calvarium BM resides in close proximity to endosteum and OBs (Lo Celso et al. 2009). Analysis of transgenic mice with activated parathyroid hormone peptide receptor showed an increase in OB numbers that was mediated by Notch ligand, Jagged 1 and in-turn led to an increase in HSCs (Calvi et al. 2003). OBs express molecules such as angioepoietin-1 (Ang-1), Tpo and osteopoietin (OPN), which are reported to influence HSC maintenance (Arai et al. 2004; Nilsson et al. 2005; Qian et al. 2007). The interaction of *Ang-1* in *Tie2*-enriched HSCs resulted in prolonged long-term reconstitution and enhanced their quiescence (Arai et al. 2004). *Tpo*-deficient mice displayed a drastic loss of HSCs in the postnatal period (Qian et al. 2007) and *OPN* played a role in localisation of HSCs in the endosteal niche and enhanced their quiescent phenotype (Nilsson et al. 2005). Hence, OBs contribute to molecules that regulate HSC maintenance

in their niche. Additional cells, such as OCs and macrophages, have also been reported to influence HSC-niche interactions (Christopher et al. 2011; Miyamoto et al. 2011). These studies together suggest the localisation of HSCs in the endosteal niche and the influence of factors secreted by the OBs on HSC functions.

On the other hand, several studies suggest the existence of vascular and perivascular HSC niche that consists of endothelial cells, BM sinusoidal endothelial cells, CAR cells and MSCs (Kiel et al. 2005; Mendez-Ferrer et al. 2010; Omatsu et al. 2010; Suda et al. 2011). It has been shown that HSCs are localised adjacent to BM sinusoidal endothelial cells (Kiel et al. 2005). Further, transgenic mice studies showed that the interaction of endothelial cells and HSCs was mediated by Notch signalling (Duncan et al. 2005). Deletion of *Scf* secreted by endothelial cells led to a reduced frequency of HSCs and resulted in their defective reconstitution potential in congenic recipients (Ding et al. 2012). CAR cells in the perivascular niche were shown to secrete *Scf* and Cxcl12 essential for HSC maintenance (Omatsu et al. 2010). Additionally, depletion of CAR cells was reported to affect HSC proliferation capability (Omatsu et al. 2010). Perivascular MSCs were shown to express high levels of various genes mediating HSC functions, such as *Cxcl12, Ang-1, IL-7, Scf* and *OPN* (Chow et al. 2011). Selective depletion of $Nestin^{+}$ mesenchymal stem cells in mice resulted in decreased primitive progenitor cells and defective repopulating ability of HSCs in lethally irradiated recipients (Mendez-Ferrer et al. 2010). A recent publication reported the identification of non-myelinating Schwann cells in the perivascular niche as the source of TGF-$\beta$ activation, thereby playing a critical role in TGF-$\beta$/Smad signalling in HSC maintenance (Yamazaki et al. 2011). Furthermore, live imaging of murine calvarial BM reported a highly vascularised endosteal niche contributing to the co-existence of endosteal and vascular niche (Lo Celso et al. 2009). This was further supported by a recent publication where quantitative imaging of femoral BM displayed a micro-vascular network in the endosteal niche (Nombela-Arrieta et al. 2013). Taken together, it is evident that the BM niche is dynamic and consists of diverse cell types regulating HSC functions and fate decisions.

A recent publication showed that *Scf*, a key HSC niche factor, was expressed exclusively by perivascular cells and that specific deletion of *Scf* from endothelial and leptin receptor (Lepr)-expressing perivascular cells led to loss of HSCs (Ding et al. 2012). Additionally, conditional deletion of *Scf* from OBs or HSCs did not affect the HSC functions (Ding et al. 2012). Further, cell type specific conditional deletion of the chemokine, *Cxcl12* from the BM niche showed that HSC mainly reside in the perivascular niche as deletion of *Cxcl12* from the endothelial and perivascular stromal cells depleted HSCs (Ding and Morrison 2013). Deletion of *Cxcl12* from OBs decreased early lymphoid progenitors without affecting HSCs or myeloerythroid progenitors, suggesting primitive lymphoid progenitors reside in the endosteal niche (Ding and Morrison 2013). These papers together revealed that HSCs and progenitor cells reside in distinct cellular niches in the BM with HSCs choosing the perivascular niche and early lymphoid progenitors in the endosteal niche (Ding et al. 2012; Ding and Morrison 2013). Hence a detailed characterisation of the BM niche and their roles in regulating HSC functions by unfolding their cellular and molecular complexities is necessary to provide a more detailed understanding of the BM microenvironment.

The importance of Hifs in maintaining cell-autonomous HSC functions is under extensive study, however the importance of Hifs in the HSC niche is poorly understood. Hif signalling is known to play a vital role in maintaining the functional microenvironment of the BM (Chan et al. 2009; Nishida et al. 2012; Rankin et al. 2012). Takubo et al showed that lethally irradiated Hif-1$\alpha^{\Delta/\Delta}$ mice (lacking *Hif-1*$\alpha$ in the BM microenvironment) transplanted with BM mononuclear cells expressing WT levels of *Hif-1*$\alpha$ successfully maintained long-term reconstitution potential suggesting that *Hif-1*$\alpha$ expression in the BM niche is not essential for HSC functions (Takubo et al. 2010). *Vegf*, regulated by hypoxia has been reported as highly expressed in perichondrial cells and chondrocytes, thus influencing HSC niche activity (Chan et al. 2009). A previous study showed that impaired expression of *Vegf*$\alpha$ (a hypoxia regulated angiogenic factor) led to increased HSC numbers, while affecting their self-renewal and differentiation properties (Rehn et al 2011). It was demonstrated that stabilisation of Hifs by *Vhl* deletion specifically in the OBs resulted in accumulation of trabecular OBs

and increased expression of *Vegf* and *Epo* in turn leading to expansion of HSCs (Rankin et al. 2012). Deletion of *Vhl* in OBs resulted in elevated *Epo* production in the BM and increased primitive erythroid progenitors and matured erythroid cells (Rankin et al. 2012). However, Hifs were proposed to mediate the haemopoietic defects, such as increased LSKs in Vhl-deficient mice as co-deletion of *Hif-1α* and *Hif-2α* reverted the defective phenotype (Rankin et al. 2012). A genetic knockout approach of membrane-type 1 matrix metalloproteinase (*MT1-MMP*) in mice caused severe pancytopenia and resulted in a defective HSC pool (Nishida et al. 2012). Also, several factors secreted by BM niche cells, such as *SDF-1*, *IL-7*, *Scf* and *Epo* were reduced in MT1-MMP-deficient mice (Nishida et al. 2012). Further, expression of *SDF-1* or *Scf* reversed the pancytopenia and bypassed the HSC proliferation defects (Nishida et al. 2012). This study also revealed that in Nestin$^{+}$ mesenchymal stem cells, *MT1-MMP* induced *Hif-1α* expression and thereby regulated postnatal haemopoiesis (Nishida et al. 2012).

Scortegagna et al studied the effect of global deletion of *Hif-2α* on the HSC microenvironment using a small number of congenic *Hif-2α* null mice that survived 1 month into adulthood generated by crossing two different inbred strains, C57BL/6 and 129S6/SvEvTac that are *Hif-2α* heterozygotes (Scortegagna et al. 2003b). These *Hif-2α* null mice showed decreased haematocrit, RBC and renal Epo production (Scortegagna et al. 2003b; Scortegagna et al. 2005). Transplantation of WT BM cells into lethally irradiated mice lacking global *Hif-2α* expression showed reduced haematocrit levels and PB cell counts with low numbers of WBC, RBC and platelets as opposed to Controls when analysed after short-term engraftment (Scortegagna et al. 2003b). The donor cells in the *Hif-2α* null mice were decreased but repopulated the irradiated recipients and were capable of inducing normal haemopoiesis (Scortegagna et al. 2003b). This study suggested that haemopoietic defects in transplanted *Hif-2α* null mice might be due to an altered BM microenvironment (Scortegagna et al. 2003b). However, the phenotype described above might represent a unique strain specific phenotype caused by systemic deletion of *Hif-2α*. Although this study attempted to explain the potential role of *Hif-2α* in the BM niche and its influence on HSC engraftment, the overall role of *Hif-2α* in the HSC

niche and its effect on HSC functions remained unclear. In this Chapter, I have therefore studied the effect of deletion of *Hif-1α* and *Hif-2α* in the niche and their importance in maintenance of HSC functions.

## 4.2 Outline of experiments described in Chapter 4

The aim of the experiments in this Chapter was to dissect out the role of *Hif-2α* alone, and *Hif-1α* and *Hif-2α* in combination, in non-cell-autonomous HSC maintenance. I generated inducible mouse models where acute deletion of the floxed *Hif-1α* and/or *Hif-2α* allele was achieved by induction of Mx1-Cre expression by pIpC. Upon confirmation of successful *Hif-1α* and/or *Hif-2α* deletion in the BM microenvironment, I performed immunophenotypic analyses of BM, spleens, thymi, PB and found a decrease in absolute numbers of HSCs and primitive progenitors in the Hif-2$\alpha^{\Delta/\Delta}$ mice compared to the Control mice. However, the numbers of primitive progenitors and HSCs were reverted back to the Control levels with additional deletion of one or both alleles of *Hif-1α* respectively. These results indicate that *Hif-2α* is essential for non-cell-autonomous maintenance of HSC functions and also indicate the previously unappreciated interplay between *Hif-1α* and *Hif-2α*.

## 4.3 Results

### 4.3.1 The analysis of mature haemopoietic cells in pIpC-treated Hif-2$\alpha^{fl/fl}$ Mx1-Cre mice.

To examine the role of *Hif-2α* in the maintenance of the haemopoietic system, I employed the loss of function approach. *Hif-2α* is essential for embryogenesis (Tian et al. 1998; Peng et al. 2000; Compernolle et al. 2002; Scortegagna et al. 2003a; Gruber et al. 2007), however the role of *Hif-2α* in adult haemopoiesis has not been fully explored. As the Hif-2α null mice are embryonic lethal (Tian et al. 1998; Peng et al. 2000; Compernolle et al. 2002), to investigate the function of *Hif-2α* in adult haemopoiesis I used the inducible gene deletion strategy using the Mx1-Cre system. Mx1-Cre is shown to efficiently delete floxed alleles in both haemopoietic cells and the surrounding BM microenvironment, but to a lesser extent also within non-haemopoietic organs, such as heart, liver, kidney and urinary bladder (Kuhn

et al. 1995; Schneider et al. 2003; Walkley and Orkin 2006; Walkley et al. 2007). I generated Hif-2$\alpha^{fl/fl}$ Mx1-Cre mice and Hif-2$\alpha^{fl/fl}$ without Mx1-Cre (Control) in which the deletion of the floxed *Hif-2$\alpha$* allele within the haemopoietic system and BM microenvironment is induced by activation of Mx1-Cre by administration of pIpC (Section 2.1.2) (Figure 4.1A). Hif-2$\alpha^{fl/fl}$ Mx1-Cre and Hif-2$\alpha^{fl/fl}$ males that were 8-12 weeks old were treated IP with 6 doses of 300ug of pIpC on alternate days. Efficient deletion of the *Hif-2$\alpha$* allele in the Hif-2$\alpha^{fl/fl}$ Mx1-Cre mice (here after referred to as Hif-2$\alpha^{\Delta/\Delta}$) in the BM haemopoietic cells and in the CD45$^-$ non-haemopoietic cell components of the BM was confirmed by PCR using genomic DNA as a template (Figure 4.1B). Mice were sacrificed at day 11 from the last pIpC injection. Immunophenotypic analyses of differentiated cells in BM and spleens of Hif-2$\alpha^{\Delta/\Delta}$ mice and Control mice revealed equal frequencies and total numbers of myeloid cells (Gr-1$^+$ Mac-1$^+$) and erythroid cells (Ter119$^+$) (Figure 4.2A, 4.2C-4.2D and 4.2F-4.2H). In the BM of Hif-2$\alpha^{\Delta/\Delta}$ mice, there was a decrease in B-cells (CD19$^+$ B220$^+$) however, spleen had equal number of B-cells in the Hif-2$\alpha^{\Delta/\Delta}$ mice and Control mice (4.2B and 4.2E). There was no difference in the total number of T-cells (CD4$^+$ or CD8$^+$) in the spleen or T-cells (CD4$^+$, CD8$^+$ and double positive) in the thymus between Hif-2$\alpha^{\Delta/\Delta}$ mice and Control mice (Figure 4.2I-4.2K). These results showed that acute deletion of *Hif-2$\alpha$* had no impact on multilineage differentiation potential of HSCs/progenitors and that *Hif-2$\alpha$* was dispensable for the maintenance of mature blood lineages in mice (at least within the first 11 days after *Hif-2$\alpha$* deletion).

### 4.3.2 The requirement for *Hif-2$\alpha$* in non-cell-autonomous HSC maintenance

The requirement of *Hif-2$\alpha$* in the non-cell-autonomous maintenance of HSC functions and its functions in the BM niche cells remained to be investigated. Hence, I looked into the effects of *Hif-2$\alpha$* deletion on HSCs and also in the BM microenvironment. To address this, I used the Mx1-Cre inducible gene deletion model that excises the floxed *Hif-2$\alpha$* allele in the BM microenvironment and in the haemopoietic system upon pIpC treatment. Hif-2$\alpha^{fl/fl}$ Mx1-Cre mice and Control mice that were between 8-12 weeks of

age were treated with sequential pIpC (6 doses, 300ug per dose). After pIpC treatment, the *Hif-2α* floxed allele was deleted efficiently within both the haemopoietic system and in non-haemopoietic $CD45^-$ cell components of the BM microenvironment (Figure 4.1B). Mice were analysed 11 days after the last pIpC injection. Immunophenotypic analyses of the BM revealed that Hif-$2\alpha^{\Delta/\Delta}$ mice had reduced frequencies and absolute numbers of LSKs, HSCs (LSK $CD150^+CD48^-$) and primitive progenitors within the LSK compartment (LSK $CD150^+CD48^+$ and LSK $CD150^-CD48^+$) compared to Control mice (Figure 4.3C–4.3F). However, there was no difference in the numbers of myeloid progenitors ($Lin^-$c-$Kit^+$, LK cells), $Lin^-$ cells (comprising HSCs and different progenitors at different stages of the differentiation hierarchy) or LSK $CD150^-CD48^-$ (ST-HSCs) between Hif-$2\alpha^{\Delta/\Delta}$ mice and Control mice (Figure 4.3A-4.3B and 4.3G). Deletion of *Hif-2α* led to a decrease in numbers of HSCs and primitive progenitors compared to Controls. Taking into account that deletion of *Hif-2α* within HSCs does not perturb normal haemopoiesis along with the above results, my data indicated that *Hif-2α* is required for maintenance of HSCs in a non-cell-autonomous manner. The results in this thesis suggested that although *Hif-2α* within the HSCs alone may be dispensable for normal haemopoiesis, *Hif-2α* in the BM microenvironment is essential for normal maintenance of HSCs and primitive progenitors.

### 4.3.3 The lack of extra-medullary haemopoiesis in Hif-$2\alpha^{\Delta/\Delta}$ mice

The above experiments showed the decrease in numbers of HSCs and primitive progenitors in the Hif-$2\alpha^{\Delta/\Delta}$ mice compared to the Control mice. This decrease could be due to mobilisation of HSCs or primitive progenitors from BM to PB and their migration to other haemopoietic organs such as the spleen. To address this, I performed immunophenotypic analyses to detect the presence of primitive progenitors in the blood and spleens of Hif-$2\alpha^{\Delta/\Delta}$ mice and Control mice. Hif-$2\alpha^{fl/fl}$ Mx1-Cre and Control mice were treated with 6 doses of pIpC on alternate days and 11 days after the last pIpC injection mice were sacrificed and tested for extra-medullary haemopoiesis in the spleen and the PB. FACS analyses revealed that there was no difference in the percentage of LSKs in the spleen or PB of Hif-$2\alpha^{\Delta/\Delta}$ mice

and in the Control (Figure 4.4). This confirmed that there was no mobilisation of the HSCs or primitive progenitors from the BM to the spleen or PB in the Hif-2$\alpha^{\Delta/\Delta}$ mice.

Next, to test if the loss of HSCs and primitive progenitor cells in the Hif-2$\alpha^{\Delta/\Delta}$ mice was due to apoptosis, I carried out a cell viability assay using Annexin V and DAPI. I performed the immunostaining of the LSKs from the BM with Annexin V and DAPI. There was no difference in the percentage of Annexin$^-$/DAPI$^-$ (alive cells), Annexin V$^+$/DAPI$^-$ (early apoptotic cells), Annexin V$^+$/DAPI$^+$ (late apoptotic cells) or AnnexinV$^-$/DAPI$^+$ (dead cells) in the BM from Hif-2$\alpha^{\Delta/\Delta}$ mice and the Control mice (Figure 4.5). This cell viability assay showed no difference in the frequency of cell death in the LSK cells in Hif-2$\alpha^{\Delta/\Delta}$ and Control mice.

### 4.3.4 The analysis of HSCs in pIpC-treated Hif-2$\alpha^{fl/fl}$ mice, Hif-1$\alpha^{+/fl}$ Hif-2$\alpha^{fl/fl}$ mice and Hif-1$\alpha^{fl/fl}$ Hif-2$\alpha^{fl/fl}$ mice

It is widely accepted that *Hif-1α* and *Hif-2α* have high sequence homology, but vary widely in their mRNA expression pattern (Gruber et al. 2007; Takubo et al. 2010). While *Hif-1α* is expressed ubiquitously and expressed at higher levels in the HSCs compared to haemopoietic progenitor and mature cells, *Hif-2α* expression is restricted to organs like the endothelium, lung, brain, neural crest derivatives and *Hif-2α* is expressed in HSCs and haemopoietic progenitors (Gruber et al. 2007; Takubo et al. 2010; Kocabas et al. 2012). *Hif-1α* and *Hif-2α* have common target genes, including *Vegf* and *Glut-1*, (Hu et al. 2003) but may also regulate the expression of distinct genes; e.g. *Hif-1α* regulates hexokinase, *PGK-1* (Hu et al. 2003) while *Hif-2α* regulates renal *Epo* (Rankin et al. 2007). The interplay between *Hif-1α* and *Hif-2α* in the HSC and BM microenvironment has yet to be fully understood. To address this, I generated Hif-1$\alpha^{+/fl}$ Hif2$\alpha^{fl/fl}$ Mx1-Cre, Hif-1$\alpha^{fl/fl}$ Hif-2$\alpha^{fl/fl}$ Mx1-Cre and Hif-1$\alpha^{fl/fl}$ Hif-2$\alpha^{fl/fl}$ (Control) mice. Upon induction of Cre by pIpC, the floxed alleles are excised resulting in acute deletion of *Hif-1α* and *Hif-2α* in the adult haemopoietic organs and BM microenvironment (Figure 4.6A and 4.6B). In my previous experiment (Chapter 4 Section 4.2.2), deletion of *Hif-2α* resulted in decreased HSCs and primitive progenitors in

the LSK compartment (LSK $CD150^{+}CD48^{+}$ and LSK $CD150^{-}CD48^{+}$, LSK $CD150^{-}CD48^{-}$). To investigate if additional deletion of *Hif-1α* would exacerbate the Hif-$2\alpha^{\Delta/\Delta}$ phenotype, Hif2$\alpha^{fl/fl}$ Mx1-Cre, Hif-$1\alpha^{+/fl}$ Hif2$\alpha^{fl/fl}$ Mx1-Cre, Hif-$1\alpha^{fl/fl}$ Hif-$2\alpha^{fl/fl}$ Mx1-Cre and Hif-$1\alpha^{fl/fl}$ Hif-$2\alpha^{fl/fl}$ (Control) mice were treated with 6 doses of pIpC injections and monitored on a daily basis. Gene deletion was confirmed in Hif-$1\alpha^{+/fl}$ Hif-$2\alpha^{fl/fl}$ Mx1-Cre mice (Hif-$1\alpha^{+/\Delta}$ Hif-$2\alpha^{\Delta/\Delta}$) and Hif-$1\alpha^{fl/fl}$ Hif-$2\alpha^{fl/fl}$ Mx1-Cre (DKO) BM cells (Figure 4.6A). There were no survival defects observed and the WBC count was normal in PB, BM and spleens in *Hif-1α* and *Hif-2α* deficient mice compared to Control irrespective of the genotypes (Figure 4.6C-4.6D). Mice were sacrificed at day 11 after the last pIpC injection and the haemopoietic organs were characterised immunophenotypically.

Surprisingly, unlike Hif-$2\alpha^{\Delta/\Delta}$ mice, Hif-$1\alpha^{+/\Delta}$ Hif-$2\alpha^{\Delta/\Delta}$ mice showed similar number of LSKs and primitive progenitors (LSK $CD150^{-}CD48^{+}$, LSK $CD150^{+}CD48^{+}$) to the Control mice (Figure 4.7C, 4,7E and 4.7F). However, the number of HSCs in Hif-$1\alpha^{+/\Delta}$ Hif-$2\alpha^{\Delta/\Delta}$ mice (i.e. mice lacking one allele of *Hif-1α* and both alleles of *Hif–2α)* was reduced compared to the Control mice (Figure 4.7D). With deletion of both alleles of *Hif-1α* and *Hif-2α*, in DKO mice there was a similar number of HSCs to Control mice compared to decreased HSCs in the Hif-$2\alpha^{\Delta/\Delta}$ (Figure 4.7D). However, DKO mice showed reduced numbers of primitive progenitors in the LSK compartment (LSK $CD150^{-}CD48^{+}$, LSK $CD150^{+}CD48^{+}$) compared to Control mice with WT levels of *Hif-1α* and *Hif-2α* (Figure 4.7E-F). The number of ST-HSCs did not show any significant changes in any of the genotypes (Figure 4.7G). There was no difference in the Lin-, LK and mature blood lineages in the spleen or thymus in Hif-$1\alpha^{+/\Delta}$ Hif-$2\alpha^{\Delta/\Delta}$, DKO, and Control mice (Figure 4.7A-4.7B and 4.8). However, in the BM of DKO mice there was an increase in the number of myeloid cells (Gr-1 Mac-1) and erythroid cells ($Ter119^{+}$) compared to the Control mice (Figure 4.8A and 4.8C). The total number of B-cells ($CD19^{+}$ $B220^{+}$) was equal to Control mice in all the genotypes (Figure 4.8B and J). These results illustrate the dose dependent interplay between *Hif-1α* and *Hif-2α* between different cell types (Table 4.1).

### 4.3.5 The analysis of HSCs in males and females of pIpC-treated Hif-2$\alpha^{fl/fl}$ mice, Hif-1$\alpha^{+/fl}$ Hif-2$\alpha^{fl/fl}$ mice and Hif-1$\alpha^{fl/fl}$ Hif-2$\alpha^{fl/fl}$ mice

Previous studies have shown the influence of sex hormones in regulating bone mass (Manolagas 2010). Studies have shown that *Hif* target genes, *Vegf* and *Angiopoietins* (*Ang1* and *Ang2*), were regulated by oestrogen and progesterone in human endometrial stromal cells (Tsuzuki et al. 2013). Also, various publications have demonstrated that sex steroid hormones, oestrogen and androgen, regulated *HIF-1$\alpha$, HIF-2$\alpha$* and *VEGF* expression in various cancer models, such as human endothelial cancer, prostrate cancer and in murine breast cancer cells (Mabjeesh et al. 2003; Boddy et al. 2005; Kazi and Koos 2007; George et al. 2012). Pregnancy or exogenous administration of oestrogen was shown to decrease haemopoietic colony forming cells and mature B-lymphocytes (Fried et al. 1974; Medina et al. 1993). A recent publication showed that estradiol treatment increased the number of HSCs in the vascular niche (Illing et al. 2012). In the previous section I have shown that *Hif-2$\alpha$* deletion in the HSCs and BM microenvironment resulted in decreased number of HSCs and primitive progenitor cells compared to Control and co-deletion of both alleles of *Hif-1$\alpha$* rectified the defective HSC phenotype (Section 4.3.2 and 4.3.4). Immunophenotypic analysis of sex-matched BM by multicolour flow cytometry showed that Hif-2$\alpha^{\Delta/\Delta}$ males displayed decreased absolute numbers of HSCs (LSK CD150$^{+}$CD48$^{-}$) and primitive progenitors within the LSK compartment (LSK CD150$^{+}$CD48$^{+}$ and LSK CD150$^{-}$CD48$^{+}$, LSK CD150$^{-}$CD48$^{-}$) compared to the Control males analysed 11 days after last pIpC administration (Figure 4.9). Further, analysis of Hif-2$\alpha^{\Delta/\Delta}$ females showed equal number of HSCs (LSK CD150$^{+}$CD48$^{-}$) and primitive progenitors within the LSK compartment (LSK CD150$^{+}$CD48$^{+}$ and LSK CD150$^{-}$CD48$^{+}$) compared to the Control (Figure 4.9). However, analysis of Hif-1$\alpha^{+/\Delta}$ Hif-2$\alpha^{\Delta/\Delta}$ males and females along with Controls for respective gender showed comparable numbers of HSCs (LSK CD150$^{+}$CD48$^{-}$) and primitive progenitors within the LSK compartment (LSK CD150$^{+}$CD48$^{+}$ and LSK CD150$^{-}$CD48$^{+}$, LSK CD150$^{-}$CD48$^{-}$) (Figure 4.10). The DKO males and females had similar number of HSCs (LSK CD150$^{+}$CD48$^{-}$) compared to respective Controls (Figure 4.10B). The absolute number of primitive progenitors within the LSK compartment (LSK

$CD150^{+}CD48^{+}$ and LSK $CD150^{-}CD48^{+}$) were decreased in DKO males compared to Control mice whereas DKO and Control females showed no difference in absolute number of primitive progenitors within the LSK compartment (LSK $CD150^{+}CD48^{+}$ and LSK $CD150^{-}CD48^{+}$) (Figure 4.10D-E). These surprising results imply that the role of Hifs in HSC maintenance is dependent on gender.

### 4.3.6 Immunophenotypic analysis of HSC niche cells lacking *Hif-2α*

It has been shown that OBs-specific *Vhl* deletion (most likely via upregulation of HIFs in the OBs) resulted in expansion of the HSC niche and increased numbers of HSCs (Rankin et al. 2012). However, there is no direct evidence of specific functions of *Hif-1α* and *Hif-2α* in the maintenance of adult HSC niche. I have shown that *Hif-2α* is essential for the maintenance of functional HSCs in a non-cell-autonomous manner specifically in males (Section 4.3.6). Also, deletion of *Hif-1α* together with *Hif-2α* rescued the HSC loss observed in Hif-$2\alpha^{\Delta/\Delta}$ males. To further dissect the role of *Hif-2α* in maintaining the BM niche and in turn number of functional HSCs, I used the CKO mouse model.

I analysed the expression of *Hif-1α* and *Hif-2α* in different populations of niche cells sorted based on the expression of immunophenotypic markers by flow cytometry (Nakamura et al 2010). *Hif-1α* expression in endothelial and endosteal cells that were $ALCAM^{+}Sca\text{-}1^{-}$ was higher compared to immature mesenchymal enriched $ALCAM^{-}Sca\text{-}1^{+}$ cells and endosteal cells that were $ALCAM^{-}Sca\text{-}1^{-}$ (Figure 4.11A). *Hif-2α* expression was highest in endothelial cells (10 folds higher) followed by similar expression levels in immature mesenchymal enriched $ALCAM^{-}Sca\text{-}1^{+}$ cells and endosteal cells that were $ALCAM^{+}Sca\text{-}1^{-}$, with lowest levels of expression in the endosteal cells that were $ALCAM^{-}Sca\text{-}1^{-}$ (Figure 4.11B). To investigate the effect of deletion of *Hif-2α* on the HSC niche in males, I analysed sex-matched Hif-$2\alpha^{fl/fl}$ Mx1-Cre and Control males. The deletion of the floxed allele was induced by pIpC administration. Mice between 8 weeks and 12 weeks of age received 6 doses of pIpC and were sacrificed at 11 days after the last pIpC injection. To isolate the BM niche cells, bone fragments from Hif-$2\alpha^{\Delta/\Delta}$ and Control mice

were treated with crude collagenase VIII. Efficient deletion of *Hif-2α* in the BM microenvironmental cells was confirmed by PCR (Figure 4.6B). The isolated cells were analysed for the expression of cell surface markers using flow cytometry to distinguish different subpopulations of the endothelial and endosteal niche (Nakamura et al 2010). There was no difference in the number of endothelial cells ($CD45^-Ter119^-CD31^+$) cells between Hif-$2\alpha^{\Delta/\Delta}$ and Control mice (Figure 4.12A). There was no difference in the number of immature mesenchymal enriched $ALCAM^-Sca\text{-}1^+$ cells (Figure 4.12B) or endosteal cells that were $ALCAM^-Sca\text{-}1^-$ and $ALCAM^+Sca\text{-}1^-$, classed as OB-enriched cells (Figure 4.12C-D), between Hif-$2\alpha^{\Delta/\Delta}$ and Control mice. Collectively, the immunophenotypic analysis of the male BM niche showed no difference in the number of endothelial, endosteal and mesenchymal niche cells in Hif-$2\alpha^{\Delta/\Delta}$ or Control mice.

### 4.3.7 The analysis of *Hif-2α* deficient BM niche in regulating HSC reconstitution potential

To test the effect of *Hif-2α* deletion on the functionality of the HSC niche, I performed a transplantation experiment. 500,000 WT $CD45.1^+$ BM cells were transplanted into lethally irradiated Hif-$2\alpha^{fl/fl}$ Mx1-Cre and Control mice. After 8 weeks of transplantation following multilineage engraftment (Figure 4.13B) mice were injected with 6 doses of pIpC on alternate days to induce the deletion of *Hif-2α*. BM was analysed 4 weeks after the last dose of pIpC (Figure 4.13A). The result suggested an equal number of HSCs and primitive progenitor cells in the Hif-$2\alpha^{\Delta/\Delta}$ and Control recipient mice (Figure 4.14). I also analysed the number of endosteal and endothelial cells in the BM niche. Consistent with the previous data shown in figure 4.13, there was no difference in the number of endothelial cells, immature mesenchymal cells or OBs in the Hif-$2\alpha^{\Delta/\Delta}$ mice or Controls (Figure 4.15). The results suggest that *Hif-2α* deletion in the HSC niche alone might not be critical for HSC maintenance and that deletion from the HSC niche together with HSCs might affect their functions. However, this experiment had insufficient numbers of Hif-$2\alpha^{\Delta/\Delta}$ males to perform statistical tests.

# 4.4 Discussion

## 4.4.1 *Hif-2α* is essential for non-cell-autonomous maintenance of HSCs specifically in males, but not females

The above experiments shed light on our current understanding of the contribution of Hifs to the non-cell-autonomous maintenance of HSCs. Analysis of different populations of BM niche cells in WT mice revealed that *Hif-2α* was expressed in all cell populations analysed with highest expression in endothelial cells (Figure 4.10B). The results described in this Chapter showed that acute deletion of *Hif-2α* in the haemopoietic system and surrounding BM microenvironment resulted in a decrease of LSKs, particularly in males and not in females. The results discussed hereafter pertain to the studies observed specifically in males unless otherwise mentioned. Analysis of Hif-2$\alpha^{\Delta/\Delta}$ mice after 11 days of pIpC was chosen as the optimum time point to overcome the interferon responsive phenotype and to analyse the effect of acute Hif-$\alpha$ deletion on HSCs. Hif-2$\alpha^{\Delta/\Delta}$ mice showed a reduced number of primitive progenitors (LSK CD48$^{+}$CD150$^{+}$, LSK CD48$^{-}$CD150$^{+}$). The frequency and total number of HSCs was reduced in mice lacking *Hif-2α* compared to Control mice. Immunophenotypic analyses of spleens and PB of Hif-2$\alpha^{\Delta/\Delta}$ mice showed no evidence of mobilised HSCs or primitive progenitors in Hif-2$\alpha^{\Delta/\Delta}$ mice compared to Control mice. Together, these results imply that *Hif-2α* signalling within the BM microenvironment contributed to the maintenance of the HSC pool and primitive progenitors. There was no difference in cell viability in primitive progenitors between Hif-2$\alpha^{\Delta/\Delta}$ and Control mice as revealed by Annexin V staining. The mature cells in the haemopoietic organs including BM, spleen and thymus were unaffected upon the deletion of *Hif-2α*. Hif-2$\alpha^{\Delta/\Delta}$ mice displayed normal haemopoiesis, which successfully differentiated into mature blood lineages. Hence, the reduced number of HSCs and primitive progenitors and the lack of phenotype in the mature blood cells in Hif-2$\alpha^{\Delta/\Delta}$ compared to Control mice additionally suggested that the effect of *Hif-2α* deletion was specific to HSC and primitive progenitor cells. This is correlated with more recent work by Rankin et al who showed an enhanced stabilisation of Hifs by Vhl deletion specifically in the endosteal niche cells, leading to expansion of

niche cells and an increase in HSC numbers (Rankin et al. 2012). It has been shown before that *Hif-2α* in mice is a major isoform of Hifs controlling Epo production in the kidney and global expression of *Hif-2α* is critical for erythropoiesis (Scortegagna et al. 2003a; Gruber et al. 2007). A previous publication suggested that *Hif-2α* in the BM microenvironment regulated repopulation potential of HSCs, but the importance of *Hif-2α* in the BM microenvironment was not fully understood (Scortegagna et al. 2003b). These results taken together with previous data showing that *Hif-2α* was dispensable for cell-autonomous HSC maintenance (Chapter 3) suggested that *Hif-2α* expression in the BM microenvironment was essential for the maintenance of HSC functions in a non-cell-autonomous manner and was gender dependent. However, since Mx1-Cre is known to recombine floxed alleles in non-haemopoietic organs like heart, liver, kidney and urinary bladder (Kuhn et al. 1995; Schneider et al. 2003; Walkley and Orkin 2006; Walkley et al. 2007) it might be possible that *Hif-2α* deletion also outside the BM microenvironment contributed to the phenotype in Hif-2$\alpha^{\Delta/\Delta}$ mice. Nonetheless, as shown by data described in figures 4.3 and 4.6B, deletion of *Hif-2α* in the BM microenvironment cells accounted at least partially for the loss of HSCs and primitive progenitors.

### 4.4.2 Additional deletion of *Hif-1α* rescues non-cell-autonomous HSC loss observed in Hif-2$\alpha^{\Delta/\Delta}$ males

Several studies have suggested that hypoxia-signalling pathways in the BM niche might play a vital role in regulating HSC functions (Rehn et al. 2011; Nishida et al. 2012; Rankin et al. 2012). Several publications showed the importance of *Hif-1α* in regulating BM niche functions (Miharada et al 2011; Nishida et al. 2012; Rankin et al 2012). *MT1-MMP* and *Cripto* play a major role in regulating HSC functions via Hif-1α-signalling (Miharada et al 2011; Nishida et al. 2012). *MT1-MMP* regulates haemopoietic Hif-dependent signalling in the BM niche cells by activating the Hif-1α pathway via FIH-1 (Nishida et al. 2012). It has been reported that reduced expression of *Cripto* resulted in decreased number of HSCs that are positive for its receptor GRP78 in the endosteal region of *Hif-1α* CKO mice (Miharada et al 2011). Increased stabilisation of HIFs by *Vhl* deletion resulted in Nestin$^{+}$ HSC niche

cell expansion and in turn increased HSCs with defective self-renewal and differentiation potential (Rankin et al 2012). However, the relationship between *Hif-1α* and *Hif-2α* signalling within the BM microenvironment remains unknown. It has been reported that *Hif-1α* deletion using Mx1-Cre within the HSC and BM microenvironment at steady state does not affect the maintenance of HSCs (Takubo et al. 2010). I have shown that deletion of *Hif-2α* specifically in males resulted in decreased numbers of HSCs and primitive progenitors compared to sex-matched controls. Hence, results from this Chapter compared to previous publications suggested that unlike *Hif-1α*, *Hif-2α* expression in the BM niche is essential for the maintenance of HSC functions specifically in males. Alternatively, the authors might have missed the short-term HSC phenotype due to compensatory mechanisms activated in Hif-1$\alpha^{\Delta/\Delta}$ mice analysed several weeks after pIpC (Takubo et al. 2010). Further, I have shown that additional deletion of one allele of *Hif-1α* together with *Hif-2α* reverted the loss of primitive progenitors (LSK CD48$^{+}$CD150$^{+}$, LSK CD48$^{-}$CD150$^{+}$), but no significant rescue of reduced HSCs that was observed in Hif-2$\alpha^{\Delta/\Delta}$ males. Notably, the loss of HSCs in Hif-2$\alpha^{\Delta/\Delta}$ mice is bypassed only upon deletion of both alleles of *Hif-1α* along with *Hif-2α*. Taken together, these results showed a cell type specific interplay between *Hif-1α* and *Hif-2α* in HSCs and primitive progenitors (Table 4.1).

Several studies shed light on the fact that Hif-signalling pathways can be critically regulated in a dose-dependent manner. For example, in the study by Takubo et al, the authors showed that deletion of one allele of *Vhl* led to increased quiescence of HSCs and their expansion, but in contrast over-stabilisation of *Hif-1α* by deletion of both alleles of *Vhl* resulted in defective HSC phenotype upon transplantation (Takubo et al. 2010). Additional studies showed a profound increase in *Hif-2α* levels in the Hif-1$\alpha^{-/-}$ HSCs (Kocabas et al. 2012). Knockdown of *Hif-2α* enhanced the stabilisation and functions of Hif-1α protein (Carroll and Ashcroft, 2006; Raval et al., 2005; Schulz et al., 2012). On the other hand, *Vhl* deletion in the endosteal niche resulted in increased HSC number and their reconstitution potential (Rankin et al. 2012). These results together suggest interplay between *Hif-1α* and *Hif-2α* and also add to the dose-dependent effect of *Hif-1α* and *Hif-2α* in different

cell types. The lack of differences in the number of mature haemopoietic lineages observed in the BM, spleen and thymus except a decrease in number of B-cells ($CD19^+$ $B220^+$) in the spleen is in agreement with previous publications showing role of Hifs in B-cell regulation (Kojima et al. 2002; Kojima et al. 2010). Taken together, the results imply that the decrease in HSCs and primitive progenitor cells caused by *Hif-2* $\alpha$–deficiency is mediated by *Hif-1*$\alpha$.

The relationship between the BM niche and HSCs has been extensively studied and alterations of BM niche cell types influence the number and functions of HSCs (Calvi et al. 2003; Chan et al. 2009; Eliasson and Jonsson 2010; Ding et al. 2012). Sex steroids, like oestrogen and androgen, affect the bone mass by regulating OBs and OCs (Samuels et al. 1999). Several human and murine studies have demonstrated the relationship between steroid hormones and hypoxia signalling pathways in several cell types, including HSCs, B- lymphocytes, thymocytes, BM niche cells, breast cancer, prostrate cancer and endocrine stromal cells (Fried et al. 1974; Medina et al. 1993; Thurmond et al. 2000; Tsuzuki et al. 2013). My data showed that acute deletion of *Hif-2*$\alpha$ reduced the number of HSCs and primitive progenitors in males compared to Control mice, but the females lacking *Hif-2*$\alpha$ or Control mice had similar numbers of HSCs and primitive progenitors. The defective HSC phenotype was specific to males lacking *Hif-2*$\alpha$ and not females. This might be due to the advantage of oestrogen in the female BM niche compared to males. Independent from the effect of oestrogen on the BM niche, a recent publication showed that estradiol treated female mice had increased numbers of LSK and increased reconstituted potential in lethally irradiated mice (Tsuzuki et al. 2013). However, continued supplement of estradiol affected the quiescent state of HSCs and increased their cell cycle rate. This resulted in exhaustion of the HSCs during serial transplantation. In the prostrate cancer (LNCaP) cells dihydrotestosterone (DHT) induced *Hif-1*$\alpha$ expression and elevated *Vegf* production through the PI3/AKT pathway (Mabjeesh et al. 2003; Kimbro and Simons 2006). Hence, additional to my result, the publications have shown that sex steroids affect the BM niche and HSCs, thus playing a role in maintenance of HSCs. The result in this Chapter suggested that the female BM niche had an advantage

over the male BM niche in the absence of *Hif-2α*. Considering the previous publications showing that estradiol elevated LSK numbers and BM mass (Tsuzuki et al. 2013), the female BM niche might have oestrogen regulated HSC maintenance resistant to *Hif-2α* deletion. However, further studies need to be carried out to dissect the relationship between sex steroids and HSC functions and also to fully explore the mechanisms governing this effect.

| BM | Hif-2α$^{\Delta/\Delta}$ Vs Control | Hif-1α$^{+/\Delta}$:Hif-2α$^{\Delta/\Delta}$ Vs Hif-2α$^{\Delta/\Delta}$ | DKO Vs Hif-2α$^{\Delta/\Delta}$ |
|---|---|---|---|
| WBC | No significant difference | No significant difference | No significant difference |
| Lin- | No significant difference | No significant difference | No significant difference |
| LK | No significant difference | No significant difference | No significant difference |
| LSK | Decrease | Increase | No significant difference |
| LSK CD150-CD48+ | Decrease | Increase | No significant difference |
| LSK CD150+CD48+ | Decrease | Increase | No significant difference |
| HSC | Decrease | No significant difference | Increase |
| ST-HSC | No significant difference | No significant difference | No significant difference |
| Myeloid cells (Gr-1, Mac-1) | No significant difference | No significant difference | No significant difference |
| Erythroid cells (Ter119) | No significant difference | No significant difference | Decrease |
| B-cells (CD19, B220) | Decrease | No significant difference | No significant difference |
| T-cells (CD4, CD8) | No significant difference | No significant difference | No significant difference |

**Table 4. 1: Summary of phenotype observed in Hif-2α$^{\Delta/\Delta}$, Hif-1α$^{+/\Delta}$ Hif-2α$^{\Delta/\Delta}$ and DKO males.**

### 4.4.3 *Hif-2α* deletion does not affect absolute numbers of the niche cell components in the BM

In this Chapter, I have employed immunophenotypic analysis to investigate the effect of *Hif-2α* deletion in the niche cells of the BM. Growing evidence indicates the importance of *Hif-1α* and its targets in HSC niche functions (Rankin et al 2012; Miharada et al 2011; Nishida et al. 2012). But, the importance of *Hif-2α* specifically in the maintenance of the HSC niche influencing HSC functions is unclear. The results shown in this chapter suggested that *Hif-2α* deletion did not affect total numbers of the niche cell components. However, the experiments are based on immunophenotypic analyses. The transplantation experiment provided a clue that the functionality of the HSC niche lacking *Hif-2α* was comparable to its Control. There was equal engraftment of HSCs in mice lacking *Hif-2α* in the niche cells as compared to the Control mice. Thus expression of *Hif-2α* in the niche cells alone might be dispensable for the maintenance of HSC pool. This is in contrast to the previous publication by Scrotegagna, where the author attributed the decrease in HSCs and reduced potential of short-term engraftment of WT cells in lethally irradiated *Hif-2α* null mice to the lack of *Hif-2α* expression in the BM niche (Scortegagna et al 2003; (Scortegagna et al. 2003b). However, the defective engraftment potential of WT HSCs in the *Hif-2α*–deficient BM niche might be specific to congenic mice developed by interbreeding two different strains and to the small experimental cohort. Therefore, the data from this Chapter suggested that deletion of *Hif-2α* in both HSCs and BM niche cells led to a decrease in HSCs and LMPPs. Whether the transplantation results indicating no difference in the number of engrafted HSCs between Hif-2$\alpha^{\Delta/\Delta}$ and Control BM niche were biologically relevant or were a consequence of small sample size remains to be addressed. Further genetic studies focusing on the effect of *Hif-1α* and *Hif-2α* deletion, singly and in combination, specifically in the niche cells will provide with more insights into the functions of HIFs in the maintenance of niche cells and HSCs.

## 4.5 Conclusion

The experiments presented in this Chapter provide evidence that *Hif-2α*-dependent signalling is essential for non-cell-autonomous HSC maintenance specifically in males and not in females. The results showed that the presence of *Hif-1α* is required for the generation of the phenotype in in Hif-2α$^{\Delta/\Delta}$ males as deletion of *Hif-1α* rescues the HSC phenotype. My data showed that loss of *Hif-2α* expression had no effect on the number of HSC niche cells as revealed by immunophenotypic analysis. Transplantation experiment suggested that the BM niche from Hif-2α$^{\Delta/\Delta}$ males maintained HSC reconstitution. Hence, lack of *Hif-2α* expression collectively in the HSC and the BM niche resulted in the decrease of HSCs and primitive progenitors in Hif-2α$^{\Delta/\Delta}$ males. The data also suggest the possibility of sex-steroid regulated Hif-pathways responsible for gender-specific HSC phenotype observed in Hif-2α$^{\Delta/\Delta}$ mice. Therefore, the results in this Chapter demonstrated that the complex interplay between *Hif-1α*- and *Hif-2α*-dependent signalling is required for adult HSC maintenance in a non-cell-autonomous manner.

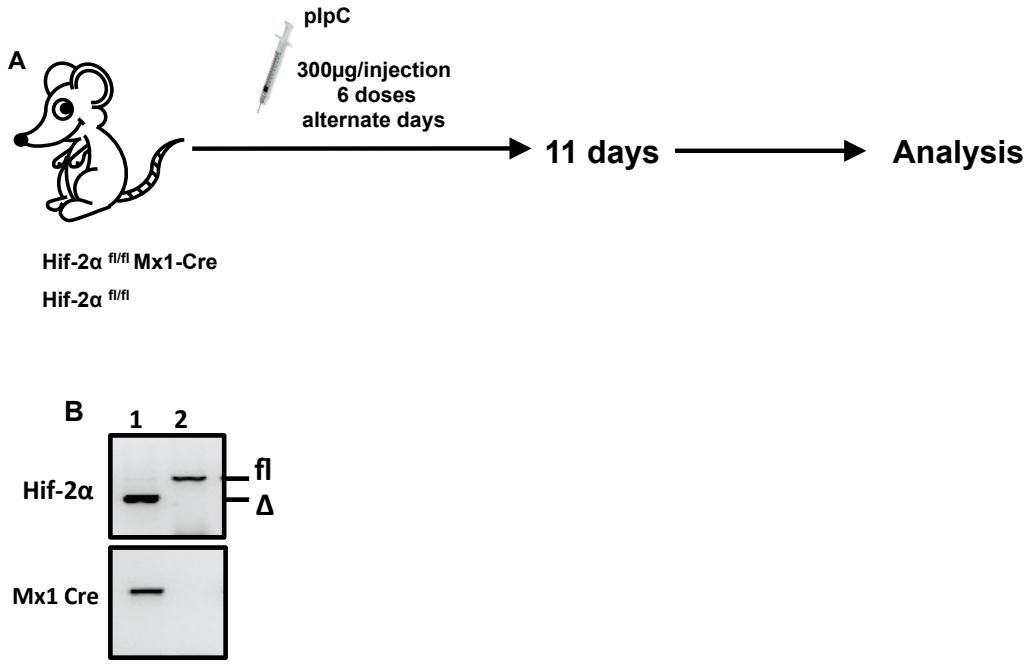



**Figure 4. 1: Conditional deletion of *Hif-2α* induced by pIpC administration**

(A) Experimental Design- Hif-2α$^{\Delta/\Delta}$ and Control mice between 8-12 weeks of age were treated with 6 doses of pIpC on alternate days. The mice were sacrificed at 11 days after the last injection (B) Confirmation of *Hif-2α* deletion in the BM cells of Hif-2α$^{\Delta/\Delta}$ mice by PCR using genomic DNA, 1: Hif-2α$^{\Delta/\Delta}$ and 2-Control, n=5.

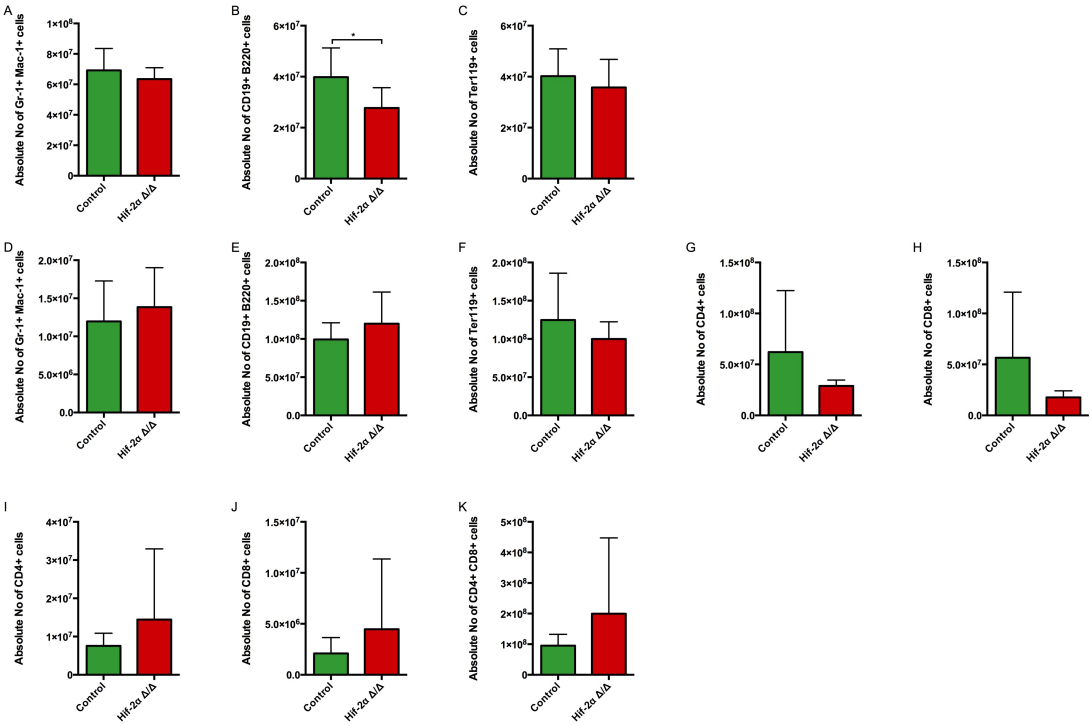



**Figure 4. 2: Analysis of mature blood lineages in Hif-2α$^{\Delta/\Delta}$ males.**

Total number of myeloid cells (Gr-1, Mac-1), erythroid cells (Ter119), B-cells (CD19, B220) and T-cells (CD4, CD8) in (A-C) BM, (D-H) spleen and (I-K) thymus of Hif-2α$^{\Delta/\Delta}$ (n=5) and Control mice (n=12) analysed 11 days after last pIpC injection. The results are presented as average number of cells ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance, $p<0.05$ (*).

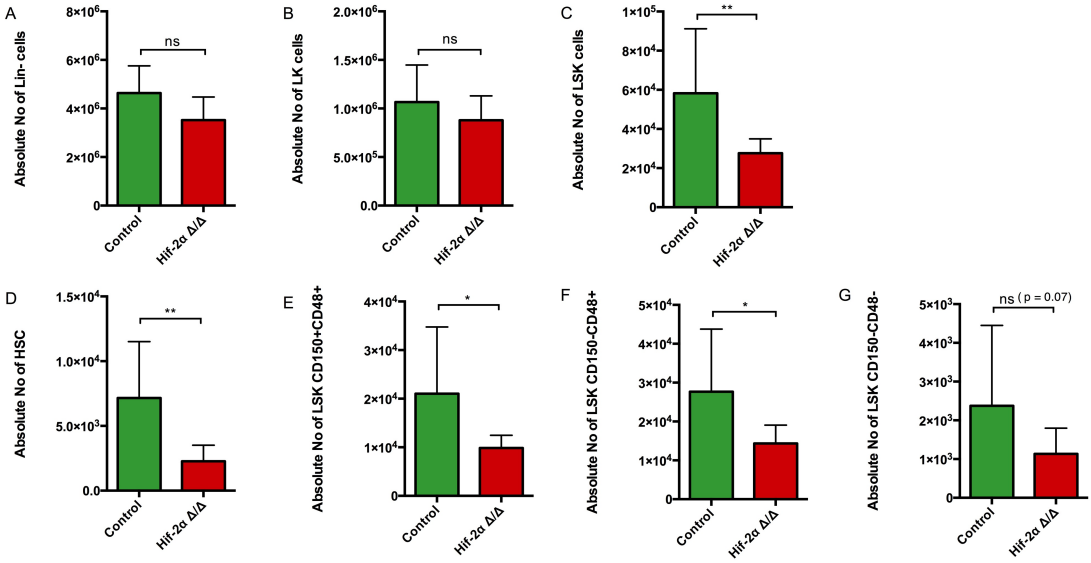



**Figure 4. 3: Conditional deletion of *Hif-2α* results in defective HSCs.**

Absolute number of (A) Lin-, (B) LK, (C) LSK, (D) HSCs, (E-F) Primitive progenitors and (G) ST-HSCs in the BM of Hif-2α$^{\Delta/\Delta}$ (n=5) and Control males (n=12) analysed after 11 days after the last pIpC injection. The results are presented as average number of cells ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance, p value ≤ 0.05 (*), p ≤ 0.005 (**) and p ≤ 0.0005 (***) and p ≥ 0.05 (ns).

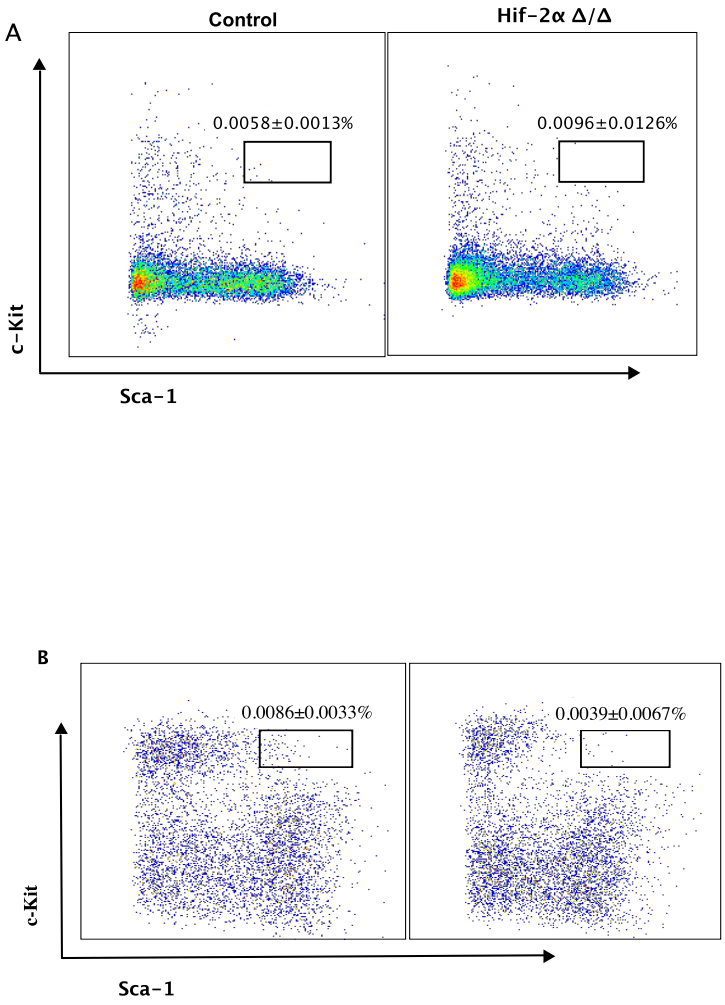



**Figure 4. 4: Analysis of PB and spleen for extramedullary haemopoiesis.**

FACS plots representing the percentage of LSKs in the (A) PB and (B) Spleen of Hif-2αΔ/Δ (n=3) and control (n=4) males. The results are mean± SD.

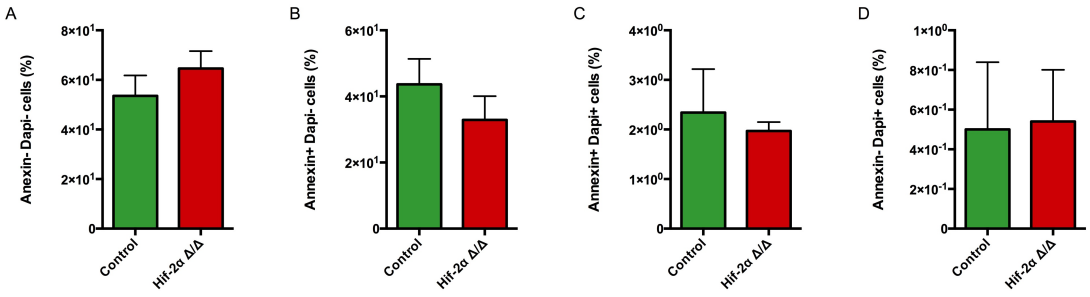



**Figure 4. 5: Analysis of apoptosis in the Hif-2α $^{\Delta/\Delta}$ males.**

Percentage of (A) Annexin$^-$/DAPI$^-$ viable cells, (B) Annexin V$^+$/DAPI$^-$ (early apoptotic cells), (C) Annexin V$^+$/DAPI$^+$ (late apoptotic cells) and (D) AnnexinV$^-$/DAPI$^+$ (dead cells) in the LSK cells from Hif-2α $^{\Delta/\Delta}$ (n=3) and Control (n=3) mice. The results are presented as average number of cells ± SD and statistical analysis was performed by two-tailed student's t test assuming unequal variance.

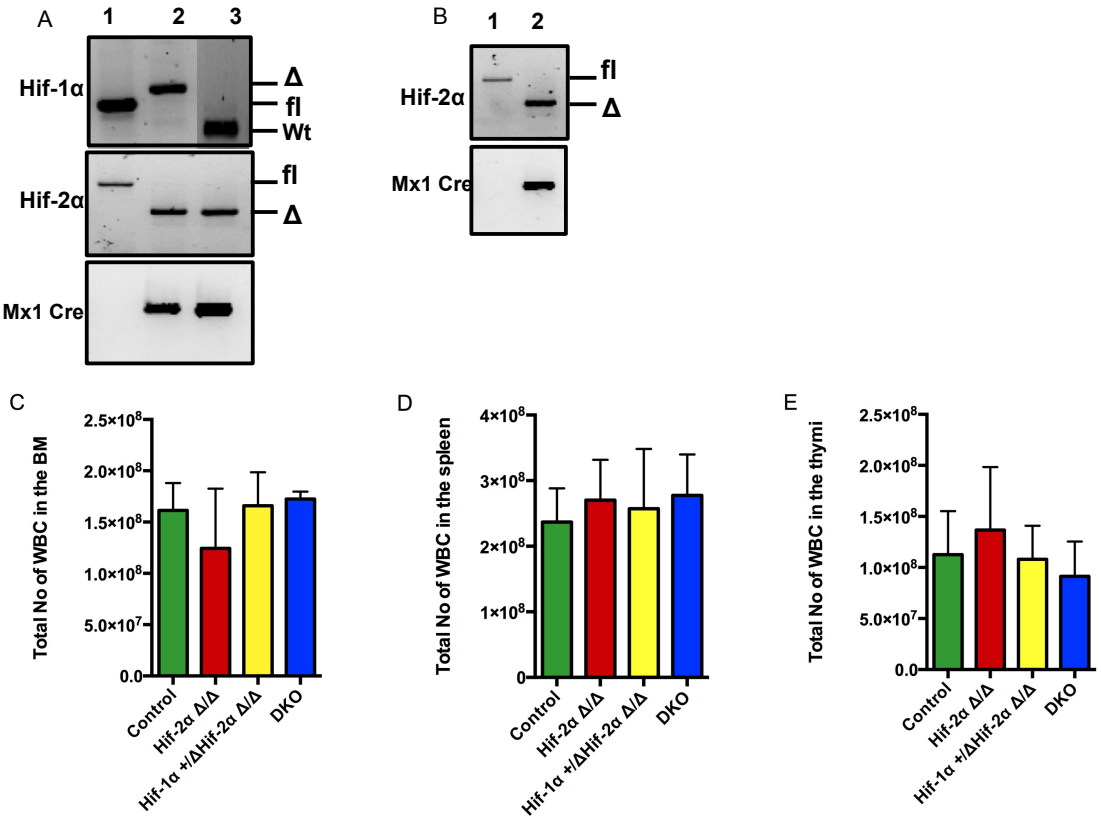



**Figure 4. 6: Conditional deletion of *Hif-1α* and *Hif-2α* induced by pIpC administration.**

Confirmation of *Hif-1α* and *Hif-2α* deletion in the (A) Haemopoetic cells of Hif-2α$^{\Delta/\Delta}$ and DKO mice (1- Control, 2-DKO and 3- Hif-2α$^{\Delta/\Delta}$) and (B) BM microenvironment of Hif-2α$^{\Delta/\Delta}$ mice by PCR using genomic DNA, 1- Control, 2- Hif-2α$^{\Delta/\Delta}$ (C) Total count of WBC in the (C) BM, (D) Spleen and (E) Thymi of Hif-2α$^{\Delta/\Delta}$ (n=5), Hif-1α$^{+/\Delta}$ Hif-2α$^{\Delta/\Delta}$ (n=7), DKO (n=9) and Control males (n=12). The results are presented as average number of cells ± SD.

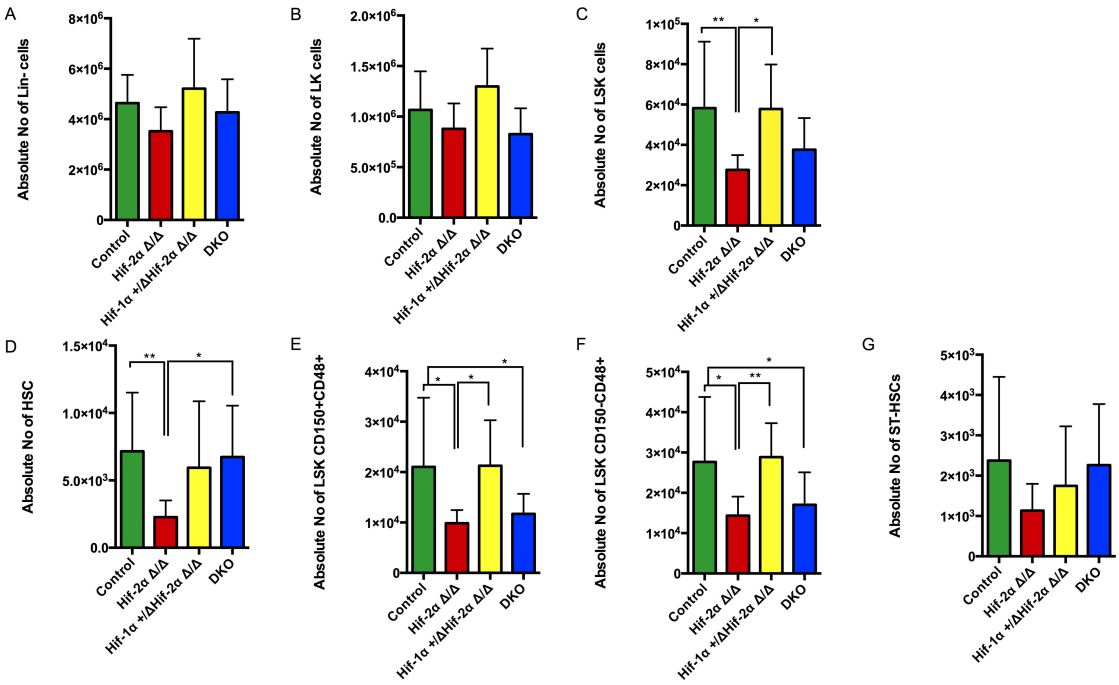



**Figure 4. 7: Immunophenotypic analysis of HSCs and progenitor compartments of Hif-2α$^{\Delta/\Delta}$, Hif-1α$^{+/\Delta}$ Hif-2α$^{\Delta/\Delta}$ and DKO males.**

Absolute number of (A) Lin-, (B) LK, (C) LSK, (D) HSC and (E-F) Primitive progenitors and (G) ST-HSC in the BM of Hif-2α$^{\Delta/\Delta}$ (n=5), Hif-1α$^{+/\Delta}$ Hif-2α$^{\Delta/\Delta}$ (n=7), DKO (n=9) and Control males (n=12) analysed 11 days after the last pIpC injection. The results are presented as average number of cells ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance, p value ≤ 0.05 (*), p ≤ 0.005 (**). The results from Figure 4.3 comprising of Hif-2α$^{\Delta/\Delta}$ and control analysis is being used for the comparison purpose.

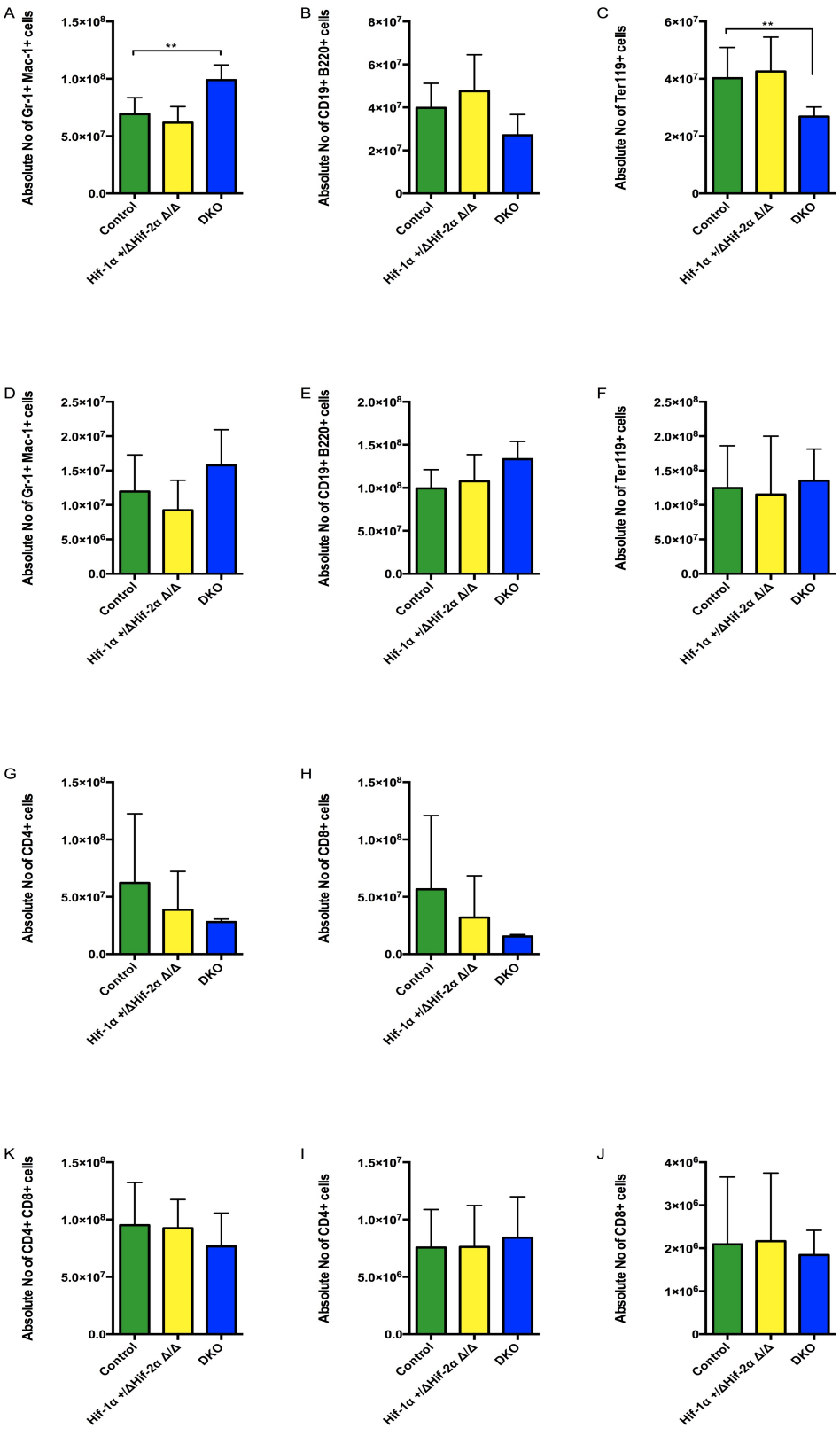



**Figure 4. 8: Analysis of mature blood lineages.**

Total number of myeloid cells (Gr-1, Mac-1), erythroid cells (Ter119), B-cells (CD19, B220) and T-cells (CD4, CD8) in (A-C) BM, (D-H) spleen and (I-K) thymus of Hif-1$\alpha^{+/\Delta}$ Hif-2$\alpha^{\Delta/\Delta}$ (n=7), DKO (n=9) and Control males (n=12). The results are presented as average number of cells ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance, $p<0.005$ (**).

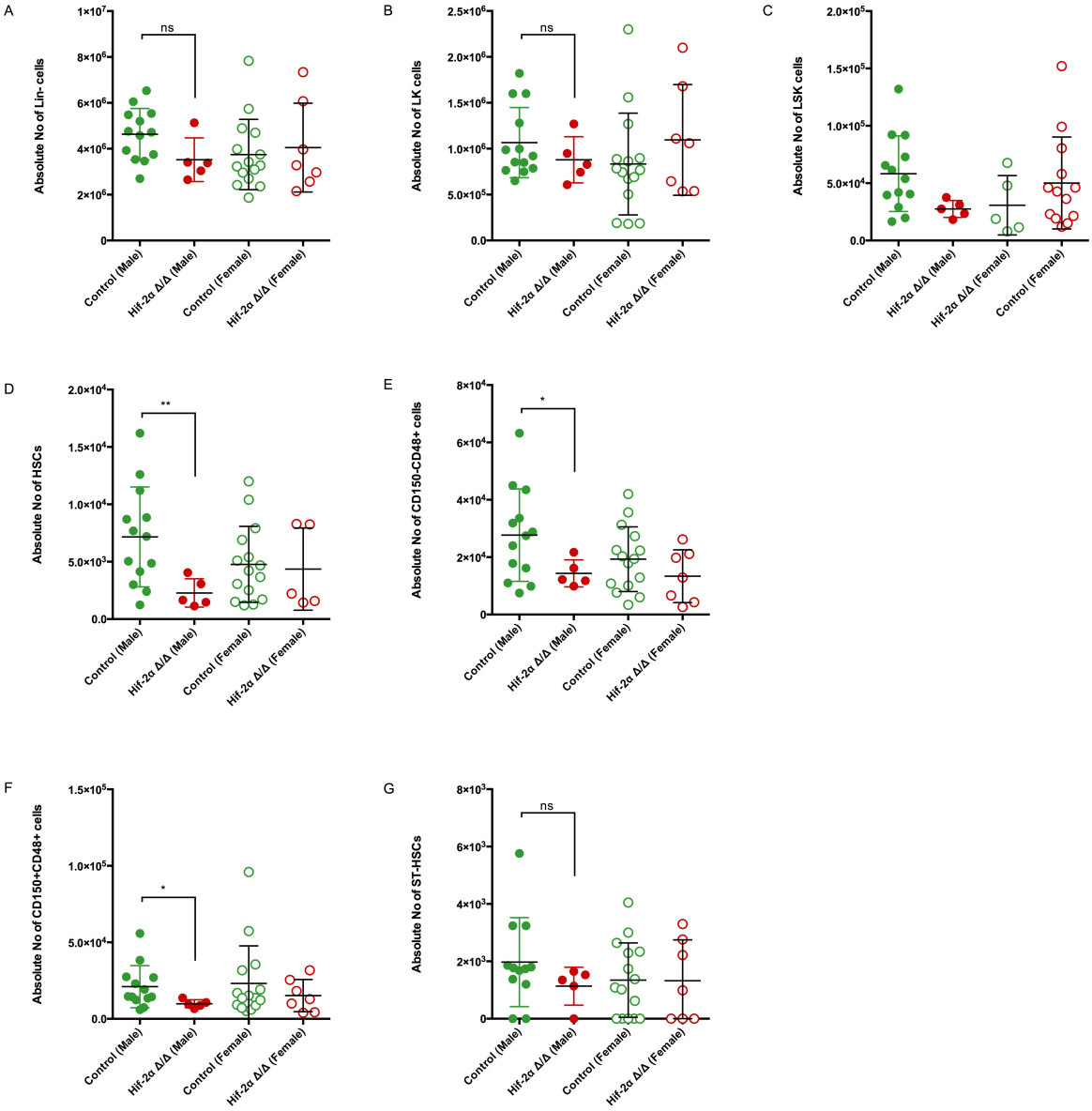



**Figure 4. 9: Gender biased effect of *Hif-2α* on HSC and primitive progenitors.**

Absolute number of (A) Lin-, (B) LK, (C) LSK, (D) HSCs, (E-F) Primitive progenitors and (G) ST-HSCs in the BM of Hif-2α$^{\Delta/\Delta}$ and Control mice analysed after 11 days of last pIpC injection, filled circles- males, empty circles- females. The results are presented as average number of cells ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance, p value ≤ 0.05 (*), p ≤ 0.005 (**) and p ≤ 0.0005 (***) and p ≥ 0.05 (ns). The data from figure 4.3 consisting of analysis of Hif-2α$^{\Delta/\Delta}$ and Control males are used for comparison with the Hif-2α$^{\Delta/\Delta}$ and Control females.

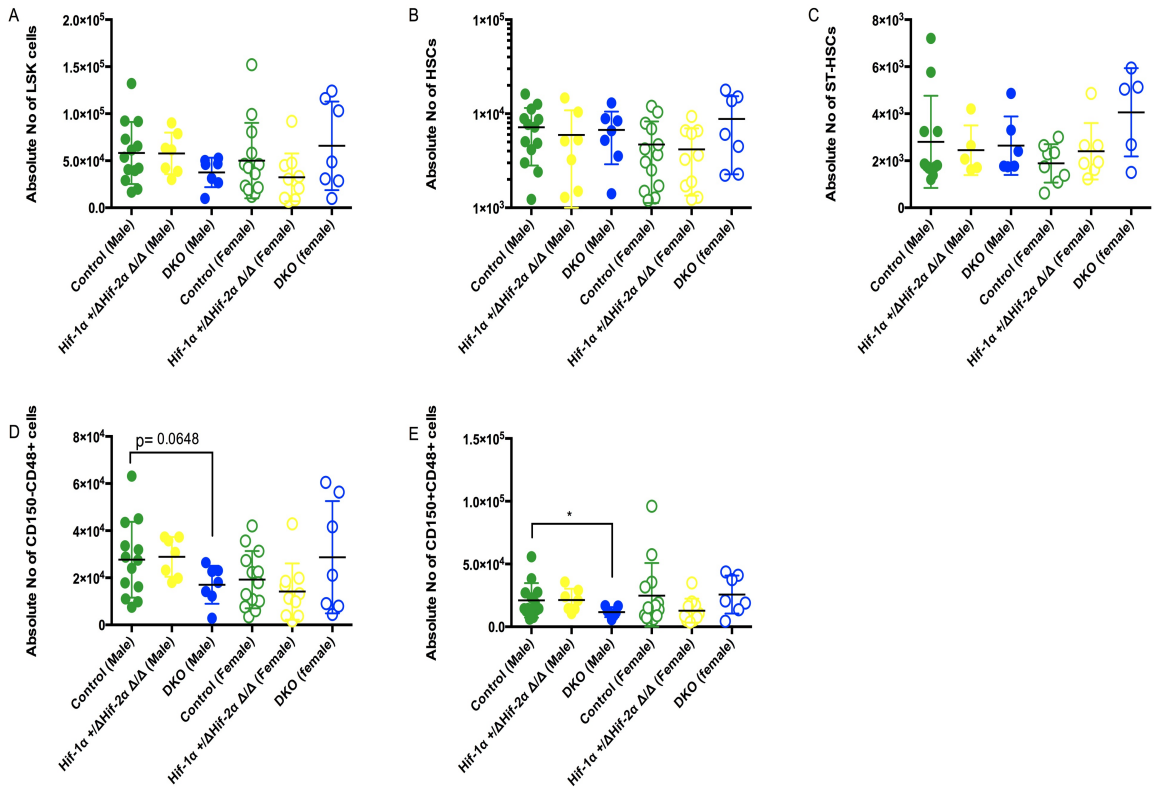



**Figure 4. 10: Gender biased effect of Hif-1α$^{+/\Delta}$ Hif-2α$^{\Delta/\Delta}$ and DKO HSC and primitive progenitors.**

Absolute number of (A) LSK, (B) HSCs, (C) ST-HSCs and (D-E) Primitive progenitors in the BM of Hif-1α$^{+/\Delta}$ Hif-2α$^{\Delta/\Delta}$, DKO and Control mice analysed after 11 days of last pIpC injection, filled circles- males, empty circles- females. The results are presented as average number of cells ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance, p value ≤ 0.05 (*). The data from figure 4.7 consisting of analysis of Hif-2α$^{\Delta/\Delta}$ and Control males are used for comparison with the Hif-2α$^{\Delta/\Delta}$ and Control females.

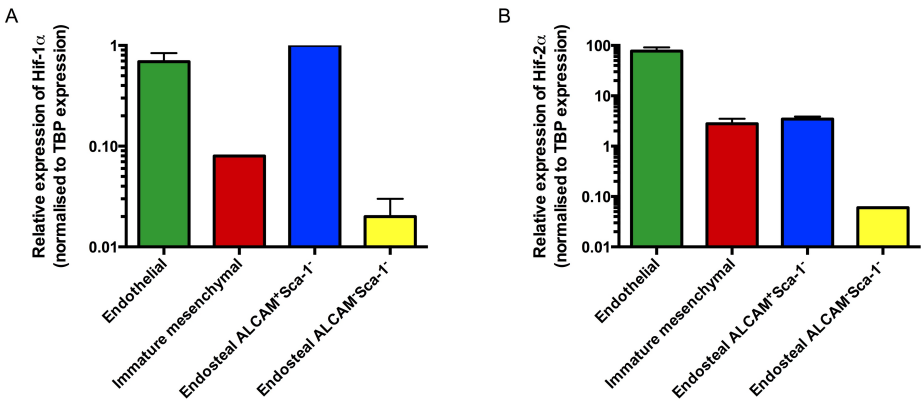



**Figure 4. 11: Relative expression of Hif-1α and Hif-2α in the BM niche cells.**

BM niche cells were sorted based on the immunophenotypic analysis by flow cytometry. Relative expression of (A) *Hif-1α and (B) Hif-2α* in endothelial cells ($CD45^-Ter119^-CD31^+$), immature mesenchymal enriched $ALCAM^-Sca\text{-}1^+$ cells, endosteal cells that are $ALCAM^-Sca\text{-}1^-$ and $ALCAM^+Sca\text{-}1^-$.

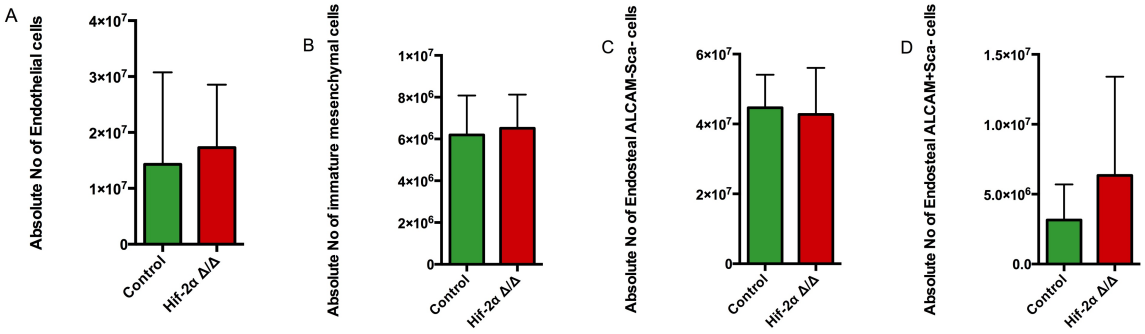



**Figure 4. 12: Immunophenotypic analysis of HSC niche cells lacking Hif-2α.**

Absolute number of (A) Endothelial cells ($CD45^-Ter119^-CD31^+$), (B) Immature mesenchymal enriched $ALCAM^-Sca\text{-}1^+$ cells, Endosteal cells that are (C) $ALCAM^-Sca\text{-}1^-$ and (D) $ALCAM^+Sca\text{-}1^-$ cells in the Hif-$2\alpha^{\Delta/\Delta}$ (n=4) and Control males (n=4). The results are presented as average number of cells ± SD and statistical analysis was performed by two-tailed student's t tests assuming unequal variance.

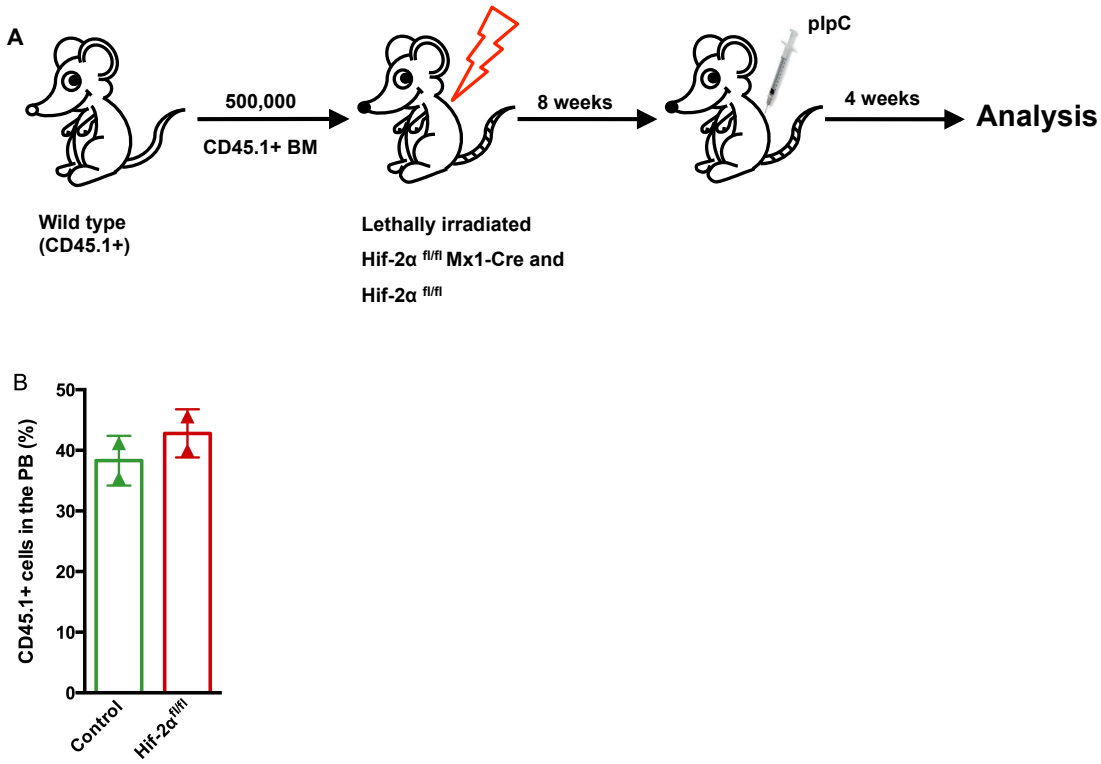



**Figure 4. 13: Analysis of reconstitution potential of BM niche lacking *Hif-2α* expression.**

(A) Experimental design - Lethally irradiated Hif-2α$^{fl/fl}$ Mx1-Cre and Control males transplanted with 500,000 WT CD45.1$^{+}$ BM cells after 8 weeks of transplantation were treated with 5 doses of pIpC and analysed 4 weeks after the last pIpC injection. (B) Percentage of CD45.1$^{+}$ donor-derived cells at 8 weeks post-transplantation from PB of Hif-2α$^{fl/fl}$ Mx1-Cre (n=2) and Control recipients (n=2) (males) transplanted with WT BM cells showing successful engraftment of donor derived cells. WT- Wild type.

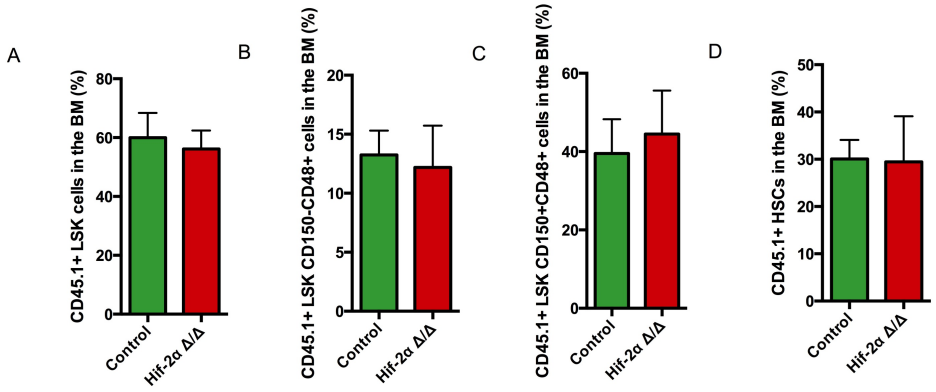



**Figure 4. 14: Immunophenotypic analysis of donor-derived HSCs and progenitor compartments in the lethally irradiated Hif-2α $^{\Delta/\Delta}$ and Control recipients.**

Percentage of donor-derived CD45.1$^{+}$ (A) LSK, (B-C) Primitive progenitors and (D) HSCs in the BM of Hif-2α$^{\Delta/\Delta}$ (n=2) and Control males (n=2) analysed after 4 weeks of last pIpC injection. The results are presented as mean ± SD.

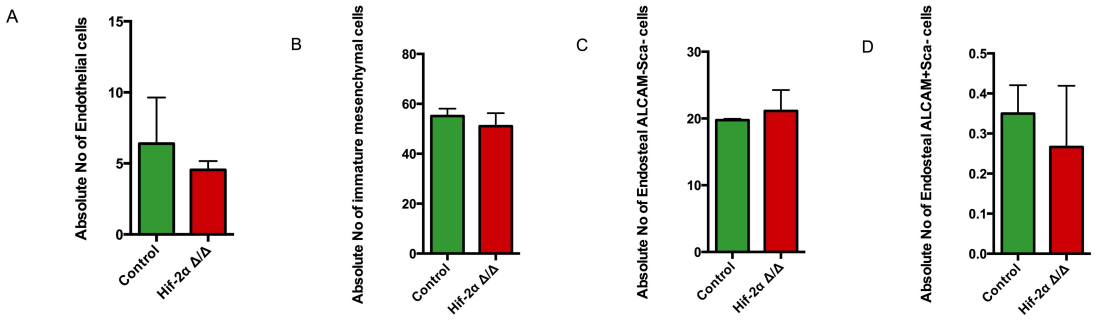



**Figure 4. 15: Analysis of BM niche cells of Hif-2α $^{\Delta/\Delta}$ and Control primary recipients**

Percentage of (A) Endothelial cells (CD45$^{-}$Ter119$^{-}$CD31$^{+}$), (B) Immature mesenchymal enriched ALCAM$^{-}$Sca-1$^{+}$ cells, Endosteal cells that are (C) ALCAM$^{-}$Sca-1$^{-}$ and (D) ALCAM$^{+}$Sca-1$^{-}$ cells in the Hif-2α$^{\Delta/\Delta}$ (n=2) and Control male (n=2) recipients analysed 4 weeks after last pIpC injection. The results are presented as mean ± SD.

# Chapter 5

# The role of *Cited2* in the generation of leukaemia

# 5.1 Introduction

## 5.1.1 *Cited2* is a hypoxia inducible transcriptional regulator

*Cited2* is a CBP/p300 interacting transactivator with glutamic acid and aspartic acid (ED)-rich tail 2 (*Cited2*). It is ubiquitously expressed nuclear protein and functions as a transcriptional co-activator of several DNA binding TFs including AP2, HNF4$\alpha$, PPAR $\alpha/\Upsilon$, and Smad2/Smad3 (Bamforth et al. 2001; Tien et al. 2004; Chou and Yang 2006; Qu et al. 2007). *Cited2* is induced by hypoxia. *Hif-1*$\alpha$ and *Hif-2*$\alpha$ control the transcriptional induction of *Cited2* by binding to HRE elements in the *Cited2* promoter region (Bhattacharya et al. 1999). *Cited2* also functions as a repressor of *Hif-1*$\alpha$ by competing for binding of CBP/p300 (Bhattacharya et al. 1999; Freedman et al. 2003; van den Beucken et al. 2007). Cited2 acts as negative regulator of Hif-1$\alpha$-mediated signalling by high affinity binding to first Cystine-histidine-rich (CH1) domain of p300 restricting the recruitment of CBP/p300 (Bhattacharya et al. 1999; Freedman et al. 2003; van den Beucken et al. 2007). Cited2 null mice died prenatally because of defective neural tube and cardiovascular abnormalities, in part due to abnormal transactivation by *Tfap2* (Bamforth et al. 2001). Another publication reported abnormal transcriptional activity of *Hif-1*$\alpha$ in Cited2 null embryos (Yin et al. 2002). Mouse embryonic studies showed increased expression of *Hif-1* target genes, such as *Vegf, Glut-1* and *PGK-1*, in E14.5 Cited2$^{-/-}$ embryonic hearts and in MEFs under hypoxic conditions (Yin et al. 2002). However, unpublished results from Prof. Kranc's lab have shown that *Cited2* deletion in MEFs did not affect the expression of *Vegf*, *Glut-1* or *PGK-1* (Unpublished data from the Kranc laboratory). The myocardial defects including abnormal cardiac vasculature, outflow tract, inter-ventricular septum and hyposplenia in embryos lacking *Cited2* were partially rescued by heterozygous *Hif-1*$\alpha$ expression (Xu et al. 2007). *Cited2* was shown to inhibit induction of Hif-1$\alpha$-mediated apoptosis, regulated by *Foxo3a*-stimulated *Cited2* expression in fibroblasts and breast cancer cells under hypoxic conditions (Bakker et al. 2007). *Foxo3a*-induced expression of *Cited2* during hypoxia led to reduced expression of pro-apoptotic *Hif-1*$\alpha$ target genes, such as *NIX* and *RTP801*,

establishing *Cited2* as a negative modulator of *Hif-1α* transcriptional activity (Bakker et al. 2007) (Figure 5.1).

### 5.1.2 *Cited2* and its role in cancer

The role of *Cited2* in cancer is unknown. A recent study showed an increase in *Cited2* expression in leukaemic cells from MLL-AF9 induced AML mouse model (Sykes et al. 2011). Experimental evidence from previous studies showed that *Cited2* was highly expressed in lung cancer patients and in Engelbreth–Holm–Swarm (EHS) tumours (Futaki et al. 2003; Chou et al. 2006; Chou et al. 2012). An elevated expression of *Cited2* was also reported in the F9 embryonic carcinoma (F9-PE) that produced abnormal amounts of extracellular matrix like collagen IV and laminins causing tumour formation (Futaki et al. 2003; Chou et al. 2006; Chou et al. 2012). In Rat1 cells, overexpression of *Cited2* resulted in tumour formation in nude mice, thereby suggesting the oncogenic potential of *Cited2* (Sun et al. 1998). In lung cancer patients, elevated expression of *Cited2* is associated with poor prognosis (Chou et al. 2012). Overexpression of *Cited2* enhanced growth of lung cancer xenografts and knockdown of *Cited2* lead to tumour shrinkage and increased survival of nude mice (Chou et al. 2012). A study by Lau et al. reported an elevated expression of *Cited2* in primary human breast tumours (Lau et al. 2010). However, the role of *Cited2* in breast cancer is not clear (Chou et al. 2006). One study showed that knockdown of *Cited2 i*n breast cancer cell lines reduced the TGF-ß-mediated tumour invasion and increased cell death induced by hypoxia by modulating the expression of MMP-9 via TGF-ß (Chou and Yang 2006). Additionally, a recent publication indicated that *Cited2* acts as a transcriptional co-activator of the oestrogen-receptor (ER) in breast cancer cell lines, thus resulting in oestrogen-independent ER activation (Lau et al. 2013). In contrast, another study showed that reduced expression of *Cited2* mRNA in breast cancer patients (van Agthoven et al. 2009). However, in human colon cancer cells, *Cited2* regulated the tumour invasion and knockdown of *Cited2* increased invasiveness of colon cancer cells *in vitro* (Bai and Merchant 2007). These findings together suggest the importance and variable role of *Cited2* in tumourigenesis. Depending on the cell type and cancer, *Cited2* appears to

act as a tumour suppressor or as an oncogene. The role of *Cited2* in leukaemogenesis and LSC functions remains to be investigated.

### 5.1.3 *Cited2* is critical for normal haemopoiesis in foetal liver and adult BM

*Cited2* is essential for murine foetal liver development (Qu et al. 2007) and normal foetal liver haemopoiesis (Chen et al. 2007; Qu et al. 2007; Du and Yang 2013). *Cited2* is expressed at high levels in HSCs, LSKs and in myeloid progenitors (LK cells) in the foetal liver (Chen et al. 2007). Chen et al reported that *Cited2* null mouse embryos had reduced numbers of LSK and LK cells with compromised reconstitution ability in lethally irradiated recipients. The number of erythroid and myeloid cells in *Cited2* null foetal liver was greatly decreased and an *in vitro* CFC assay showed reduced colony forming potential of foetal liver cells from Cited2$^{-/-}$ embryos (Chen et al. 2007). Cited2 null progenitor cells showed decreased expression of self-renewal genes such as *Bmi1* and Wnt signalling molecules like LEF-1 and Notch-1 (Chen et al. 2007). Also, Cited2$^{-/-}$ cells exhibited reduced levels of *Gata2* that play an important role in HSC/progenitor cell maintenance (Rodrigues et al. 2005; Rodrigues et al. 2008) and primitive erythrocytes formation thus establishing the role of *Cited2* in the molecular regulation of haemopoiesis during embryogenesis (Chen et al. 2007).

*Cited2* is essential for adult haemopoiesis (Kranc et al. 2009; Du et al. 2012). Acute deletion of *Cited2* in adult mice resulted in the loss of HSCs and fatal BM failure leading to haemopoietic specific lethality (Kranc et al. 2009). The loss of HSC in mice lacking *Cited2* was, in part, due to elevated levels of *p19Arf* and *p53*, leading to increased apoptosis (Kranc et al. 2009). Additional deletion of *Ink4a/Arf* or *Trp53* along with *Cited2* restored HSC functions and repopulation potential of Cited2 knockout mice, indicating the importance of *Cited2* in HSC maintenance, at least partially via the *p19Arf-p53* pathway (Kranc et al. 2009). Haemopoiesis-specific deletion of *Cited2* resulted in defective HSC functions including reduced quiescence and additional deletion of *Hif-1*$\alpha$ partially rescued HSC quiescence and reconstitution capacity (Du et al. 2012). In contrast to these findings, unpublished results from Prof. Kranc's lab showed that deletion of *Hif-1*$\alpha$ in

HSCs lacking *Cited2* did not rescue HSC functions (Guitart et al, unpublished data). Therefore, the role of *Cited2* and its downstream targets in regulating HSC functions needs further investigation. *Cited2* functions as a regulator of primitive haemopoietic cells is conserved in humans as supported by *in vitro* knockdown studies of *CITED2* in human $CD34^+$ cells (Kranc et al. 2009). *Cited2* is also essential for the maintenance of MEFs (Kranc et al. 2003). Deletion of *Cited2* led to the increased expression of the cell-cycle inhibitors *p16Ink4a* and *p19Arf*, causing premature cellular senescence. Deletion of *Ink4a/Arf* or *p53* in MEFs lacking *Cited2* restored their proliferation (Kranc et al. 2003). Kranc et al showed that *Cited2* regulated proliferation of fibroblasts and expression of *Ink4a/Arf* in part via *Bmi1* and *Mel18* (Kranc et al. 2003). Therefore, Cited2 is crucial for physiological and pathological conditions, including tumour development.

### 5.1.4 *Mcl-1*, an anti-apoptotic gene and its role in normal and malignant haemopoiesis

*Mcl-1* (Myeloid cell leukaemia-1) is an anti-apoptotic gene and a member of the BCL-2 family. *Mcl-1* expression is induced by growth factor and cytokine signalling, such as by IL-7 in lymphoid progenitors (Opferman et al. 2003), *Scf* and IL-5 in murine HSPCs and human BM cell lines (Huang et al. 2000; Opferman et al. 2005). *Mcl-1* expression is critical for embryonic development and deletion of *Mcl-1* resulted in defective pre-implantation development and implantation leading to embryonic lethality (Rinkenberger et al. 2000). In the haemopoietic system, *Mcl-1* plays crucial role in maintenance of HSC functions (Opferman et al. 2005; Campbell et al. 2010b). *Mcl-1* is highly expressed in mouse and human HSCs compared to primitive progenitors (Opferman et al. 2005; Campbell et al. 2010a). Mice with induced deletion of *Mcl-1* were severely anaemic, had BM failure and were moribund (Opferman et al. 2005). They had dramatic loss of HSCs and progenitor cells, which failed to engraft lethally irradiated host mice (Opferman et al. 2005). *Mcl-1* was shown to be required for the survival of HSPCs *in vitro* (Opferman et al. 2005). Analysis of expression of BCL-2 members in de novo isolated and *in vivo* reconstituted human haemopoietic cells revealed specifically high expression of *MCL-1* in primitive HSCs (Campbell et al. 2010a). Mice with conditional deletion of *Mcl-1* in

neutrophils and macrophages showed survival defects and enhanced apoptotic granulocytes without affecting macrophage viability (Dzhagalov et al. 2007).

Overexpression of *Mcl-1* is associated with increased transformation potential (Zhou et al. 1998; Dzhagalov et al. 2008; Campbell et al. 2010a; Glaser et al. 2012). *In vivo* pharmacological targeting of BCL-2 family members, including *MCL-1* resulted in decreased self-renewal and regenerative potential of human HSCs and LSCs (Campbell et al. 2010a). Human *MCL-1* transgene expression in transgenic mice resulted in splenic enlargement, increased CFC colony formation from BM cells and enhanced survival of mature B and T cells as well as myeloid cells (Zhou et al. 1998; Dzhagalov et al. 2008). Under *in vitro* culture conditions, overexpression of *MCL-1* transgene increased the immortalisation of myeloid cells (Zhou et al. 1998). Among the Bcl-2 family members, specific deletion of *Mcl-1* resulted in apoptosis of transformed murine AML cells both *in vitro* and *in vivo*, via a Bim-dependent apoptotic pathway (Glaser et al. 2012). Functional inactivation of *Mcl-1* by lentiviral expression of $Bim_S$-derived BH3 variant ligand, $Bim_S$2A, that selectively neutralises *MCL-1*, showed that blockade of *MCL-1* significantly decreased the viability of primary human AML and human AML-derived cell lines (Glaser et al. 2012).

### 5.1.5 AML mouse model – Retroviral transformation and transplantation

The RTTA mouse model is widely employed to understand the molecular mechanisms governing AML (Lavau et al. 1997; Kroon et al. 1998; Cozzio et al. 2003; Lessard and Sauvageau 2003; Yan et al. 2006; Somervaille et al. 2009; Yan et al. 2009; Wang et al. 2010; Gibbs et al. 2012; Kamezaki et al. 2013). Ectopic expression of oncogenes using the retroviral expression vector, MSCV to infect HSPCs results in rapid development of AML (Zeisig and So 2009). Isolated BM cells are enriched for HSPCs by positive selection for c-Kit expression (Zeisig and So 2009). These cells are transduced with retrovirus expressing oncogenic fusions, such as AML1-ETO9a, MLL-ENL or Meis1 and Hoxa9 and cultured in cytokine (IL-3, IL-6 and *Scf*) supplemented media to support proliferation and survival of primitive cells (Zeisig and So

2009). RTTA is explained in detail in the section 2.2.3, briefly this involves serial re-plating of HSPCs, transduced with an oncogenic fusion and selected for antibiotic resistance into methylcellulose (supplemented with SCF and cytokines supporting myeloid differentiation) to generate pre-LSCs that are capable of generating LSCs and transplantable AML once transplanted into lethally irradiated recipients (Zeisig and So 2009). Non-transformed cells exhaust during initial plating in methylcellulose (CFC-1, Colony forming cell-1), while transformed cells successfully re-plate with colonies exhibiting transformed morphology (Zeisig and So 2009). At CFC-3, cells are capable of giving rise to transplantable AML when transplanted and are termed pre-LSCs, that eventually acquire additional mutations thus forming LSCs (Zeisig and So 2009).

The AML1-ETO9a fusion transcript is reported in AML patients with the t(8; 21) translocation (Yan et al. 2006; Yan et al. 2009; Kamezaki et al. 2013). Previous study on murine model showed that transplantation of AML1-ETO9a transduced foetal liver cells into lethally irradiated recipients resulted in anaemia with high WBC count, elevated primitive progenitor cells and defective differentiation (Yan et al. 2006). Further, the study reported that mice transplanted with AML1-ETO9a pre-LSCs developed leukaemia with in 16 weeks of transplantation with enlarged spleens and livers, pale femurs and elevated blast cells in PB (Yan et al. 2006). However, lymphnodes or thymi were not affected (Yan et al. 2006). Flow cytometric analysis showed lack of mature lineage markers with elevated LSKs resembling human AML without maturation (Yan et al. 2006).

MLL-ENL fusion protein results from the chromosomal translocation t(11; 19) (Lavau et al. 1997). Retroviral transformation of HSPCs with MLL-ENL resulted in morphologically compact transformed colonies at CFC-2 and CFC-3 that exhibited enhanced self-renewal and proliferative potential (Lavau et al. 1997; Cozzio et al. 2003). Mice transplanted with MLL-ENL transduced BM cells developed AML similar to M4 subtype (Acute myelomonocytic leukaemia) in human AML, with a latency of 73-118 days, with mice exhibiting increased WBC counts, infiltration of immature myeloid cells in the spleen liver, kidney and PB (Lavau et al. 1997; Cozzio et al. 2003; Somervaille et al. 2009). Flow cytometric analysis revealed that LSCs

expressed immature markers, including CD43 and c-Kit, as well as myeloid markers such as Mac-1 and Gr-1 (Lavau et al. 1997; Cozzio et al. 2003; Somervaille et al. 2009).

Meis1 and Hoxa9 when co-expressed in HSPCs and transplanted into mice resulted in the rapid onset of leukaemia with a latency of 49-70 days (Kroon et al. 1998). Clinical signs included infiltration into other organs, including spleen, lymphnodes, BM and thymus and elevated WBCs in the PB and BM (Kroon et al. 1998). The leukaemic cells expressed the immature c-Kit cell surface marker and myeloid markers, Gr-1 and Mac-1 (Kroon et al. 1998; Lessard and Sauvageau 2003; Wang et al. 2010; Gibbs et al. 2012). These murine models have given insight into the molecular factors and signalling pathways governing AML development (Lessard and Sauvageau 2003; Wang et al. 2010).

## 5.2 Outline of experiments described in Chapter 5

*Cited2* has been shown to be critical for the maintenance of HSC functions (Kranc et al. 2009) however, the oncogenic potential of *Cited2* in leukaemogenesis and in the maintenance of LSC functions remains to be investigated. The aim of this Chapter was to study the role of *Cited2* in AML development. I generated CKO mice: Cited2$^{fl/fl}$ Vav-iCre with haemopoietic specific *Cited2* deletion and Cited2$^{fl/fl}$ Mx1-Cre with pIpC induced *Cited2* deletion and respective Controls. To investigate the transformation ability of HSPCs lacking *Cited2* expression, I employed *in vitro* RTTA with ectopic expression of fusion genes MLL-ENL, AML1-ETO9a or co-expression of *Meis1* and *Hoxa9*. I have shown that HSPCs lacking *Cited2* displayed reduced transformation potential and failure to generate transplantable AML in lethally irradiated syngeneic recipients. Additionally, overexpression of *Mcl-1* in Cited2$^{\Delta/\Delta}$ cells bypassed their defective transformation potential. Hence, the data in this Chapter provide evidence that *Cited2* is essential for leukaemic transformation at least in part via *Mcl-1* regulation.

# 5.3 Results

## 5.3.1 Investigating the transformation potential of HSPCs lacking *Cited2*

To investigate the potential of HSPCs lacking *Cited2* to generate transformed colonies, I employed RTTA. I generated mice lacking *Cited2* specifically in the haemopoietic cells using the Vav-iCre system that efficiently deletes the floxed gene during early haemopoietic development in the embryos (de Boer et al. 2003b; Mortensen et al. 2011; Du et al. 2012). BM cells from the Cited2$^{fl/fl}$ Vav-iCre mice (referred as Cited2$^{\Delta/\Delta}$) and Cited2$^{fl/fl}$ mice without Vav-iCre (referred as Control) were isolated and enriched for HSPCs by positive selection for the expression of c-Kit (CD117) using microbeads (Figure 5.2). The HSPCs were transduced with retroviruses carrying the fusion genes (MLL-ENL, AML1-ETO9a) or co-expressing Meis1 and Hoxa9 and Control vector (MSCV neo) by a centrifugation process called spinoculation (detail description in section 2.5.1). During spinoculation, primary cells were cultured in cytokines (SCF-20ug/ml, IL-3-10ug/ml and IL-6-10ug/ml) supplemented culture media (IMDM, 10%FBS, Penicilin-100U/ml, Streptomycin-100ug/ml) that promotes cell division. The cells transduced with fusion genes were positively selected for puromycin and/or neomycin resistant gene cassette and plated into methycellulose medium supplemented with specific cytokines (GM-CSF, SCF, IL-3, IL-6) and puromycin or neomycin. The number and morphology of transformed colonies generated were analysed after 6-7 days and then the colonies were serially re-plated to examine transformation potential *in vitro* (Zeisig and So 2009).

HSPCs from Cited2$^{fl/fl}$ without Vav-iCre (Control) mice were transduced with empty vector (MSCV neo) along with MLL-ENL or Meis1-Hoxa9. HSPCs transduced with MSCV neo formed less colonies compared to the number of colonies formed by MLL-ENL or Meis1-Hoxa9 transduced HSPCs (Figure 5.3A). The colonies generated by HSPCs transduced by MSCV neo lacked the transformation morphology and failed to generate colonies during serial re-plating, whereas MLL-ENL and Meis1-Hoxa9 transduced cells generated compact, immature transformed colonies and retained the continued ability

of serial re-plating (Figure 5.3B). These results showed that the HSPCs upon retroviral transduction of oncogenic fusions generated transformed colonies and retained the potential for serial re-plating *in vitro*.

Cited2$^{\Delta/\Delta}$ HSPCs transduced with retrovirus expressing MLL-ENL yielded a smaller number of neomycin resistant colonies compared to Control at first plating into methycellulose (CFC-1) (Figure 5.4A). The colonies formed by MLL-ENL transduced cells lacking *Cited2* appeared morphologically non-transformed and failed to generate colonies upon re-plating (Figure 5.4D). MLL-ENL transduced Control cells successfully generated compact transformed CFC-blast colonies and maintained the ability to generate colonies through serial re-plating (Figure 5.4A and 5.4D). Number of compact transformed colonies generated by Cited2$^{\Delta/\Delta}$ HSPCs transformed by either Meis1-Hoxa9 or AML1-ETO9a oncogenic fusions were reduced to a great extent compared to Control (Figure 5.4B and 5.4C). Meis1-Hoxa9 or AML1-ETO9a transduced Cited2$^{\Delta/\Delta}$ cells gave rise to less compact colonies (Figure 5.4D). The colonies with AML1-ETO9a, lacking *Cited2*, eventually expired at CFC-3, while transformed Control cells continued forming colonies during serial re-plating (Figure 5.4C). *In vitro* RTTA using three different oncogenic fusions shows the lack of transformation potential of cells lacking *Cited2* compared to its Control. Collectively, the results indicate that *Cited2* is essential for transformation of cells *in vitro* and formation of pre-LSCs.

### 5.3.2 Immunophenotypic characterisation of transformed colonies

Meis1-Hoxa9 induced leukaemia is categorised as M2 (i.e. AML with maturation) under the FAB classification (Kroon et al. 1998). The leukaemic cells are characterised by high expression of c-Kit and co-expression of Gr-1 and/or CD11b cell surface markers (Kroon et al. 1998; Lessard and Sauvageau 2003; Wang et al. 2010). To examine the immunophenotypic characteristics of pre-LSCs generated by *in vitro* RTTA, Meis1-Hoxa9 transformed HSPCs forming puromycin- and neomycin-resistant colonies were harvested at each CFC assay and analysed for pre-LSC markers (c-Kit, CD11b and Gr-1)(Kroon et al. 1998; Lessard and Sauvageau 2003; Wang et al.

2010) using flow cytometry. Meis1-Hoxa9 transduced HSPCs from colonies lacking *Cited2* and Control were stained for expression of primitive cell marker c-Kit and mature myeloid markers CD11b and Gr-1. HSPCs from both Cited2$^{\Delta/\Delta}$ and Control colonies expressed c-Kit and were positive for both myeloid markers CD11b and Gr-1 (Figure 5.5A and 5.5B). The percentage of c-Kit$^{+}$ cells isolated from transformed colonies lacking *Cited2* was similar to the Control (Figure 5.5A). There was no difference in the percentage of c-Kit$^{+}$/Gr-1$^{+}$/CD11b$^{+}$ cells derived from colonies lacking *Cited2* and colonies with WT levels of *Cited2* expression (Figure 5.5B). Though a decrease in percentage of c-Kit$^{+}$Gr-1$^{+}$CD11b$^{+}$ cells in the Meis1-Hoxa9 transformed Cited2$^{\Delta/\Delta}$ cells compared to Control cells was observed, it was not statistically significant. These results show that there was no significant difference in the immunophenotypic characteristics of pre-LSCs lacking *Cited2* compared to Control. *Cited2*-deficient cells transformed with Meis1-Hoxa9 resulted in immunophenotypically similar, but reduced numbers of transformed colonies with diverse self-renewal potential compared to Control. This might possibly be due to defects in apoptosis or in self-renewal properties of HSPCs lacking *Cited2*.

### 5.3.3 Investigating the transformation potential of cells lacking *Cited2* by complementation of *Cited2*

In the previous section (5.3.1), I have shown that transduction of HSPCs lacking *Cited2* with co-expression of Meis1 and Hoxa9 leads to decreased numbers of colonies and eventually exhaustion of colony forming potential. To further investigate if the reduced ability of leukaemic transformation *in vitro* is due to *Cited2* deletion, I co-transduced HSPCs from Cited2$^{\Delta/\Delta}$ mice and Cited2$^{fl/fl}$ Control mice (without Vav-iCre) with Meis1-Hoxa9 and lentivirus carrying *Cited2* or empty vector (Figure 5.6A). This experiment also ruled out the possibility of loss of HSPCs in mice lacking *Cited2* and hence no target cells available for leukaemic transformation. Puromycin and neomycin resistant Meis1-Hoxa9 transformed cells were further sorted for GFP$^{+}$ (Cited2 overexpression or empty vector) and serially re-plated to assess the *in vitro* transformation potential. Cells lacking *Cited2* and Control co-transduced with empty vector and Meis1-Hoxa9 generated transformed colonies during the first three re-platings (Figure 5.6B). Eventually, the

number of colonies formed by transformed Cited2$^{\Delta/\Delta}$ cells reduced compared to Control (Figure 5.6B). However, cells with WT level of *Cited2* expression co-transduced with empty vector and Meis1-Hoxa9 continued to generate compact transformed colonies through serial re-plating (Figure 5.6B). Cited2$^{\Delta/\Delta}$ and Control HSPCs co-transduced with Meis1-Hoxa9 and *Cited2* transgene successfully generated compact and transformed CFC-blast colonies (Figure 5.6B). Ectopic expression of *Cited2* in Cited2$^{\Delta/\Delta}$ HSPCs yielded compact CFC-blast colonies and was able to continuously transform cells *in vitro* in serial re-plating assay compared to colonies generated by transduced HSPCs lacking *Cited2* that appeared non-transformed (Figure 5.6C). These results together imply that *Cited2*-deficient HSPCs failed to generate transformed colonies and ectopic expression of *Cited2* in Cited2$^{\Delta/\Delta}$ HSPCs generated transformed colonies and retained the potential to self-renew *in vitro*. Therefore, *Cited2* expression is critical for transformation of HSPCs and to generate pre-LSCs.

### 5.3.4 Lack of transformation potential of *Cited2* deficient cells to generate AML *in vivo*

To dissect the role of *Cited2* in the maintenance of AML *in vivo*, I employed a genetic knockout approach by using inducible Cre. I generated Cited2$^{fl/fl}$ Mx1-Cre mice where inducible acute deletion of *Cited2* is achieved by pIpC treatment. Upon pIpC treatment, Mx1 promoter is activated resulting in Cre-mediated deletion of *Cited2*. Following *Cited2* deletion, *Cited2* promoter controls the expression of LacZ in pIpC treated Cited2$^{fl/fl}$ Mx1-Cre cells demonstrating efficient gene deletion (Kranc et al. 2009). To generate pre-LSCs with the potential to develop disease resembling human AML in the mouse model, HSPCs isolated from untreated Cited2$^{fl/fl}$ Mx1-Cre mice and Cited2$^{fl/fl}$ without Mx1-Cre (Control) were transduced with Meis1-Hoxa9 using the spinoculation protocol described in section 2.2.3. Transduced cells were positively selected for puromycin and neomycin resistance and plated onto methylcellulose to produce transformed colonies. The cells harvested from transformed colonies were serially re-plated until CFC-3 to generate pre-LSCs (Figure 5.7A).

Meis1-Hoxa9 transformed cells from Cited2$^{fl/fl}$ Mx1-Cre and Cited2$^{fl/fl}$ colonies that expressed c-Kit and myeloid markers Gr-1 and Mac-1 that are termed as pre-LSCs were assessed for their leukaemogenic potential. At CFC-3, cells were isolated and sorted for expression of c-Kit, Gr-1 and CD11b using multi-colour flow cytometry. 200,000 sorted cells from untreated Cited2$^{fl/fl}$ Mx1-Cre and Control colonies along with 500,000 support-BM cells were transplanted into lethally irradiated syngeneic B6.SJL (CD45.1$^{+}$) recipients (Figure 5.7A). 9 out of 16 mice transplanted with pre-LSCs derived from Meis1-Hoxa9 transduced Control HSPCs developed AML with a latency period of 6-10 months, while none of the mice transplanted with pre-LSCs derived from untreated Cited2$^{fl/fl}$ Mx1-Cre mice developed leukaemia (Figure 5.7B). Mice transplanted with pre-LSCs from Meis1-Hoxa9 transduced Control cells showed appearance of leukaemic cells expressing c-Kit, Gr-1 and CD11b in the PB as characterised by immunopheneotypic analysis. Mice developing leukaemia were sacrificed when they appeared moribund and analysed for AML phenotype. Mice with AML displayed organ infiltration in the spleen and immunophenotypic analysis of the blood, BM and spleen from leukaemic mice showed abundance of leukaemic cells characterised by expression of c-Kit$^{+}$, Gr-1$^{+}$ and CD11b$^{+}$ (Figure 5.7D-F). However, there was no trace of mobilised leukaemic cells in the PB of mice transplanted with pre-LSCs from untreated *Cited2*$^{fl/fl}$ Mx1-Cre mice (Figure 5.7F). Pre-LSCs from CFC-3 of untreated Cited2$^{fl/fl}$ Mx1-Cre mice had partial deletion of *Cited2* shown by LacZ staining (Figure 5.7C). This partial deletion of *Cited2* might be induced by the cytokines and stressful culture conditions and was sufficient to modulate the disease development potential of pre-LSCs. Additional deletion could have been triggered *in vivo* by irradiation that activates the interferon type I/II signalling (Leong 1996; Burnette et al. 2011). These results show that HSPCs transformed with retrovirus containing Meis1 and Hoxa9 induced transplantable AML *in vivo* and partial deletion of *Cited2* in these cells reduced their ability to generate disease upon transplantation.

### 5.3.5 *Mcl-1* enhances the ability of *Cited2*-deficient cells to undergo leukaemic transformation

Next, I wished to identify the molecular mechanism through which *Cited2* functions during leukaemic transformation. *Cited2* deletion in progenitor cells resulted in significant 4.2 fold decreased expression of *Mcl-1* suggesting defective survival of HSCs in turn led to their loss in *Cited2*-deficient foetal liver (Chen et al. 2007). Also, unpublished work from Prof. Kranc's lab found *Mcl-1* down-regulated in Cited2$^{\Delta/\Delta}$ cells (Dr. Guitart, unpublished data and Prof. Kranc personal communication). Notably, deletion of *Mcl-1*, similar to the deletion of *Cited2*, results in the inability of haemopoietic progenitors to undergo leukaemic transformation (Glaser et al. 2012). Therefore, I tested the hypothesis that *Mcl-1* over-expression can bypass defective transformation of Cited2$^{\Delta/\Delta}$ c-Kit$^{+}$ cells. To investigate the requirement for *Cited2* in the development, establishment and maintenance of LSCs, I employed a mouse model of AML. In this model, HSPCs are transduced with retroviruses expressing oncogenic fusions, undergo serial re-plating and generate pre-LSCs, which upon transplantation give rise to LSCs causing leukaemia (Figure 5.8A). To this end, I crossed Cited2$^{fl/fl}$ Vav-iCre mice to *Mcl-1* transgenic mice in which the over expression of *Mcl-1* is driven by haemopoiesis-specific Vav promoter (in collaboration with Prof. Suzanne Cory, WEHI) (Campbell et al. 2010b). These crosses gave rise to Cited2$^{fl/fl}$ Vav-iCre mice, Cited2$^{fl/fl}$ Vav-iCre Mcl-1$^{+}$ and Control mice. BM c-Kit$^{+}$ cells from these mice transduced with oncogenic fusions MLL-ENL, AML-ETO9a or co-expressing Meis1 and Hoxa9 were subjected to the serial re-plating transformation assay as described in Figure 5.8A. While Control cells gave rise to transformed colonies efficiently as expected, Cited2$^{\Delta/\Delta}$ cells failed to generate transformed colonies (Figure 5.8B). However, Cited2$^{\Delta/\Delta}$ cells over-expressing *Mcl-1* transgene could be serially re-plated and generated colonies with a transformed phenotype (Figure 5.8B). Cited2$^{\Delta/\Delta}$ colonies over expressing *Mcl-1* were morphologically indistinguishable from Control transformed colonies (Figure 5.8C). Additionally, ectopic expression of *Mcl-1* in Cited2$^{\Delta/\Delta}$ cells led to enhanced transformation potential when co-transduced with Meis1 and Hoxa9 (Figure 5.9A) and the cells formed morphologically transformed compact colonies similar to Control HSPCs

(Figure 5.9B). Therefore, these results demonstrated that over expression of *Mcl-1* bypassed the defective transformation potential of Cited2$^{\Delta/\Delta}$ HSPCs.

# 5.4 Discussion

## 5.4.1 *Cited2* is essential for the transformation of HSPCs *in vitro* and AML generation *in vivo*

*Cited2* is induced by various stimuli including cytokines, growth factors and serum (Sun et al. 1998). *Cited2* has a critical role in regulating embryogenesis and adult haemopoiesis (Bamforth et al. 2001; Bamforth et al. 2004; Chen et al. 2007; Qu et al. 2007; Kranc et al. 2009; Du et al. 2012; Du and Yang 2013). Impaired expression of *Cited2* (increased/decreased expression based on the type of cancer) is associated with various solid tumours (Sun et al. 1998; Futaki et al. 2003; Chou et al. 2006; Bai and Merchant 2007; Chou et al. 2012). However, the function of *Cited2* in cancer formation and leukaemogenesis is unclear.

Alongside *Cited2* being critical for regulating functions of adult HSCs (self-renewal, apoptosis and quiescence) (Kranc et al. 2009; Du et al. 2012), a recent study showed an increase in *Cited2* expression in leukaemic cells from MLL-AF9 induced AML mouse model (Sykes et al. 2011). Also, elevated levels of *CITED2* are observed in CD34$^{+}$ human AML samples compared to normal CD34$^{+}$ cells (Prof. K. Kranc, personal communication). Taken together these preliminary results suggest a potential role of *Cited2* in AML. I investigated if expression of *Cited2* is essential in the generation and maintenance of AML LSCs both *in vitro* and *in vivo*, using a murine model generated by RTTA. Transformation of HSPCs from Control mice with oncogenic fusions, MLL-ENL, AML1-ETO9a or co-expression of Meis1-Hoxa9 successfully generated compact transformed colonies *in vitro* compared to Control HSPCs transduced with MSCV-neo (Control vector). This validated the transformation potential of HSPCs retrovirally transduced by oncogenic fusions (MLL-ENL, Meis1-Hoxa9, AML1-ETO9a) in agreement with previous publications (Cozzio et al. 2003; Lessard and Sauvageau 2003; So et al. 2003; Somervaille and Cleary 2006; Gibbs et al. 2012). HSPCs lacking *Cited2* displayed defective transformation potential *in vitro*. *Cited2*-deficient

HSPCs transduced with oncogenic fusions generated dramatically reduced numbers of colonies which appeared morphologically non-transformed (not dense) compared to transformed HSPCs from Control mice (which looked very compact), irrespective of the oncogenes. However, the severity of the transformation defects was dependent on the oncogenic fusions. Meis1-Hoxa9-transduced cells lacking *Cited2* formed colonies that retained the ability to generate transformed colonies during initial re-plating, but eventually expired after CFC-5. Conversely, MLL-ENL transduced HSPCs lacking *Cited2* had a striking phenotype, i.e. they formed few colonies at CFC-1 and failed to generate colonies when re-plated, exhibiting impaired self-renewal ability assessed by serial re-plating assay compared to its Control. Immunophenotypic analyses of both *Cited2* deficient and Control HSPCs transformed by Meis1-Hoxa9 showed an equal percentage of pre-LSCs phenotype with expression of c-Kit, Gr-1 and CD11b. These results confirmed that presence of the target cells for oncogenic transformation in mice lacking Cited2. Further, the Cited2 deficiency resulted in their defective transformation ability.

Acute deletion of *Cited2* results in severe loss of HSCs and primitive progenitors causing fatal BM failure (Kranc et al. 2009). To rule out the possibility that Cited2$^{\Delta/\Delta}$ HSPCs were apoptotic due to lack of Cited2 and hence failed to survive during transformation resulting in reduced colony number, I ectopically expressed *Cited2* along with Meis1 and Hoxa9 in the Cited2$^{\Delta/\Delta}$ HSPCs. Additional expression of *Cited2* in Meis1-Hoxa9 transduced Cited2$^{\Delta/\Delta}$ HSPCs regained the ability to transform and successfully generate CFC-blast like colonies in serial re-plating. This showed that transformation defects in the Cited2$^{\Delta/\Delta}$ cells were specific for the *Cited2* deletion and could be rescued by *Cited2* complementation. Previous publications showed that *Cited2* deletion resulted in survival defects of HSPCs (Kranc et al. 2009; Du et al. 2012). Therefore, *Cited2* overexpression studies and the immunophenotypic analysis of Cited2-deficient colonies from section 5.3.3 demonstrated that the target cells (HSPCs) for the oncogeneic transformation were available in Cited2-deficient mice (from the Cited2 Vav-iCre) and also showed that these cells were able to regain the transformation potential in the presence of *Cited2*. Additionally, primitive

haemopoietic cells lacking *Cited2* showed decreased *Mcl-1* expression (Preliminary data from Prof. Kranc's lab). The reduced expression of *Mcl-1* in Cited2$^{\Delta/\Delta}$ cells might therefore explain the lack of their transformation ability.

Furthermore, to investigate the requirement of *Cited2* in the generation of AML *in vivo* I conducted transplantation experiments. In a period of 6 months, more than 50% of the recipients transplanted with Meis1-Hoxa9 transduced pre-LSCs from Control mice developed AML. The spleen infiltration, elevated PB counts and leukaemic blast cells in PB, BM and spleen that were immunophenotypically stained for c-Kit, Gr-1 and Mac-1 confirmed AML development. However, none of the recipients of pre-LSCs from Meis1-Hoxa9 transduced with untreated Cited2$^{fl/fl}$ Mx1-Cre mice (inducible CKO mouse) developed AML in the time frame of 6 months. Retrospectively, the analysis of gene deletion in pre-LSCs from CFC-3 showed partial deletion (30%-40%) of *Cited2* as analysed by LacZ expression that was sufficient to prevent the generation of AML *in vivo*. The partial deletion of *Cited2* in untreated Cited2$^{fl/fl}$ Mx1-Cre pre-LSCs, when transplanted into the lethally irradiated mice, could possibly have further Cre activation triggering additional deletion due to stimulation of type I/II interferon signalling due to irradiation or superficial infection of mice (Leong 1996; Onoguchi et al. 2007; Burnette et al. 2011).

Studies show that *Foxo3a* is essential for the maintenance of self-renewal properties of HSCs and also inhibits Hif1-induced apoptosis by stimulating *Cited2* expression in fibroblasts and breast cancer cell lines (Bakker et al. 2007; Miyamoto et al. 2007). It is known that *Cited2* functions as a negative feedback loop controlling the *Hif-1*$\alpha$ activity and that deletion of *Hif-1*$\alpha$ on a background of *Cited2* deletion partially rescued the defective HSC functions (Bhattacharya et al. 1999; Xu et al. 2007; Du et al. 2012). Together, these previous studies and the results presented in this Chapter suggest the possibility that deletion of *Cited2* may have resulted in accumulation of *Hif-1*$\alpha$ levels, in turn leading to increased *Hif-1*$\alpha$-induced apoptosis. This revealed that the possible block of AML generation in *Cited2* deficient pre-LSCs is due to the *Cited2*-mediated inhibitory effects of

Foxo3a on *Hif-1*$\alpha$-induced apoptosis. It is noteworthy that *Cited2* deletion in HSCs affect other TFs including cyclin-dependent kinase inhibitor p57, Erg1, Stat5 and Myc that play a critical role in the maintenance of HSC quiescence and survival properties (Wang et al. 2009; Du et al. 2012). Further studies need to be carried out investigating the mechanistic pathway mediated by *Cited2* during AML transformation.

### 5.4.2 Transformation defect in HSPCs lacking *Cited2* is rescued by overexpression of *Mcl-1*

*Cited2* deletion results in the loss of HSCs and defective multilineage haemopoietic potential (Kranc et al. 2009; Du et al. 2012). This HSC defect is attributed at least in part to the increased p53 activity (Kranc et al. 2009). HSPCs lacking *Cited2* failed to generate transformed colonies *in vitro* and did not induce AML in the *in vivo* murine models. *Mcl-1*, a BCL-2 family member is an anti-apoptotic gene essential for the survival of HSCs and AML LSCs (Opferman et al. 2005; Campbell et al. 2010a; Glaser et al. 2012). HSPCs overexpressing *Mcl-1* in the background of *Cited2* deficiency were investigated for their transformation potential. Ectopic expression of *Mcl-1* in Cited2$^{\Delta/\Delta}$ HSPCs co-transduced with Meis1 and Hoxa9 regained the transformation potential compared to Cited2$^{\Delta/\Delta}$ cells. Meis1 and Hoxa9 transduced HSPCs from Control mice generated similar numbers of colonies compared HSPCs overexpressing *Mcl-1*, therefore *Mcl-1* overexpression did not seem to have an advantage over Control HSPCs. RTTA experiments showed that MLL-ENL, AML1-ETO9a or Meis1-Hoxa9 transduced HSPCs from Cited2$^{\Delta/\Delta}$ Mcl-1$^{+}$ mice generated increased numbers of morphologically transformed CFC-blast like colonies compared to colonies generated by Cited2$^{\Delta/\Delta}$ cells and restored the self-renewal potential as assessed by serial re-plating.

*Cited2* and *Mcl-1* have similar expression profiles, both are highly expressed in HSCs and their expression is decreased in more differentiated progenitor cells (Opferman et al. 2005; Kranc et al. 2009). Inducible deletion of *Cited2* or *Mcl-1* in the CKO mouse model resulted in similar phenotypes, including fatal BM failure, loss of haemopoietic progenitor population and failure of engraftment in lethally irradiated recipients (Opferman et al. 2005; Kranc et

al. 2009). I have shown in the previous section that deletion of *Cited2* results in inability of transformation of HSPCs transduced with oncogenic fusions. Similarly, deletion of *Mcl-1* resulted in increased death rate in HSPCs transformed using oncogenic fusions in culture and knockdown of *Mcl-1* cleared AML cells resulting in prolonged survival of the mice (Glaser et al. 2012). Previous studies (Opferman et al. 2005; Kranc et al. 2009; Glaser et al. 2012) taken together with the results described in this Chapter indicate that *Cited2* is essential for leukaemic transformation of HSPCs at least in part by regulating the expression of *Mcl-1*. However, at this point we cannot rule out the possibility that apoptotic HSPCs in Cited2$^{\Delta/\Delta}$ mice display defective leukaemic transformation potential that is bypassed by overexpression of anti-apoptotic gene *Mcl-1*, independent of *Cited2*. Gene analyses studies will give more insight into molecular mechanisms of Cited2-Mcl-1 pathway.

## 5.5 Conclusion

In this Chapter, I have shown that *Cited2* plays a critical role in the transformation ability of HSPCs. Deletion of *Cited2* results in reduced transformation potential of HSPCs and failure to generate transplantable AML in lethally irradiated syngeneic recipients. Additionally, my results show that *Mcl-1* overexpressing cells are able to bypass the transformation defects in Cited2$^{\Delta/\Delta}$ cells. This supports the idea that *Cited2* is essential for leukaemic transformation at least in part by regulating *Mcl-1*.

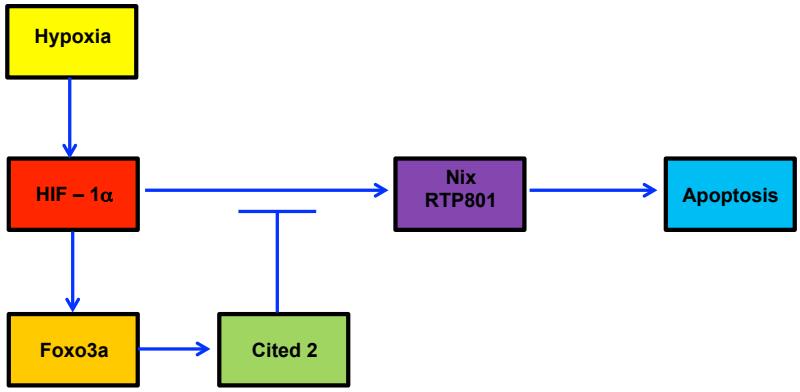



**Figure 5. 1: Schematic representation of Hif-mediated apoptotic pathway regulated by hypoxia and *Foxo3a***

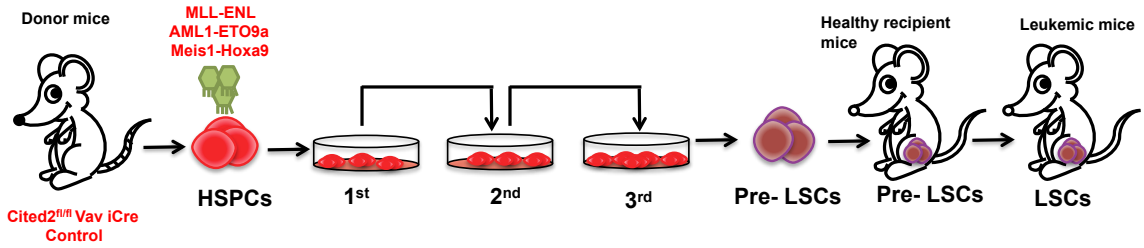



**Figure 5. 2: Schematic representation of RTTA.**

BM cells from the Cited2$^{fl/fl}$ Vav-iCre and control mice are enriched for haemopoietic stem and progenitor cells (HSPCs), transduced with retrovirus expressing fusion genes AML1-ETO9a, MLL-ENL or co-expression of Meis1 and Hoxa9 are serially re-plated to form pre-LSCs. At CFC-3, pre-LSCs are injected in to lethally irradiated mice that generate LSCs and transplantable AML.

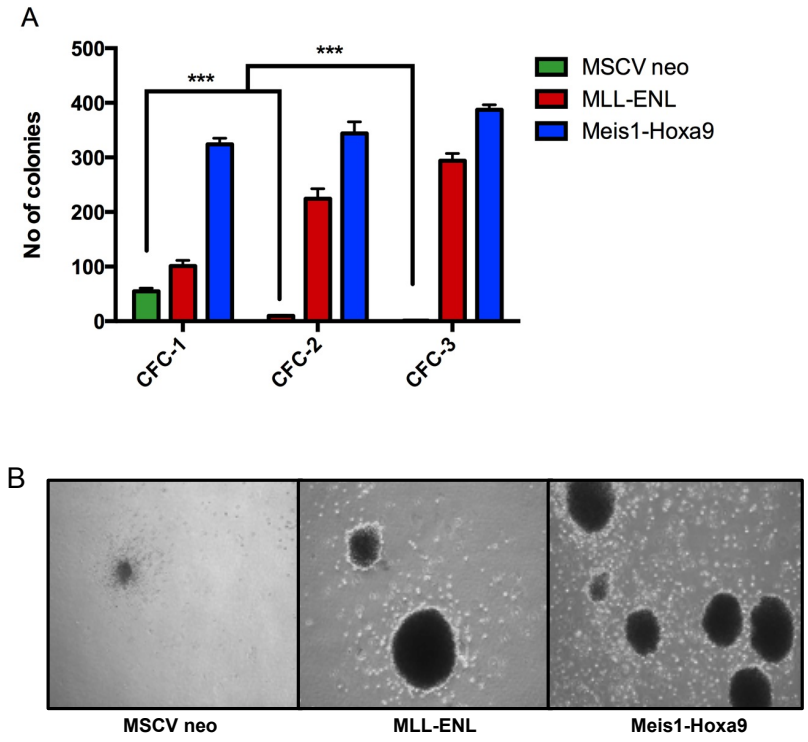



**Figure 5. 3: Retroviral transformation of HSPCs from wild type (WT) mice.**

(A) Number of colonies formed during serial re-plating of WT HSPCs transformed with MSCV neo, MLL-ENL and Meis1-Hoxa9. Results are represented as mean ± SD, n=3 and statistical analysis was performed by two-tailed student's t tests assuming unequal variance, p value ≤ 0.0005 (***). (B) Representative images of colonies formed by WT HSPCs transformed with MSCV neo, MLL-ENL and Meis1-Hoxa9 at CFC-3.

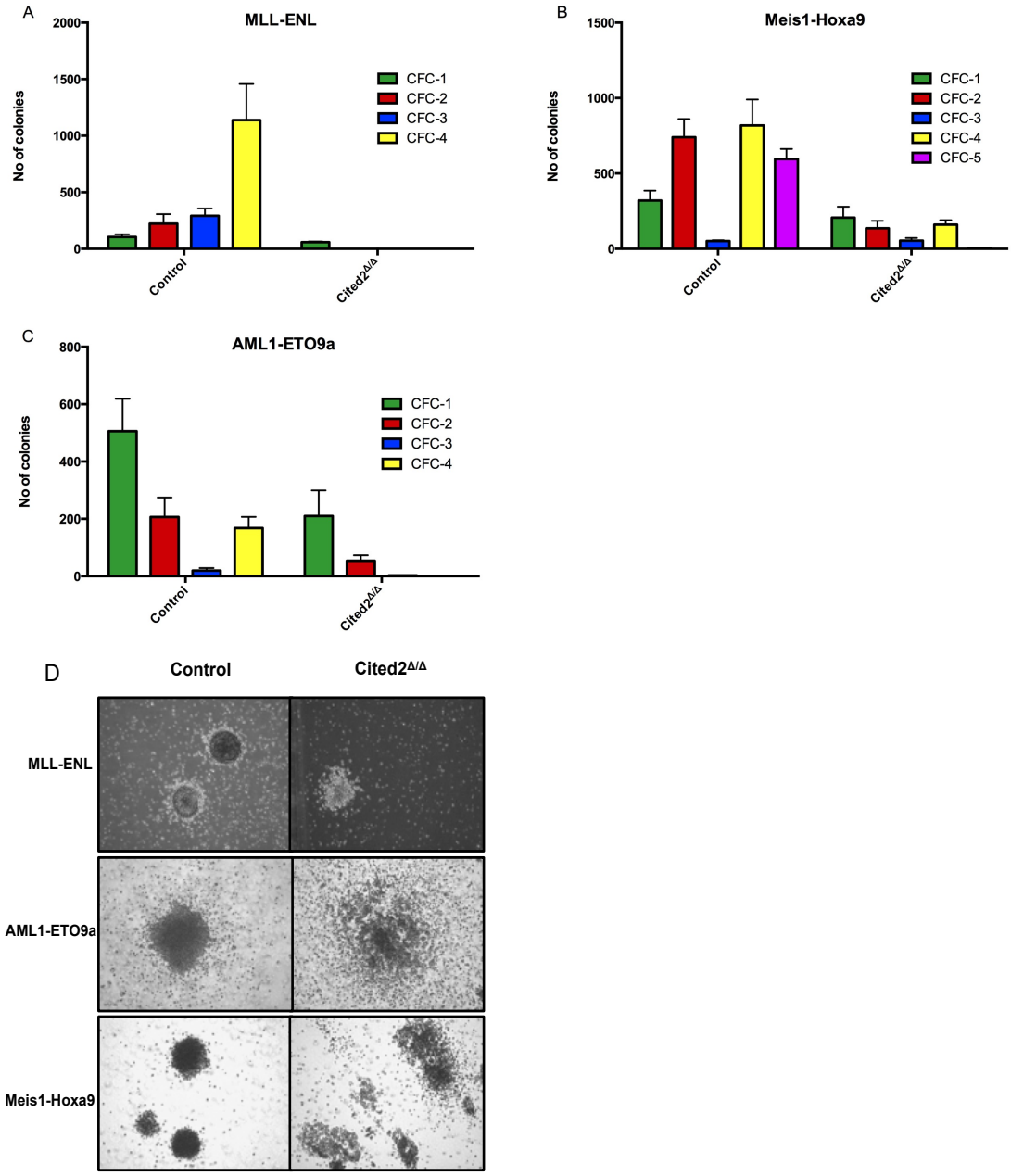



**Figure 5. 4: Retroviral transformation of HSPCs from Cited2$^{\Delta/\Delta}$ and Control mice.**

Number of colonies formed during serial re-plating of Cited2$^{\Delta/\Delta}$ and Control HSPCs transformed with (A) MLL-ENL (B) Meis1-Hoxa9 and (C) AML1-ETO9a. Results are represented as mean ± SD, n=3 and statistical analysis was performed by two-tailed student's t tests assuming unequal variance, p value ≤ 0.005 (**) and p ≤ 0.0005 (***). (B) Representative images of colonies formed by Cited2$^{\Delta/\Delta}$ and Control HSPCs transformed with MLL-ENL at CFC-1, AML1-ETO9a and Meis1-Hoxa9 at CFC-3.

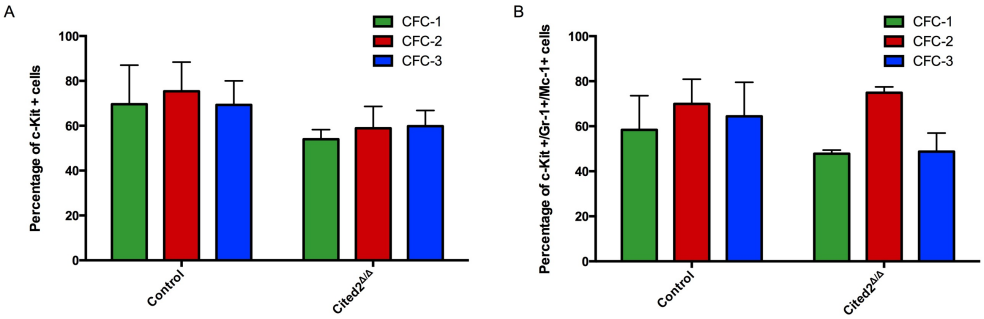



**Figure 5. 5: Immunophenotypic characterisation of Meis1 and Hoxa9 transformed colonies.**

Percentage of (A) c-Kit$^{+}$ cells and (B) c-Kit$^{+}$, Gr-1$^{+}$ and Mac-1$^{+}$ cells from Cited2$^{\Delta/\Delta}$ and Control HSPCs transformed with Meis1-Hoxa9. Results are represented as mean ± SD, n=3.

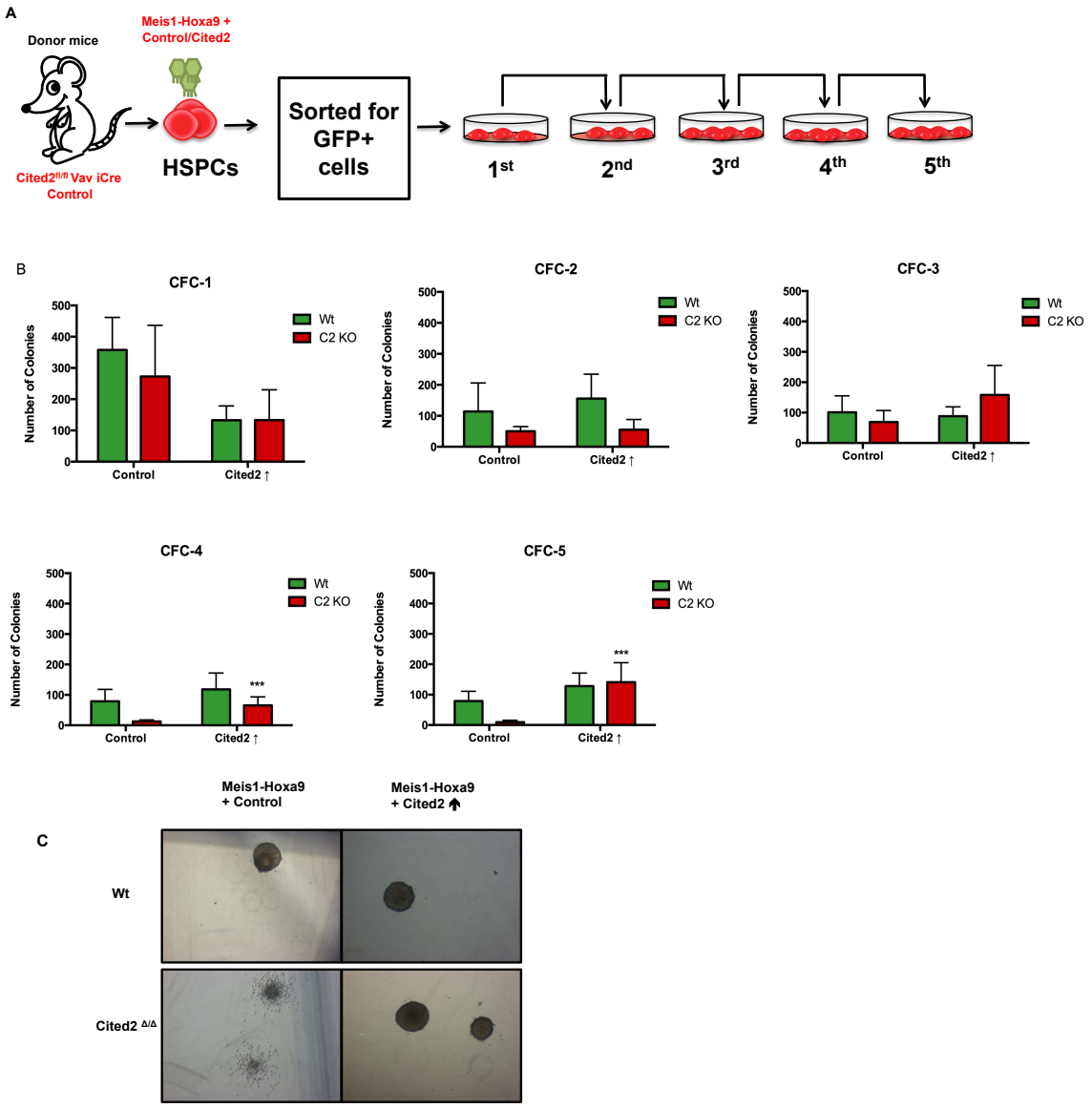
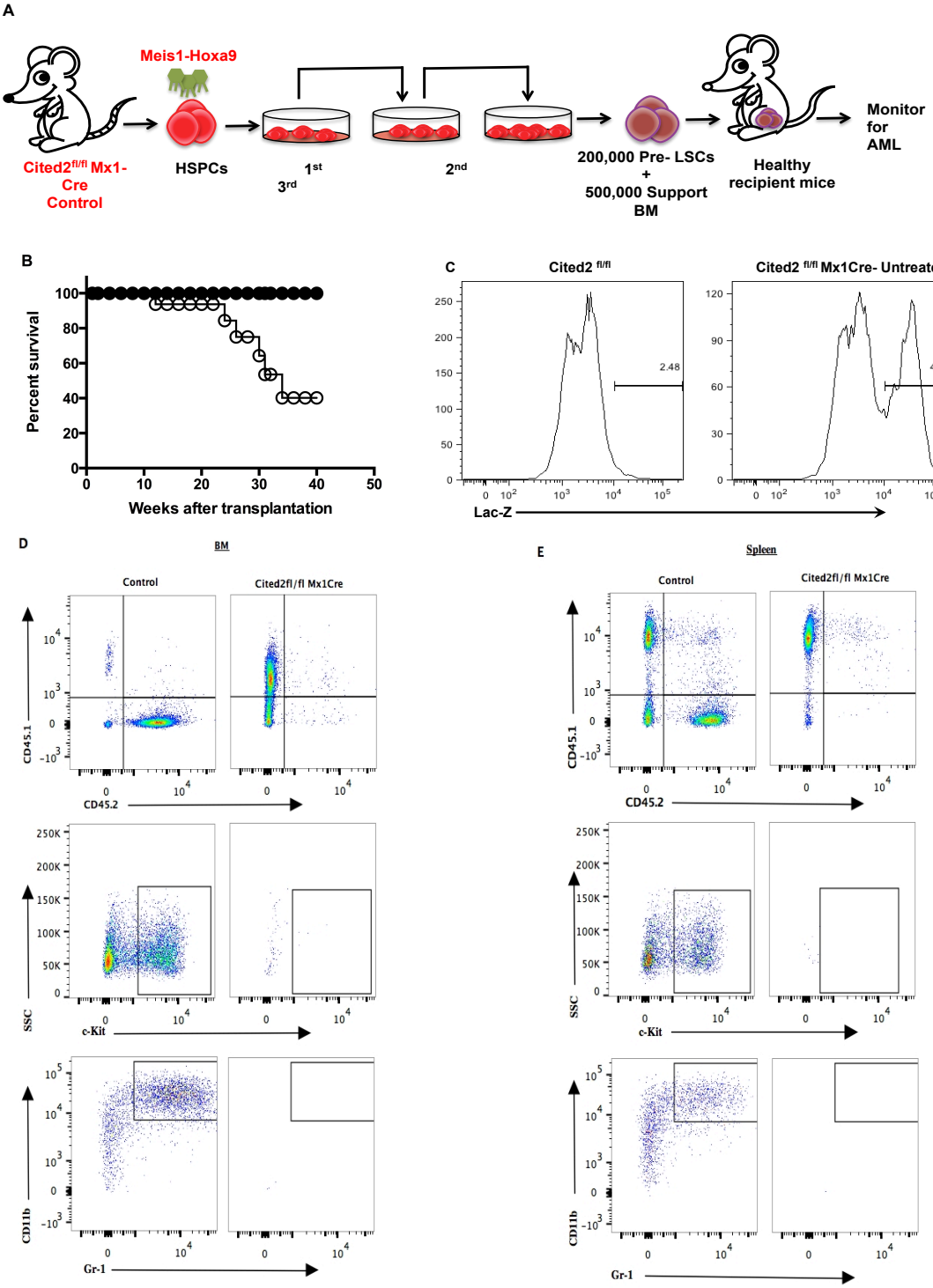



**Figure 5. 6: Transformation potential Cited2$^{\Delta/\Delta}$ cells with complimentary expression of *Cited2***

(A) Schematic representation of RTTA of Cited2$^{\Delta/\Delta}$ and Wt HSPCs transformed with Meis1-Hoxa9 with ectopic expression of Cited2. Cited2$^{\Delta/\Delta}$ and Wt HSPCs are co-transduced with Meis1-Hoxa9 and Cited2 or Control vector expression GFP. Meis1- Hoxa9 transformed cells positively selected for puromycin and neomycin resistance are further sorted for GFP by flow cytometry and serially re-plated to test the transformation potential. (B) Number of transformed colonies generated during serial re-plating of Cited2$^{\Delta/\Delta}$ and Wt HSPCs with ectopic expression of Cited2 and Control vector transformed with Meis1-Hoxa9. Results are represented as mean ± SD, n=3. (C) Representative images of colonies formed by Meis1-Hoxa9 transformed Cited2$^{\Delta/\Delta}$ and Wt HSPCs selected for expression of Cited2 and Control vector at CFC-3.


A
Meis1-Hoxa9
Cited2fl/fl Mx1-Cre Control
HSPCs
1st
3rd
2nd
200,000 Pre- LSCs + 500,000 Support BM
Healthy recipient mice
Monitor for AML
B
Percent survival
Weeks after transplantation
C
Cited2 fl/fl
Cited2 fl/fl Mx1Cre- Untreated
2.48
40.6
Lac-Z
D
BM
Control
Cited2fl/fl Mx1Cre
CD45.1
CD45.2
SSC
c-Kit
CD11b
Gr-1
E
Spleen
Control
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CD45.1
CD45.2
SSC
c-Kit
CD11b
Gr-1

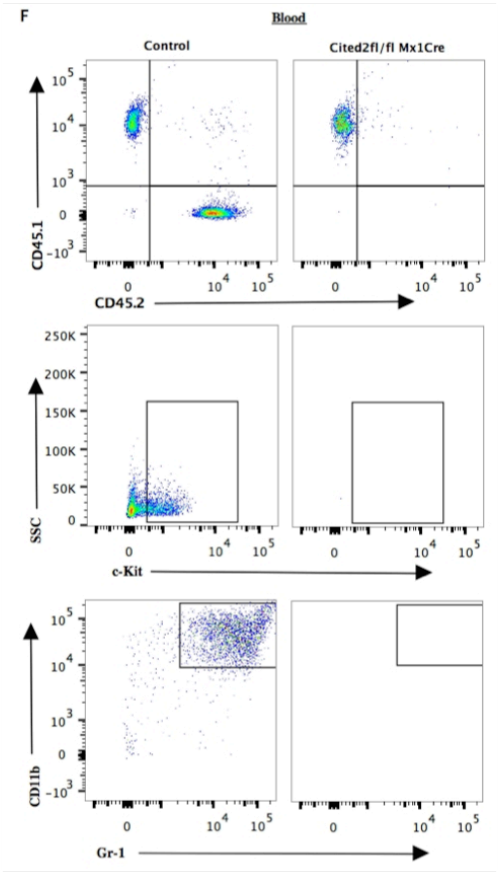
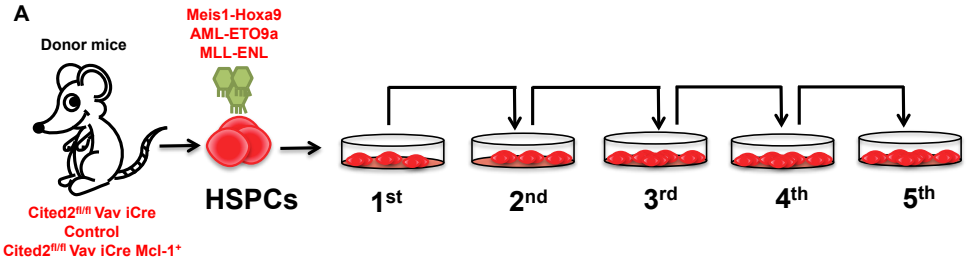
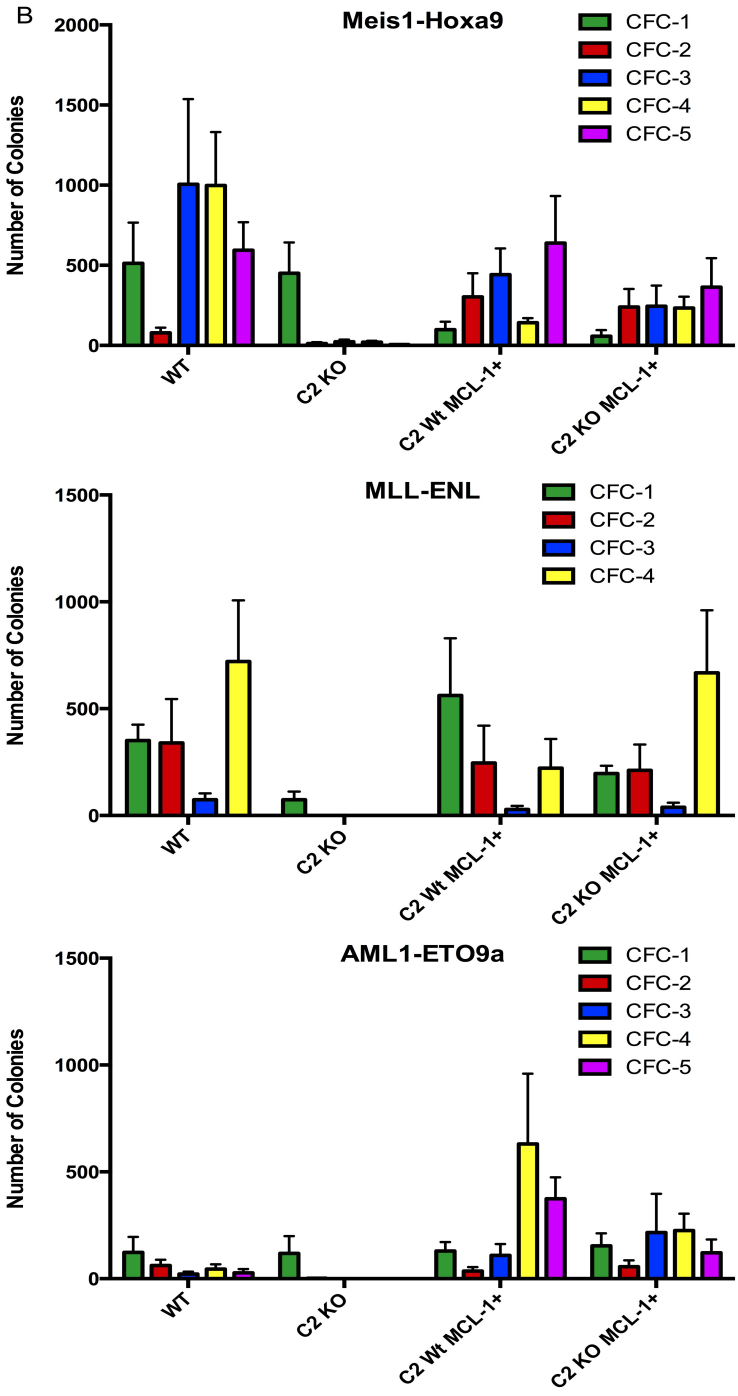



**Figure 5. 7: AML development potential of Cited2$^{\Delta/\Delta}$ pre-LSCs in vivo**

(A) Schematic representation of RTTA of Cited2$^{\Delta/\Delta}$ and Control HSPCs transformed with Meis1-Hoxa9, sorted for pre-LSCs and transplanted into lethally irradiated recipients. Cited2$^{\Delta/\Delta}$ and Control HSPCs co-transduced with Meis1-Hoxa9 were serially re-plated until CFC-3 to generate pre-LSCs. The pre-LSCs were further sorted for cell surface markers (c-Kit, Gr-1 and CD11b) by flow cytometry and 200,000 sorted pre-LSCs along with 500,000 support BM was transplanted into lethally irradiated recipients (B) Kaplan Meier survival curve representing the percentage of survival of recipients transplanted with Cited2$^{\Delta/\Delta}$ and Control pre-LSCs. Blocked dots represent recipients with Cited2$^{\Delta/\Delta}$ pre-LSCs (n=14) and empty dots represent recipients with Control pre-LSCs (n=16). (C) Percentage of LacZ+ pre-LSCs from CFC-3 that were stained for FDG expression. Representative FACS plots of (D) BM, (E) Spleen and (F) PB from the recipients transplanted with Cited2$^{\Delta/\Delta}$ (n=3) and Control (n=3) pre-LSCs.


A
Donor mice
Meis1-Hoxa9
AML-ETO9a
MLL-ENL
Cited2fl/fl Vav iCre
Control
Cited2fl/fl Vav iCre Mcl-1+
HSPCs
1st
2nd
3rd
4th
5th


B
Meis1-Hoxa9
MLL-ENL
AML1-ETO9a
CFC-1
CFC-2
CFC-3
CFC-4
CFC-5
Number of Colonies
2000
1500
1000
500
0
WT
C2 KO
C2 Wt MCL-1+
C2 KO MCL-1+

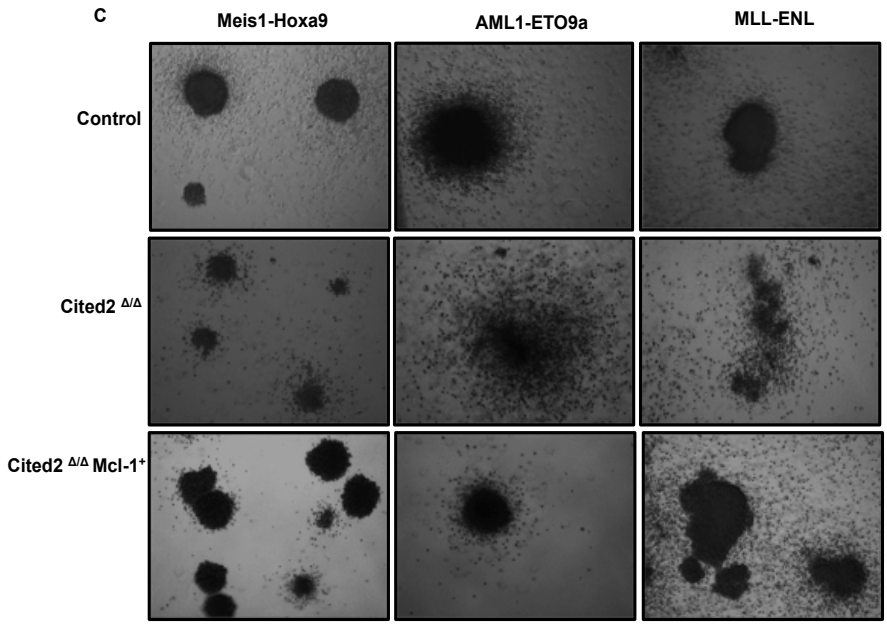



**Figure 5. 8: Transformation potential Cited2$^{\Delta/\Delta}$, Cited2$^{\Delta/\Delta}$ Mcl-1$^{+}$, Cited2$^{+/+}$Mcl-1$^{+}$ and Control cells**

(A) Schematic representation of RTTA of Cited2$^{\Delta/\Delta}$, Cited2$^{\Delta/\Delta}$ Mcl-1$^{+}$, Cited2$^{+/+}$Mcl-1$^{+}$ and Control mice transformed with MLL-ENL, Meis1-Hoxa9 and AML1-ETO9a (B) Number of transformed colonies generated during serial re-plating of Cited2$^{\Delta/\Delta}$, Cited2$^{\Delta/\Delta}$ Mcl-1$^{+}$, Cited2$^{+/+}$Mcl-1$^{+}$ and Control HSPCs transformed with Meis1-Hoxa9, MLL-ENL and AML1-ETO9a. Results are represented as mean ± SD, n=2. (C) Representative images of colonies formed by Meis1-Hoxa9 and AML1-ETO9a transformed Cited2$^{\Delta/\Delta}$, Cited2$^{\Delta/\Delta}$ Mcl-1$^{+}$ and Control HSPCs at CFC-3 and MLL-ENL at CFC-1.

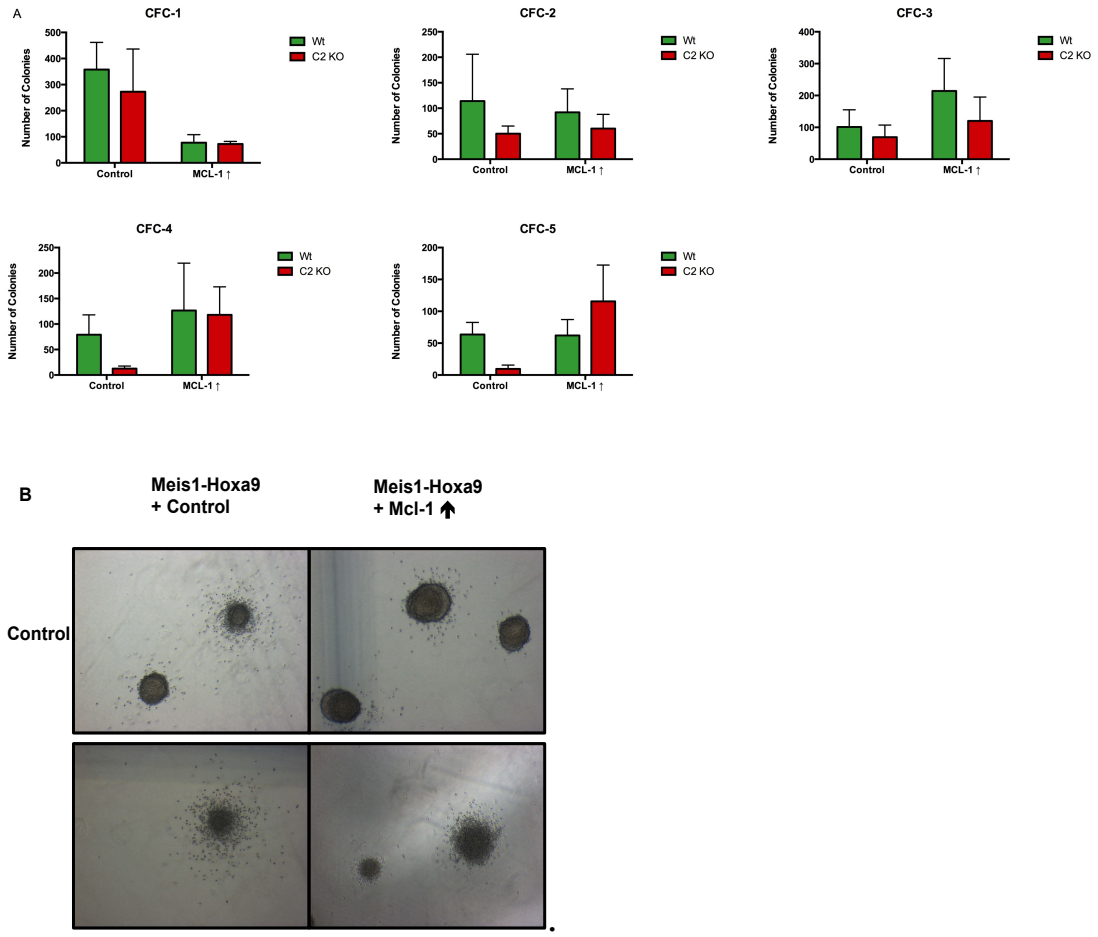



**Figure 5.9: Transformation potential Cited2$^{\Delta/\Delta}$ cells with complimentary expression of *Mcl-1***

(A) Number of transformed colonies generated during serial re-plating of Cited2$^{\Delta/\Delta}$ and Wt HSPCs with ectopic expression of Mcl-1 and Control vector transformed with Meis1-Hoxa9. Results are represented as mean ± SD, n=3. (B) Representative images of colonies formed by Meis1-Hoxa9 transformed Cited2$^{\Delta/\Delta}$ and Wt HSPCs selected for expression of Mcl-1 and Control vector at CFC-3.

# Chapter 6

# Final discussion and future directions

## 6.1 Final discussion

Hypoxia and hypoxia signalling pathways are believed to play an important role in the regulation of HSC functions (Parmar et al. 2007; Kranc et al. 2009; Simsek et al. 2010; Takubo et al. 2010; Miharada et al. 2011; Kocabas et al. 2012; Singh et al. 2013; Gezer et al. 2014). Both isoforms of Hif-$\alpha$ and their target genes (e.g. *Vegf, Meis1, Cited2*) have been shown to be important in murine embryonic development (Maltepe et al. 1997; Adelman et al. 1999; Bamforth et al. 2001; Gruber et al. 2007; Chen et al. 2008). Takubo and colleagues demonstrated evidence suggesting that *Hif-1*$\alpha$ expression is important in the maintenance of HSC functions under stressful conditions of serial transplantation and aging (Takubo et al. 2010). However, the role of the other isoform of Hif-$\alpha$, *Hif-2*$\alpha$, in HSC functions was unknown. Hence, the following work focused on understanding the importance of *Hif-2*$\alpha$ and the interplay between *Hif-1*$\alpha$ and *Hif-2*$\alpha$ in regulating HSC functions. Results presented in this thesis show that expression of *Hif-2*$\alpha$, or both *Hif-1*$\alpha$ and *Hif-2*$\alpha$, within HSCs is dispensable for their normal functions under the stressful conditions of serial transplantation and aging. However, *Hif-2*$\alpha$ is essential for the maintenance of HSC functions in a non-cell-autonomous manner, specifically in males and possibly regulated via *Hif-1*$\alpha$.

In a separate, but related study, the current work examined the role of the hypoxia-inducible gene, *Cited2,* in the development of AML. The data presented in this thesis strongly suggest that *Cited2* is required for the formation of LSCs and in the development of AML. Additionally, overexpression of *Mcl-1*, an anti-apoptotic gene (Dzhagalov et al. 2008; Campbell et al. 2010b; Glaser et al. 2012), increases the leukaemic transformation potential of the cells lacking *Cited2*. Hence, *Cited2* is required for the transformation potential of HSPCs potentially via *Mcl-1*.

### 6.1.1 *Hif-2α* is dispensable for the maintenance of cell-autonomous HSC functions

To dissect the role of *Hif-2α* in the maintenance of HSC functions in a cell-autonomous manner, mice with conditional deletion of *Hif-2α* were studied. The results demonstrated that HSCs without *Hif-2α* sustained normal multilineage haemopoiesis and self-renewal properties (Chapter 3). Under stressful conditions of serial transplantation, HSCs lacking *Hif-2α* successfully repopulated secondary and tertiary recipients (Chapter 3). Furthermore, *Hif-2α*-deficient HSCs were examined for age induced stress response. Lethally irradiated recipients were transplanted with BM cells lacking *Hif-2α* and were analysed at 24 weeks of age. This experiment showed that age-induced stress in *Hif-2α*-deficient HSCs did not affect the maintenance of HSC functions (Chapter 3). These results, taken together, demonstrate that cell-autonomous expression of *Hif-2α* in HSCs is dispensable for their normal functions. This is in agreement with recent published evidence where we showed that a mouse model with *Hif-2α* deletion during embryogenesis in the haemopoietic cells (Vav-iCre) successfully maintained adult HSC functions and retained steady state multilineage haemopoiesis (Guitart, Subramani et al, Blood, 2013).

Previously it was shown that global deletion of *Hif-2α* severely affected survival of mice with increased perinatal lethality and multiple organ pathologies, including pancytopenia (Scortegagna et al. 2003a). Another study showed that *Hif-2α* was essential for regulation of *Epo* in the kidney and maintenance of erythroid progenitors in adult mice (Gruber et al. 2007). A recent publication provided evidence that high levels of systemic *Epo* expression resulted in altered fate-choices of HSCs and primitive progenitors with increased erythroid-biased lineages and suppressed non-erythroid lineages (Grover et al. 2014). Given that *Hif-2α* regulates *Epo* expression, which has downstream effects on HSC/progenitor fate choices, it is intriguing that the current work using serial transplantation studies demonstrates that acute deletion of *Hif-2α* is dispensable for the maintenance of HSC functions in a cell-autonomous manner. The serial

transplantation studies conducted in Chapter 3 demonstrated that HSCs lacking *Hif-2α* were capable of long-term multilineage reconstitution in lethally irradiated recipients. The current work also showed that age-induced stress in HSCs lacking *Hif-2α* did not affect their potential of multilineage haemopoiesis. Additional evidence demonstrated that HSCs from Hif-2$\alpha^{fl/fl}$ Vav-iCre successfully maintained long-term reconstitution properties when transplanted into lethally irradiated mice (Guitart, Subramani et al, Blood, 2013). Hence, taken together, the results from Chapter 3 showed that unlike *Hif-1α* (Takubo et al. 2010), *Hif-2α* is not critical for regulating HSC functions in a cell-autonomous manner including stressful conditions of aging and serial transplantation.

Gathered from the previous publications, global deletion of *Hif-2α* in mice is embryonic lethal (Scortegagna et al. 2003a; Gruber et al. 2007). Hence, the current study employed a CKO approach with inducible-Cre (Mx1-Cre) that allowed for acute deletion of *Hif-2α* in adult mice in haemopoietic cells, BM microenvironment (Kuhn et al. 1995; Schneider et al. 2003; Walkley and Orkin 2006; Walkley et al. 2007), thus bypassing the embryonic lethality. The use of Cre-inducible deletion of conditional alleles of *Hif-2α* provided a valuable tool to address the haemopoietic-specific role of *Hif-2α*. Importantly, the administration of pIpC and resultant acute deletion of *Hif-2α* allowed for HSC-specific deletion within transplanted Hif-2$\alpha^{\Delta/\Delta}$ donor derived BM cells with flexibility of pIpC-induced deletion following the successful engraftment of HSCs from Hif-2$\alpha^{\Delta/\Delta}$ mice into the recipient BM, thus, addressing the function of *Hif-2α* in regulating the long-term HSC reconstitution. pIpC has been reported to interfere with cell-cycle status (Lundberg et al. 2007) and altering the BM microenvironment, thus creating an additional stress. Taking into account the possible stress that is induced by pIpC, the data in the Chapter 3 showed that HSCs did not require cell-autonomous *Hif-2α* to maintain their normal functions under stressful conditions of altered BM niche due to pIpC administration.

A recent study on human HSPCs showed that knockdown of *HIF-2α* using shRNA (short-hairpin RNA) resulted in defective long-term repopulation potential (Rouault-Pierre et al. 2013). The *HIF-2α* knockdown in human

CD34+ cells resulted in a decrease in proliferative capability and erythroid colony formation *in vitro*, coupled with impaired reconstitution capacity in part due to an increase in ROS production and increased ER stress resulting in subsequent apoptosis (Rouault-Pierre et al. 2013). However, this study had several caveats such as the use of a single shRNA to knockdown *HIF-2α* whose specificity for *HIF-2α* was not validated. Currently, there are numerous publications highlighting the importance of off-target effects in shRNA studies (Grimm et al. 2006; Jackson and Linsley 2010; Kaelin 2012). In addition, all experiments were conducted in normoxia, further adding complexity to the interpretation of the resultant data. Collectively the current work and that of colleagues within the field (Rouault-Pierre et al. 2013) suggest distinct roles of *Hif-2α*-dependent signalling in mouse and human HSCs/HSPCs. Nevertheless, further studies are required to rule out the possibility of system-dependent discrepancies (haematopoiesis-specific conditional gene knockout in mice versus shRNA mediated knockdown in human ex vivo samples). Indeed the field would benefit from a detailed comparative study of *Hif-2α* in regulating HSC functions in both human and mouse models to further clarify the system-dependent variability in the requirement of *Hif-2α* in HSC maintenance.

### 6.1.2 *Hif-1α* and *Hif-2α* are dispensable for maintenance of cell-autonomous HSC functions

Given that *Hif-2α* deletion did not affect the cell-autonomous HSC functions (Chapter 3) and the lack of *Hif-1α* had a mild phenotype under steady state (Takubo et al. 2010), we decided to delete both *Hif-1α* and *Hif-2α* in haemopoietic system. HSCs lacking the expression of both *Hif-1α* and *Hif-2α* maintained repopulation potential and successfully generated mature haemopoietic lineages (Chapter 3). Serial transplantation experiments showed that *Hif-1α*– and *Hif-2α*–defecient HSCs retained self-renewal potential and long-term multilineage haemopoiesis in secondary and tertiary recipients (Chapter 3). Additionally, age-induced stress in lethally irradiated mice transplanted with BM cells lacking both *Hif-1α* and *Hif-2α* did not affect the maintenance of HSC functions (Chapter 3). In contrast to

published work (Takubo et al. 2010), the current work demonstrated that cell-intrinsic Hif-α-regulated hypoxia signalling is not absolutely essential for the maintenance of HSC functions even under stressful conditions of serial transplantation and aging.

*Hif-1α* and *Hif-2α* have been shown to be important in regulating cellular and systemic hypoxia (Semenza 1999; Coleman and Ratcliffe 2007; Gruber et al. 2007; Kaelin and Ratcliffe 2008). Both *Hif-1α* and *Hif-2α* are critical for embryogenesis and early development (Maltepe et al. 1997; Iyer et al. 1998; Ryan et al. 1998; Tian et al. 1998; Adelman et al. 1999; Adelman et al. 2000; Peng et al. 2000; Compernolle et al. 2002; Scortegagna et al. 2003a; Ramirez-Bergeron et al. 2006; Gruber et al. 2007). *Hif-2α* is induced by ROS and has been shown to be important in regulating the oxidative-stress response, while *Hif-1α* is believed to be a master regulator of metabolism (Gruber et al. 2007; Simsek et al. 2010; Takubo et al. 2010; Kocabas et al. 2012). Previously, *Hif-1α* has been shown to be important in regulating HSC cell cycle quiescence (Takubo et al. 2010). Although, HSCs lacking *Hif-1α* maintained normal steady-state haemopoiesis, during stressful conditions of serial transplantation and aging, loss of HSCs and their cell cycle quiescence in a p16Ink4a/p19Arf-dependent manner was reported (Takubo et al. 2010). Results from Chapter 3 showed that additional deletion of *Hif-2α* along with *Hif-1α* did not mimic the LSK chimerism or loss of HSCs during serial trasplantation and aging observed in Hif-1$\alpha^{\Delta/\Delta}$ mice (Takubo et al. 2010), suggesting that increased *Hif-2α* expression may be driving the loss of quiescent HSC phenotype in the absense of *Hif-1α*. In support, a more recent study reported increased levels of *Hif-2α* expression in the *Hif-1α*-deficient cells (Kocabas et al. 2012). However, a more recent study suprisingly revealed that expression of *Hif-1α* in HSCs/progenitors remains stable reglardless of their cell-cycle status (Nombela-Arrieta et al. 2013). Another recent publication studied the effect of *Hif-1α* deletion in the vascular-endothelial cadherin (VEC)-expressing endothelial cells, which are the precursors to HSCs using VEC-Cre (Imanirad et al. 2014). VEC-Cre deleted *Hif-1α* during early embryogenesis and displayed reduced donor-derived LSK cells in primary recipients, but successfully reconstituted

secondary recipients (Imanirad et al. 2014). Thus providing data that are contradicting to previously published work by Takubo et al (Imanirad et al. 2014). The Hif-1$\alpha^{\Delta/\Delta}$ (VEC-Cre) mice had reduced haemopoietic progenitor cells in the AGM region at E10, but these mice matured to adulthood and displayed normal adult haemopoiesis (Imanirad et al. 2014). Furthermore, studies in the human system, using *HIF-1$\alpha$* knockdown in haemopoietic stem and progenitor cells, showed that these cells efficiently maintained their self-renewal properties during serial transplantation (Rouault-Pierre et al. 2013). Hence, the role of *Hif-1$\alpha$* in regulation of HSC self-renewal under stress conditions is unclear and merits further investigation.

Several downstream *Hif-1$\alpha$* and *Hif-2$\alpha$* targets, including *Cited2, Vegf, Cripto* and *Foxo3a,* were shown to be critical for cell-autonomous HSC maintenance (Chen et al. 2007; Miyamoto et al. 2007; Kranc et al. 2009; Takubo et al. 2010; Miharada et al. 2011; Rehn et al. 2011). These Hif-target genes are likely to be regulated by other Hif-independent pathways and this possibly explains the lack of functional defects in HSCs lacking both *Hif-1$\alpha$* and *Hif-2$\alpha$* expression. Supporting the role of Hif-independent pathways regulating the transcriptional activation of Hifs, a study showed that inducible deletion of *Meis1*, a transcriptional activator of *Hif-1$\alpha$* (Simsek et al. 2010), in murine HSCs led to loss of HSC functions, including loss of BM repopulation following transplantation, with decreased levels of *Hif-1$\alpha$* and *Hif-2$\alpha$* expression (Kocabas et al. 2012). However, the defective HSC phenotype was not rescued with stabilisation of *Hif-1$\alpha$* and *Hif-2$\alpha$* by cobalt chloride (Kocabas et al. 2012). Collectively, the results in Chapter 3 demonstrated that the Hif-2$\alpha$ was dispensible in cell autonomous manner for normal HSCs functions. Hence, suggesting that the loss of HSCs in *Meis1*-deficient mice was due to the increased level of ROS and other pathways regulated by *Meis1* and not because of the defective *Hif-1$\alpha$* and *Hif-2$\alpha$* expression.

### 6.1.3 *Hif-2α* plays an essential role in non-cell-autonomous HSC maintenance in a gender-specific manner

To examine the role of *Hif-2α* in the BM niche regulating non-cell-autonomous HSC functions, acute deletion of *Hif-2α* was studied using an inducible Mx1-Cre mouse model. The use of Mx1-Cre system allowed for the possibility of induced *Hif-2α* deletion in fully developed adult BM and haemopoietic cells and thus provided a valuable tool to understand the effect of acute deletion of *Hif-2α* on HSC functions. The analysis of the expression of *Hif-1α* and *Hif-2α* in various BM niche cells showed that *Hif-2α* is expressed at high levels in endothelial cells compared to OBs and mesenchymal-like cells (Chapter 4). The current work demonstrated that induced deletion of *Hif-2α* in the BM niche and in HSCs resulted in decreased HSCs and progenitor cells, particularly in males but not in females (Chapter 4). The lack of *Hif-2α* specifically impaired HSCs and primitive progenitors and did not affect mature cell numbers or functions. This is in agreement with a previous study by Scrotegagna et al that showed impaired repopulation potential and reduced WBC count in mice lacking *Hif-2α* expression (Scortegagna et al. 2003a). However, at this point we cannot rule out the possible influence of *Hif-2α* deletion outside the BM niche (e.g. kidney, liver or other interferon responsive organs) on the HSC and progenitor phenotype that is observed in *Hif-2α*–deficient males. Further, the data demonstrated that there was no extramedullary haemopoiesis or apoptotic defects identified in the *Hif-2α*–deficient HSCs. It is plausible that lack of *Hif-2α* expression might result in increased differentiation of HSCs and progenitor cells accounting for reduced HSPCs. A detailed study of the cell cycle status of HSCs and progenitor compartments will reveal the mechanisms of Hif-α regulation of HSC differentiation state in the BM niche. Therefore, the results from Chapter 4 showed that although the BM lacking *Hif-2α* successfully maintained long-term multilineage reconstitution properties, expression of *Hif-2α* in the BM niche is necessary in a non-cell-autonomous manner for maintenance of HSCs in males.

Results in this thesis revealed a surprising gender biased requirement of *Hif-2α* for HSC maintenance in a non-cell-autonomous manner. Several studies have demonstrated the correlation between steroid hormones and hypoxia signalling in various cell types, including HSCs and BM niche (Fried et al. 1974; Medina et al. 1993; Samuels et al. 1999; Thurmond et al. 2000; Tsuzuki et al. 2013). Females treated with oestradiol displayed elevated LSKs and increased repopulation potential (Tsuzuki et al. 2013). This effect of oestrogen on LSKs might equip HSCs with resistance to lack of *Hif-2α* expression, particularly in females explaining the impaired HSCs in Hif-2$\alpha^{\Delta/\Delta}$ males shown in Chapter 4.

The hypoxia inducible gene, *Vegf*, is highly expressed in perichondrial cells and chondrocytes of the HSC niche and helps to maintain HSC functions (Chan et al. 2009). Furthermore, Rankin and colleagues showed that enhanced stabilisation of α-subunits of Hifs by *Vhl* deletion, specifically in the endosteal niche cells, led to the expansion of niche cells and an increase in HSC numbers (Rankin et al. 2012). This defective HSC phenotype is mediated by Hifs as co-deletion of *Hif-1α* and *Hif-2α* rescued the haemopoietic defects (Rankin et al. 2012). Rankin et al. showed that *Epo* was one of the important components of the HSC niche and deletion of *Vhl* or *MT1-MMP* (matrix metalloproteinase) in the BM niche affected *Epo* levels and resulted in defective HSC functions (Nishida et al. 2012; Rankin et al. 2012). Hence it is possible that *Hif-2α*, a master regulator of *Epo*, is critical for the maintenance of HSC functions in the BM niche. *MT1-MMP* has also been shown to induce *Hif-1α* expression and to be important for postnatal haemopoiesis (Nishida et al. 2012). Gathered from these previous publications and the results presented in this thesis, it is evident that Hifs play a key role in maintaining HSC functions in a non-cell-autonomous manner by regulating their niche.

### 6.1.4 *Hif-2α* plays an essential role in the regulation of non-cell-autonomous HSC functions in a *Hif-1α*-dependent manner

Additional deletion of one allele or both alleles of *Hif-1α* together with *Hif-2α* specifically rescued decreased progenitors and HSCs observed in Hif-2α$^{\Delta/\Delta}$ males (Chapter 4). However, deletion of one allele of *Hif-1α* was sufficient to rescue the reduced progenitor cells in Hif-2α$^{\Delta/\Delta}$ males. In contrast, deletion of both alleles of *Hif-1α* did not have an effect on the decreased progenitor cells in Hif-2α$^{\Delta/\Delta}$ males (Chapter 4). These results suggested that in the absence of *Hif-2α, Hif-1α* plays a dose-dependent role in maintaining HSC functions in a non-cell-autonomous manner that is gender biased. From the previous study by Takubo and colleagues it is evident that deletion of one allele of *Vhl* stabilised *Hif-1α* and induced quiescence in HSCs and progenitor cells, but over-stabilisation of *Hif-1α* in Vhl$^{\Delta/\Delta}$ mice resulted in defective HSC repopulation potential and apoptosis (Takubo et al. 2010). However, *Cited2*, a negative regulator of *Hif-1α* is critical for the maintenance of HSC function partly via *Ink4a*/*Arf* and *Trp53* (Kranc et al. 2009). These studies support the dose-dependent regulation of HSC functions by Hifs. Further, the results in Chapter 4 showed that the deletion of *Hif-1α* and *Hif-2α* results in a cell-type-specific phenotype and Hif-α signalling is influenced by various factors, including gender contributing to the complexity of the mechanism.

Various publications have shown evidence that the BM is hypoxic and harbours a hypoxic gradient (Parmar et al. 2007; Kubota et al. 2008; Lo Celso et al. 2009). Concordantly, a recent finding measured the $pO_2$ levels in the BM niche and showed that the BM niche is hypoxic (Spencer et al. 2014). This study demonstrated the existence of an oxygen gradient in the BM niche with peri-sinusoidal being most hypoxic and endosteal being least hypoxic, questioning the previous theories postulating that the endosteal niche is the least oxygenated (Spencer et al. 2014). However, another recent publication argues against the idea of existence of hypoxic gradient in the BM niche analysed by the imaging studies and a routinely used

hypoxic dye (Pimo) (Nombela-Arrieta et al. 2013). Interestingly, it was reported that exposure to irradiation or chemotherapeutic stress resulted in increase of $pO_2$ levels and no detectable hypoxic gradient across BM niches after 2 days of irradiation (Spencer et al. 2014). Considering this recent finding, there is a possibility that the effect of irradiation on the hypoxic state might influence the expression and stabilisation of Hifs and other hypoxic targets in the irradiated BM niche. However, the disappearance of the hypoxic gradient in the irradiated BM niche was limited to short time (2 days) read out after irradiation. The lack of the oxygen gradient was attributed to the reduced BM cellularity and the demand for ample blood flow (Spencer et al. 2014). Hence, this might be a temporary environment created for short time span after irradiation due to the loss of BM cellularity. To address this, a detailed investigation of hypoxic status of the irradiated niche at various time points tracking cell recovery and oxygen levels is essential. Moreover, the lack of hypoxic gradient did not affect the homing of HSPCs (Spencer et al. 2014). It is also not clear if HSCs dictate the oxygen gradient of their niche by cell-intrinsic hypoxia signalling. Therefore, a more detailed analysis of long-term effects of irradiation on the hypoxic nature of the BM niche and on HSCs will facilitate with the understanding of its influence on serial transplantation analysis. Additionally, published evidence suggested that Hif expression in HSPCs was independent of oxygen concentration in the BM niche (Nombela-Arrieta et al. 2013). Notably *HIF-1*$\alpha$ expression was reported in $CD34^+$ cells from normoxic culture (Rouault-Pierre et al. 2013). These studies suggested that Hif-$\alpha$ isoforms are induced and stabilised by oxygen-independent pathways. Therefore, a more detailed understanding of the BM hypoxic gradient and its effect on hypoxia signalling together with its influence on HSC niche functions is essential.

### 6.1.5 Proposed model of HSC niche

The HSC niche is heterogenous and has been subdivided into two main groups, the vascular and the endosteal niche (Scadden 2006; Parmar et al. 2007; Morrison and Spradling 2008; Lo Celso et al. 2009; Chow et al. 2011; Ding et al. 2012; Nishida et al. 2012; Rankin et al. 2012; Nombela-Arrieta et al. 2013; Spencer et al. 2014). The localisation of HSCs in the BM niche is

currently under debate. Previous studies, including imaging studies provided evidence supporting the possibility of the osteoblastic niche serving as the HSC niche (Calvi et al. 2003; Lo Celso et al. 2009; Miyamoto et al. 2011). Alternatively, another study showed that deletion of *Scf* specifically from the endothelial and perivascular BM niche resulted in the depletion of HSCs (Ding et al. 2012). This study supports the close proximity of HSCs to endothelial and perivascular niche. A more recent study shed light on the distinct niches supporting HSCs and progenitor cells (Ding and Morrison 2013). Endothelial cell-specific deletion of chemokine, Cxcl12 in mice showed depletion on HSCs, while deletion of Cxcl12 in perivascular cells depleted HSCs and progenitor cells (Ding and Morrison 2013). The evidence of the hypoxic status of the BM niche is unclear. Previous studies demonstrated the existence of oxygen gradient in the BM niche by using Pimo and measuring the expression of *Hif-1*$\alpha$ (Parmar et al. 2007; Kubota et al. 2008; Suda et al. 2011). These studies suggested that the osteoblastic niche was the most hypoxic and endothelial niche the least hypoxic region (Parmar et al. 2007; Kubota et al. 2008; Suda et al. 2011). However, more recently although the hypoxic nature of the BM was confirmed (32mm Hg), levels of $pO_2$ gradient have been shown to be different. In particular, peri-sinusodial region had lowest $pO_2$, while highest $pO_2$ was reported in the endosteal region (Spencer et al. 2014). The current work demonstrated that *Hif-2*$\alpha$ is highly expressed in the endothelial cells compared to osteoblastic or mesenchymal-like cells in the BM niche (Chapter 4). Taken together, the evidence from previous literature and the results from Chapter 4 suggest that it is more likely the *Hif-2*$\alpha$ is regulating the HSC functions through the endothelial niche. Hence, taking into account the recent publications (Ding and Morrison 2013), HSCs and progenitors have distinct niches. HSCs are residing close to the vasculature and vascular niche that is highly hypoxic and has high levels of *Hif-2*$\alpha$ expression (Chapter 4) and the progenitors are in close proximity to the osteoblastic niche that is less hypoxic with low levels of *Hif-2*$\alpha$ expression (Figure 6.1).

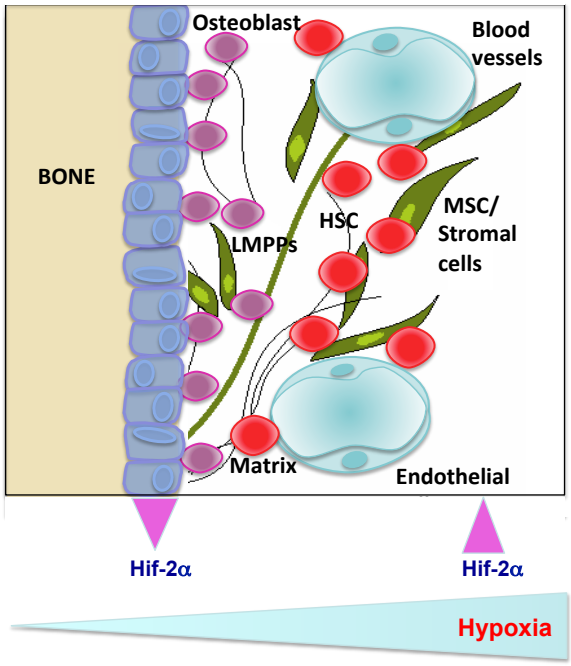



**Figure 6. 1: A proposed model of HSC niche.**

### 6.1.6 The role of *Cited2* in leukaemic transformation

*Cited2*, a hypoxia-inducible gene, has been shown to be critical during embryogenesis and in the regulation of adult HSC functions, including self-renewal, apoptosis and quiescence (Bhattacharya et al. 1999; Chen et al. 2007; Qu et al. 2007; Kranc et al. 2009; Du et al. 2012; Du and Yang 2013). While some studies supported the role of *Cited2* as a tumour suppressor (Bai and Merchant 2007; van Agthoven et al. 2009), other studies provided evidence for oncogenic properties of *Cited2* (Sun et al. 1998; Futaki et al. 2003; Chou et al. 2006; Sykes et al. 2011; Chou et al. 2012). Preliminary data from our lab suggested that *CITED2* expression is elevated in $CD34^{+}$ cells from human AML cells (Personal communication, Prof. Kranc). Concordantly, another study reported an increase in the expression of *Cited2* in MLL-AF9-induced LSCs (Sykes et al. 2011). In order to understand the role of *Cited2* in leukaemogenesis, the current work examined the transformation potential of *Cited2*-deficient HSPCs by retroviral introduction of an oncogenic fusion in an *in vitro* AML model. Results from Chapter 5 showed that $Cited2^{\Delta/\Delta}$ HSPCs retrovirally transduced with MLL-ENL, AML1-ETO9a or Meis1 and Hoxa9 displayed defective transformation potential *in vitro* and failed to generate colonies during serial re-plating (Chapter 5). Additionally, Meis1-Hoxa9 transduced HSPCs with partial

deletion of *Cited2* failed to generate AML in a period of 6 months when 50% of the control developed AML in lethally irradiated syngeneic recipients. Survival defects have been reported in Cited2$^{\Delta/\Delta}$ HSCs (Kranc et al. 2003; Kranc et al. 2009). Hence both HSCs and LSC lacking *Cited2* might share the defective survival phenotype that could explain the transformation defects of HSPCs lacking *Cited2*.

Several TFs, such as *Bmi-1*, *Meis1* and *Pu.1* have been shown to be important in regulating HSC functions and their expression is critical for generation of LSCs and transplantable AML (Lessard and Sauvageau 2003; Rosenbauer et al. 2004; Azcoitia et al. 2005; Iwasaki et al. 2005; Wong et al. 2007; Smith et al. 2011; Kocabas et al. 2012). The results from Chapter 5 showed that *Cited2*, an essential regulator of HSC functions, is also required for the transformation and generation of LSCs. To overcome the drawback that lack of HSCs and progenitor cells in the Cited2$^{fl/fl}$ Mx1-Cre mice, I used a haemopoietic-specific deletion of Cited2 in Cited2$^{fl/fl}$ Vav-iCre mice that lacked the *Cited2* expression from early embryogenesis throughout adulthood. Cited2$^{fl/fl}$ Vav-iCre mice had depleted HSCs, but similar number of primitive progenitors compared to the controls (Guitart et al. unpublished data). Therefore, Cited2$^{fl/fl}$ Vav-iCre mice, with unaffected number of primitive progenitor population provided target cells for the leukaemic transformation.

The severity of the transformation defects observed in Cited2$^{\Delta/\Delta}$ HSPCs in Chapter 5 was dependent on the oncogenic signalling. Cited2$^{\Delta/\Delta}$ HSPCs transduced with MLL-ENL that is upstream of multiple Hox genes (Smith et al. 2011), failed to generate colonies during initial re-plating, while Cited2$^{\Delta/\Delta}$ HSPCs transformed with Meis1-Hoxa9 managed to serially re-plate but with fewer colonies compared to the Control. This suggests that MLL fusion induced activation of multiple non-Hox (Smith et al. 2011), that could be potentially dependent on *Cited2*, thus regulating LSC self-renewal. However, a downstream target of MLL, such as Meis1-Hoxa9-induced leukaemic transformation is less dependent on *Cited2* signalling compared to MLL-ENL. Hence, the *Cited2* deletion has a drastic effect on transformation of HSPCs by MLL fusion that activate downstream target genes including, *Meis1* and *Hoxa9* affecting a broad spectrum of TFs.

Conversely, deletion of *Cited2* in HSPCs had less severe effects on their transformation potential when co-transduced by Meis1 and Hoxa9, downstream targets of MLL.

### 6.1.7 A possible role of the Cited2-Mcl-1 pathway in leukaemic transformation

*Mcl-1*, an anti-apoptotic gene, is down regulated in Cited2$^{\Delta/\Delta}$ embryos and in adult HSCs (Chen et al. 2007)(Guitart et al, unpublished data and Prof. Kranc personal communications). *Mcl-1*, with similar expression profile to *Cited2,* has been shown to be important for leukaemic transformation and AML generation potential (Zhou et al. 1998; Dzhagalov et al. 2008; Campbell et al. 2010b; Glaser et al. 2012). Hence, to test the possible *Cited2*-Mcl-1 pathway during AML development, Cited2$^{\Delta/\Delta}$ HSPCs with overexpression of *Mcl-1* were transformed using oncogenic fusion proteins. Overexpression of *Mcl-1* in Cited2$^{\Delta/\Delta}$ HSPCs resulted in increased numbers of transformed colonies and restored self-renewal potential assessed by serial re-plating (Chapter 5). Collectively, the data suggest that *Cited2* is essential for leukaemic transformation and for generation of LSCs *in vivo*, at least in part by regulating the expression of *Mcl-1*.

Studies have shown that *Hif-1*$\alpha$-mediated apoptotic pathway is regulated by *Cited2* via *Foxo3a* (Bakker et al. 2007). *Cited2* deletion has been reported to cause survival defects and decreased expression of *Mcl-1* (Chen et al. 2007; Kranc et al. 2009). The elevated expression of Cited2 is reported in AML (Sykes et al. 2011). Moreover, *MCL-1* has also been associated with poor prognosis and drug resistance in AML, ALL, CLL and melanoma (Beroukhim et al. 2010; Schwickart et al. 2010). Therefore, the current work focused on deciphering the role of *Cited2* and *Mcl-1* in leukaemic transformation. The results in this thesis demonstrated that the survival defects in Cited2$^{\Delta/\Delta}$ HSPCs was bypassed by the overexpression of *Mcl-1* enabling their transformation potential (Chapter 5). Hence, this study suggests that *Cited2* regulates the transformation potential of HSPCs potentially via *Mcl-1* (Figure 6.2). More research is essential to explore the molecular mechanisms regulating LSC generation via the Cited2-Mcl-1 pathway.

## 6.2 Future studies

### 6.2.1 Hif-signalling in regulating HSC niche

*Hif-2α* is essential for the HSC functions in a non-cell-autonomous manner possibly via *Hif-1α* in a gender-dependent manner. However, the specific roles of *Hif-1α* or *Hif-2α* in heterogeneous HSC niche cell maintenance and in influencing HSC functions are unclear. The HSC niche is thought to be hypoxic, with an oxygen-gradient existing in different niches (Parmar et al. 2007; Kubota et al. 2008; Simsek et al. 2010; Takubo et al. 2010; Rehn et al. 2011; Spencer et al. 2014). Studies showed that HSCs reside in the close proximity to the vasculature, which is relatively highly hypoxic (Lassailly et al. 2013; Nombela-Arrieta et al. 2013; Spencer et al. 2014). However, recent imaging study showed that HSCs are hypoxic irrespective of their location in the niche and retained *Hif-1α* expression suggesting hypoxia as a cell-intrinsic property of HSCs (Nombela-Arrieta et al. 2013). Hence, further understanding of the expression pattern of *Hif-1α* or *Hif-2α* and hypoxic nature of the HSCs and their niche is essential to rectify the contradictions within the field. This can be achieved by live imaging of the HSC niche and gene expression analysis of various niche cells by microarray experiments. Additionally, metabolic studies have shown that under hypoxic conditions, cells adapt to the low oxygen tension through predominant glycolytic metabolism over Krebs cycle (Simsek et al. 2010; Luo et al. 2011; Suda et al. 2011). Therefore, a detailed analysis of the metabolic status of the niche cells will provide with additional evidence of their hypoxic status. Further understanding of Hif-α in regulating the HSC niche could be achieved by conditional gene knockout studies by the deletion of *Hif-1α* or *Hif-2α* in niche cells that express the highest levels of the Hif proteins and activate Hif-dependent signalling pathways. To achieve conditional gene deletion specifically in endothelial cells, Tie2-Cre deleter and Lepr-Cre will be employed. Cxcl12 (Cxcl12-Cre) and PDGFR-β (PDGFR-β Cre) deleter will allow specific deletion in perivascular cells. Additionally, use of inducible Cre (Osterix-Cre) or Collagen (Col2.3-Cre) deleter in osteoblastic cells and Nestin-Cre deleter to delete in MSCs will address the requirement of Hifs in HSC niche functions. These further studies will shed light on the

characterisation along with the functionality of Hif-α in regulating the heterogeneous population of HSC niche and in-turn HSC functions.

### 6.2.2 The mechanisms of Hif-α signalling in the HSC niche that is differentially regulated in males and females

Detrimental effects of *Hif-2α* deletion from the BM microenvironment on the HSCs and progenitor cells specifically in males in a non-cell-autonomous manner (Chapter 4) suggest a discrepancy between the male and female HSC niche. This is supported by research on the relationship between Hif signalling and sex steroids that affect the BM niche to eventually influence HSC functions (Fried et al. 1974; Medina et al. 1993; Thurmond et al. 2000; Tsuzuki et al. 2013). However, this surprising gender-biased requirement of *Hif-2α* in non-cell-autonomous HSC maintenance has to be explored to understand the complex Hif signalling pathways. It would be useful to determine the expression of *Hif-1α* or *Hif-2α* in male and female HSC niches by performing a gene expression analysis of HSC niche cells from male and female mice along with niche cells that lack *Hif-1α* and/or *Hif-2α* expression in specific BM niches using inducible Cre mouse models. Recent advancement in the imaging techniques, such as two-photon phosphorescence lifetime microscopy is a valuable tool (Lo Celso et al. 2009; Spencer et al. 2014). Live imaging of mouse models for the expression of Hif-α in HSCs and their BM niche will provide more understanding of physiological hypoxia signaling. Additionally, functional analyses of male and female HSC niches by studying their potential to support HSC functions under physiological and stressful conditions of transplantations and aging will uncover the gender variability in HSC niche.

### 6.2.3 The role of Hifs in regulating LSCs and their niche in haematological malignancies

Hypoxia and HIF signalling are important in malignant haemopoiesis (Semenza 2009; Luo et al. 2011; Zhang et al. 2012). Elevated expression of *HIF-1α* is often associated with poor prognosis of various human cancers (Keith et al. 2012). *Hif-1α* has been shown to be selectively required for

survival of LSCs (Wang et al. 2011). *HIF-2α* knockdown in human AML samples resulted in decreased engraftment of human AML cells as shown in xenotransplant experiments (Rouault-Pierre et al. 2013). However, the mechanism governing AML development by Hif signalling is unclear. Additionally, the interaction of the niche and LSCs in regulating AML has not been explored. Some evidence suggested a cytokine-mediated effect on the osteoblastic niche in leukaemia (Reynaud et al. 2011; Frisch et al. 2012). More research is necessary to discover the unique features of the leukaemic niche in promoting tumourigenesis. This can be achieved by retroviral transduction transformantion AML model, where pre-LSCs generated by transduction of oncogenes or oncogenic fusions are transplanted into syngeneic recipients lacking *Hif-α* expression particularly in the niche cells. By monitoring their ability to develop AML and detailed analysis of Leukaemic mice will provide valuable data in understanding the influence of Hifs in the generation of LSCs and their niche. Additionally, functional analysis of the BM niche in transgenic AML mouse models (Flt3-ITD knock-in or MLL-AF9 knock-in) will uncover the mechanistic role of HSC niche components and Hif signalling on LSC generation and maintenance.

### 6.2.4 The role of *Cited2* in leukaemogenesis

*Cited2* expression is critical for embryogenesis, adult haemopoiesis and leukaemogenesis (Bamforth et al. 2001; Chen et al. 2007; Kranc et al. 2009; Sykes et al. ; Chou et al. 2012). *Cited2* is essential for LSC formation and generation of transplantable AML (Chapter 5). ChIP sequencing for *Cited2* in FDCP-mix cells has shown that Cited2 is binding the promoter region of *Mcl-1* (Preliminary data, Prof. Kranc's lab). Unpublished data showed that deletion of *Cited2* reduced the expression of *Mcl-1* expression in HSCs (Guitart et al, Preliminary data). *Mcl-1* overexpression bypassed transformation defects of *Cited2*-deficient HSPCs suggesting *Mcl-1* as a downstream target of *Cited2*. However, the binding partners of Cited2 remain unknown. Hence, ChIP-sequencing on pre-LSCs (generated by transformation of HSPCs with oncogenic fusions) will identify the region where Cited2 binds Mcl-1 and further sequencing studies will be carried out to identify the binding partners of Cited2 and Mcl-1. Given that *Cited2* is a

critical gene in regulating various physiological and pathological conditions, discovery of its interactors and downstream targets are of prime importance. ChIP-sequencing experiment will reveal the downstream targets of *Cited2* that will be functionally validated by gene knockout and knock-in studies. Furthermore, mass spectrometric analysis of Cited2 protein performed using primitive haemopoietic cells and pre-LSCs will identify the interactors of Cited2, thus uncovering the mechanistic pathways regulated by Cited2 in the maintenance of HSC and LSC functions. Despite deletion of *Cited2* being detrimental on HSCs, heterozygous deletion of *Cited2* does not result in HSC loss or damage its functions (Subramani et al, unpublished data). Interestingly, partial deletion of *Cited2* resulted in failure to develop AML as shown in Chapter 5. Therefore, understanding the mechanistic pathways regulated by Cited2 will explore the potential of Cited2 as a therapeutic target for AML. Additionally, AML development and generation of LSCs serve as a paradigm for understanding the mechanism behind various tumour developments and provides with new therapeutic approaches to tackle tumourigenesis. Collectively, current study provided functional evidence for Cited2 regulating the transformation properties of HSPCs possibly via *Mcl-1*. The potential Cited2-Mcl-1 pathway needs to be further validated by mechanistic studies (Figure 6.2).

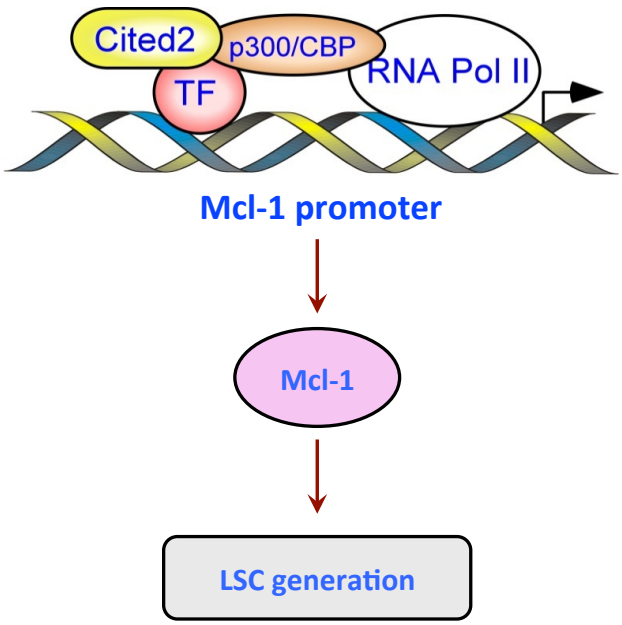
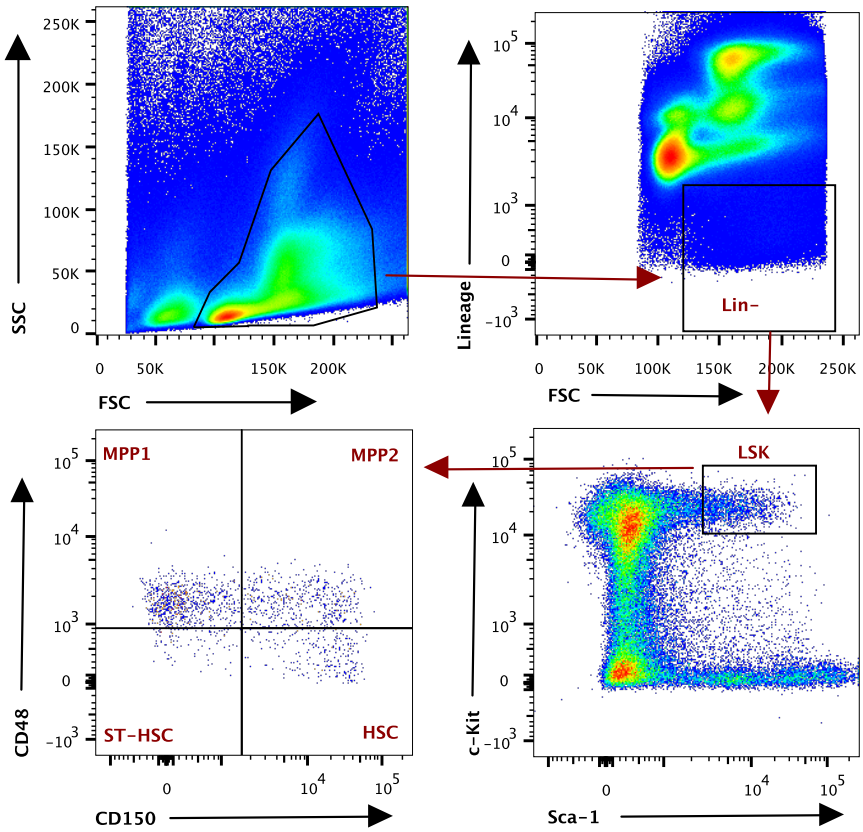
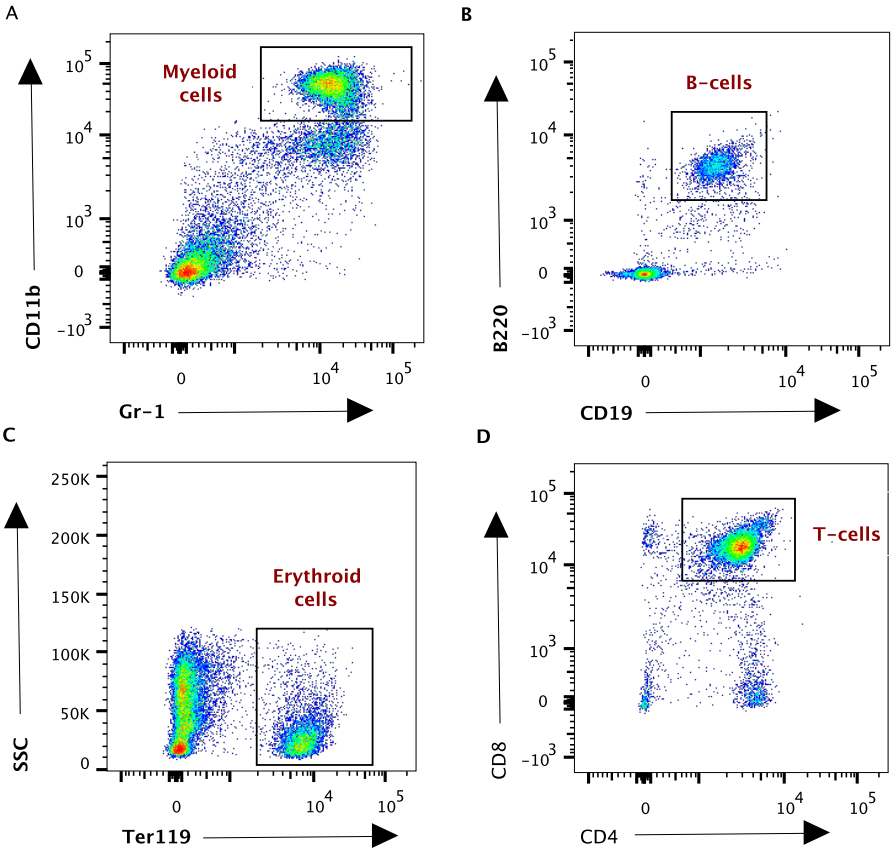



**Figure 6. 2: A proposed model of Cited2-Mcl-1 pathway in LSC generation.**

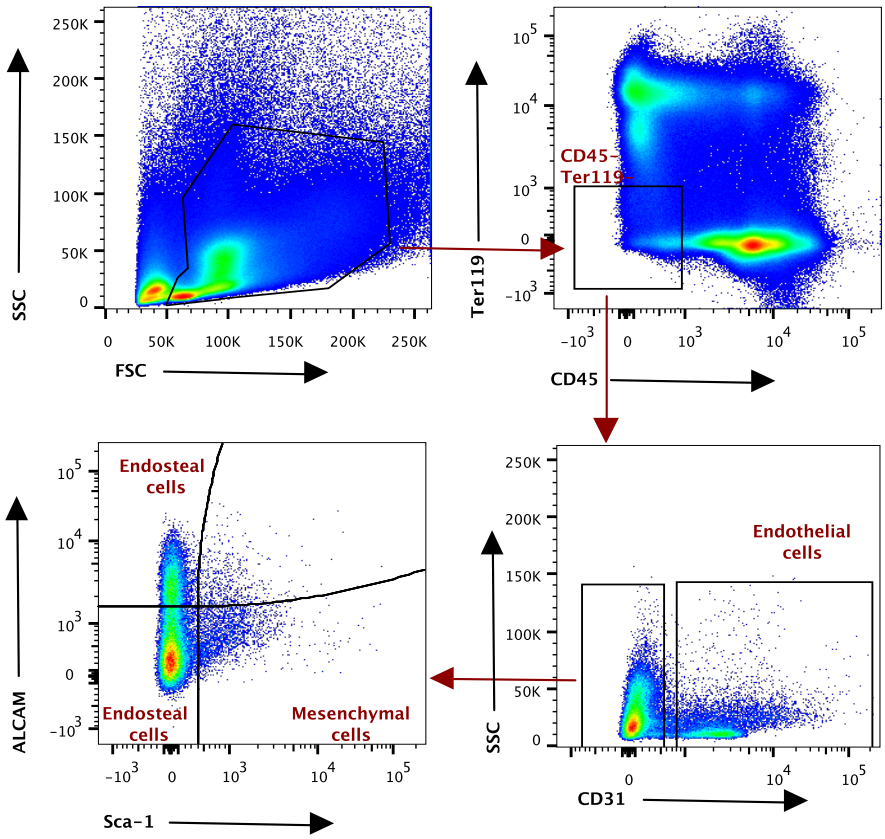
# Appendices




**Appendix 1: Representative FACS plot with gating strategy for the analysis of HSCs and primitive progenitors.**




**Appendix 2: Representative FACS plot with gating strategy for the analysis of mature lineages. (A) Myeloid cells, (B) B-cells and (C) Erythroid cells in the BM and (D) T-cells in the thymus of WT mice.**




**Appendix 3: Representative FACS plot with gating strategy for the analysis of BM niche from WT mice.**

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