THE ROLE OF PROLYL HYDROXYLASES IN THE FUNCTION OF LEUKAEMIC STEM CELLS AND THE DEVELOPMENT OF LEUKAEMIA

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Abstract

Leukaemic stem cells (LSCs) are believed to have originated from haemopoietic stem cells (HSCs) or early progenitors, which reside within a hypoxic niche in the bone marrow. Hypoxic adaption in HSCs is mediated by hypoxia-inducible factor-1 (HIF-1), which, under hypoxic conditions, acts as a transcription factor to induce the expression of hypoxic response genes. Under normoxic conditions, HIF-α is targeted for degradation by Prolyl-4-hydroxylases (PHDs). HIF-1α has been shown to play an essential role in maintaining LSCs. Negative regulator PHD has been implicated in various forms of cancer, although the role of PHD in leukaemia has not been clarified.

The overall aim of this study was to investigate the role of PHD isoforms in the transformation and function of LSCs. A mouse model lacking PHD isoforms was used (PHD KO), allowing the collection of PHD KO haemopoietic stem and progenitor cells (HSPCs). HSPCs were then transformed *in vitro* to produce pre-LSCs which were assessed for cellular function under optimal conditions, hypoxic conditions and in response to chemotherapy.

This study has indicated that under optimal conditions pre-LSCs lacking PHD1, PHD2 or both PHD1 and PHD2 had a reduced growth rate. Pre-LSCs lacking PHD2 also showed a significant increase in apoptosis. Following treatment with chemotherapy or radiation, pre-LSCs lacking PHD1, PHD2 or PHD1 and PHD2 responded better to chemotherapy and radiation treatment and had an increased frequency of cell death.

In vivo experiments were performed in which pre-LSCs were prepared and transplanted to lethally irradiated mice, allowing us to identify if the absence of PHD isoforms had an effect on the leukaemic potential of pre-LSCs. Overall we report that mice transplanted with WT pre-LSCs had impaired survival and developed leukaemia at a higher rate than mice transplanted with pre-LSCs lacking PHD1, PHD2 or PHD1 and PHD2.

Overall these results indicate that PHD isoforms may play a role in the development of leukaemia and the response to treatment and warrants further investigation into the role of PHD in leukaemia.

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Author's declaration

I declare that, except where reference is made to the contribution of others, that the work reported in this thesis is my own and has not been submitted for any other degree at the University of Glasgow or any other institution.

List of Abbreviations

2-ME	2-mercaptoethanol
ALL	Acute lymphoid leukaemia
AML	Acute myeloid leukaemia
APC	Allophycocyanin
ARA-C	Arabinosylcytosine (Cytarabine)
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
bHLH-PAS	Basic helix-loop-helix- PER-ARNT-SIM
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CFC	Colony forming cell
cKO	Conditional knock out
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukaemia
CMP	Common myeloid progenitor
CSC	Cancer stem cell
CTAD	C-terminal activation domain
DAPI	4’6-Diamidino-2-phenylindole dihydrochloride
DMEM	Dulbecco’s Modified Eagle Medium
DMOG	Dimethyloxaloylglycine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxirubicin
DTT	Dithiothreitol
EC	Endothelial cells
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EPO	Erythropoietin
ESC	Embryonic stem cell
FAB	French-American-British
FACS	Florescence activated cell sorting
FCS	Foetal Calf Serum

FH	Fumarate hydratase
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
HBSS	Hank's buffered salt solution
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid
HIF	Hypoxia inducible factor
HRE	Hypoxia response element
HSPC	Haemopoietic stem and progenitor cells
IDH	Isocitrate dehydrogenases
IL	Interleukin
IMDM	Iscove's Modifided Dulbecco Medium
iPS	Induced pluripotent stem cell
KO	Knock out
LDA	Limiting dilution assay
LIC	Leukaemia initiating cell
LSC	Leukaemic stem cell
LT-HSC	Long-term haemopoitic stem cell
$MgCl_2$	Magnesium chloride
MLL	Mixed lineage leukaemia
MPP	Multi-potent progenitor
MSCV	Mouse stem cell virus
NADH	Nicotinamide adenine dinucleotide
NEO	Neomycin
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NH_4Cl	Ammonium chloride
NO	Nitric oxide
NSCLC	Non-small cell lung cancer
NTAD	N-terminal activation domain
ODD	Oxygen dependent domain
PB	Pacific Blue

PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PE	Phycoerithrin
PHD	Prolyl Hydroxylase
PIMO	Pimonidazole
PS	Phosphatidylserine
PURO	Puromycin
PVDF	Polyvinylidene fluoride
pVHL	von-Hippel Lindeu protein
(R)-2HG	(R)-enantiomer of 2-hydroxyglutarate
RBC	Red blood cell
ROS	Reactive oxygen species
SCF	Stem cell factor
SDH	Succinate dehydrogenase
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
SL-IC	SCID leukaemia-initiating cell
ST-HSC	Short-term haemopoitic stem cell
TBE	Tris/Borate/EDTA
TCA	Tricarboxylic acid
TE	Tris EDTA
TGF	Tumour growth factor
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WBC	White blood cell
WHO	World Health Organisation
WT	Wild type

1 Introduction

1.1 The Haemopoietic System

The haemopoietic or blood system is responsible for the maintenance and protection of every tissue in the body. The cells that comprise the haemopoietic system are generated in a strictly regulated process called haemopoiesis. In adult systems, multipotent haemopoietic stem cells (HSCs) located in the bone marrow, are responsible for the production of all the cell types within the haemopoietic system.

1.1.1 Organisation of the stem cell compartment

The haemopoietic system exists as a cellular hierarchy with cells progressing through a number of differentiated states and becoming transiently more mature. HSCs lack the mature markers that are used to identify mature cells but they do express cell markers that are specific to undifferentiated, primitive cells. Primitive cells also show differences in their behavioural patterns and these can be used to organise primitive populations into a hierarchy based on functionality, as well as the expression of cell markers.

Long-term (LT)-HSCs are known to be the most primitive cells within the stem cell hierarchy and to have the unique ability to self renew through symmetrical division, allowing the production of two identical HSCs, whilst also possessing the ability to undergo asymmetrical division to produce one stem cell and one differentiated cell (Suda et al, 2011). This allows the pool of HSCs to be maintained in the bone marrow, preventing them from becoming depleted, during the production or renewal of blood cells, in response to infection or injury. Self renewal is a unique ability in HSCs that is not shared by differentiated or mature cells and is one of the defining qualities of a stem cell. Short-term (ST)-HSCs are considered to be the second most primitive cell population. Like LT-HSCs, ST-HSCs are known to contribute to the generation of lineage positive cells.

Throughout the hierarchy, cells become transiently more mature, eventually committing to a specific lineage of mature blood cell.

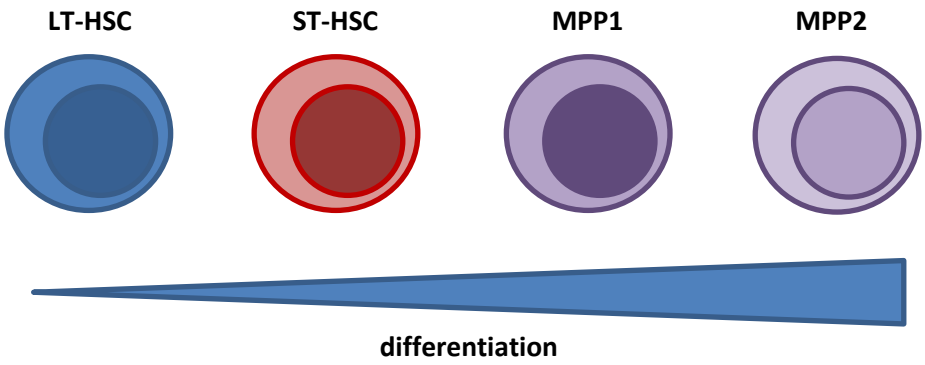


Figure 1.1. Organisation of the stem cell hierarchy.

The haemopoietic system is organised into a cellular hierarchy. Cells can be identified based on the expression of cell surface markers or by their behaviour. LT-HSCs are considered the most primitive cell type within the hierarchy and possess the unique ability to self renew, as well the generation of mature cells. ST-HSCs are also known to contribute to haemopoiesis. MMPs (Multipotent progenitor cells) are considered part of the stem cell hierarchy although they have lost the ability to self-renew. MMPs remain multipotent with an ability to differentiate into all mature cell lineages. Cells are believed to become transiently more mature throughout the hierarchy

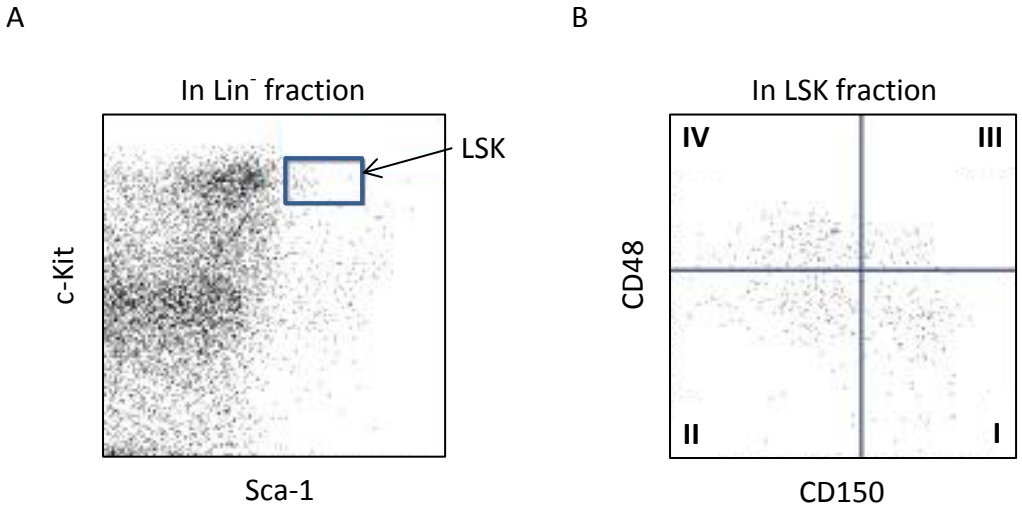
1.1.2 Identification and Isolation of stem cells by stem cell markers

Stem cells can be identified based on the expression of various cell surface markers (Savona & Talpaz, 2008; Benveniste et al, 2009). Identification of these markers also makes it possible to isolate specific populations within the stem cell compartment, in order to study the similarities or the differences between these cells. CD34 is a cell surface marker which is commonly used to identify HSCs. LT-HSCs are known to express low levels of CD34 ($CD34^{lo}$), whereas ST-HSCs are known to express high levels of CD34

($CD34^{hi}$). This difference is used to distinguish these populations and is often used in the study of the human haemopoietic system. The expression of CD34 can be analysed by staining cells with anti-CD34 antibody and determining the expression of CD34 by flow cytometry. LT-HSCs or ST-HSCs populations can also be separated by fluorescent activated cell sorting (FACS), which allows populations to be isolated based on positive staining with specific fluorescent antibodies.

There are several surface markers and specific combinations of surface markers that are used to study HSCs in the mouse (Challen et al, 2009; Wang et al, 2009, Kiel et al, 2005). Cells within the stem cell compartment, including LT-HSCs, ST-HSC and Multi Potent Progenitors (MMPs), are known to lack the expression of lineage markers. As cells become more committed, lineage markers will be expressed dependent on the lineage to which that the cell is committed. However, primitive cells are not committed to any lineage and do not express any lineage markers. Antibody cocktails can be used to stain cells with antibodies specific for most known lineage markers. Cells can then be analysed by flow cytometry and those that are negative for the expression of any lineage markers (Lin-) can then be selected through gating the population. Cells within the stem cell compartment are also known to express high levels of CD117 (c-Kit) and Sca1. Cells within the Lin- fraction can be further gated for the expression of Sca1 and c-Kit, resulting in the identification of the stem cell compartment. This is a common method and is referred to as LSK or KLS gating ($Lin^-Sca^+c\text{-}Kit^+$ or $c\text{-}Kit^+Lin^-Sca^+$). For the purpose of this report, this strategy will be referred to as LSK gating. The stem cell compartment can then be scrutinised further by identifying LT-HSCs, ST-HSCs and MMPs based on the expression of markers CD150 and CD48, commonly referred to 'SLAM' (Signalling Lymphocyte Activation Molecule) staining (Challen et al, 2009; Calaminus et al, 2012). Figure 1.2 represents an example of SLAM staining, depicting how primitive cells can be distinguished. LT-HSCs are known to be $CD48^+CD150^-$ and are therefore seen in the bottom right quadrant of the plot. ST-HSCs are known to be $CD48^-CD150^-$ and are visualised in the bottom left quadrant of the plot. Cells that are $CD48^-CD150^+$ and $CD48^+CD150^+$ are referred to as MMP1 and MMP2 respectively. This method of identifying stem cells is commonly used to isolate populations that can be used in further

studies to assess the properties of specific cells within the stem cell compartment. For example, LT-HSCs or ST-HSCs can be isolated to assess the self renewal potential of individual populations by utilising the reconstitution assay.



(Figure taken from Calaminus et al, 2012)

Figure 1.2. Example of SLAM staining strategy for the isolation of the stem cell compartment.

The stem cell compartment can be analysed following antibody staining and FACS analysis. Cells can be stained with a cocktail of antibodies against common lineage markers to determine the fraction of the cell population that is primitive and is not expressing lineage markers (Lin-). A. Lin- cells can then be analysed for expression of Sca-1 and c-Kit to identify the LSK fraction. B. Within the LSK fraction, cells can be further separated into different quadrants of the stem cell compartment depending on the expression of CD150 and CD48. Dot plot represents typical SLAM gating showing LT-HSCs (I), ST-HSCs (II), MMP1 (III) and MMP2 (IV).

1.1.3 Studying the properties of the stem cell compartment

HSCs also possess other qualities that distinguish them from less primitive cells such as quiescence. HSCs are known to be largely non-dividing with only 1-3% of the HSC population actively proliferating (Venezia et al, 2004). Quiescence in HSCs is considered to be essential to maintain the stem cell pool over long periods. The reduced proliferation

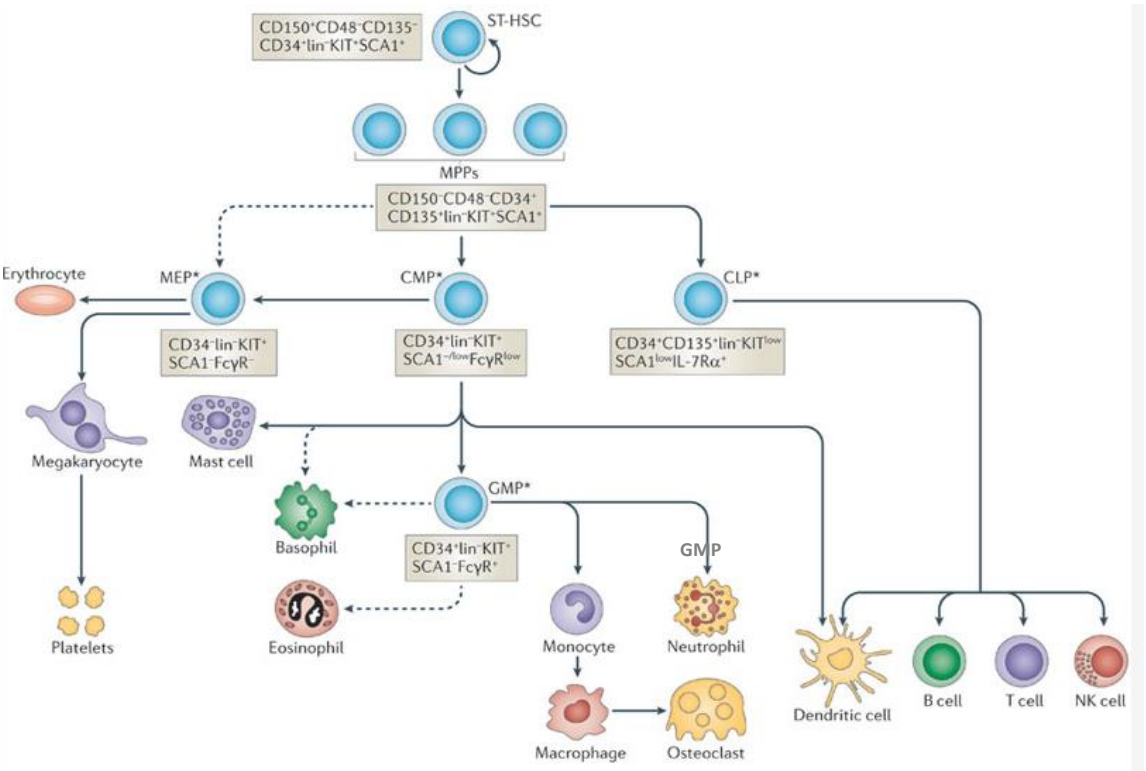
helps to prevent the exhaustion of HSCs, as well as minimising the accumulation of random mutations, which are acquired through proliferation (Thoren et al; 2008; Li, 2011). The bone marrow is thought to contain two pools of HSCs; one pool which divides approximately every 145 days and which is responsible for maintaining the HSC pool, and another which is actively dividing and is responsible for the generation of lineage cells (Forristal et al, 2013).

There are several assays that are used routinely to study the self renewal capacity of HSCs. A reconstitution assay can be used to assess the reconstitution ability or 'stemness' of HSCs following transplantation into recipient mice, which is considered a measure of self renewal ability. HSCs are responsible for the reconstitution of the entire haemopoietic system. HSCs can be isolated by FACS and transplanted to lethally irradiated mice to test their ability to reconstitute the haemopoietic system of the recipient mice. However, the long term survival of the mouse would also depend on the self renewal of HSCs to prevent exhaustion of the HSC pool, diminishing the production of new cells. Failure of the cells to reconstitute the haemopoietic system may be due to a reduced ability to maintain the stem cell pool. This assay is also commonly used in studies which aim to determine the requirement of specific genes in the maintenance of stem cells by assessing the self renewal of HSCs in absence of target genes. Single cell reconstitution assays can be used to examine whether a single HSC has the ability to reconstitute the entire haemopoietic system (Ema et al, 2006). This involves transplanting a single HSC, along with support bone marrow, into a lethally irradiated mouse and monitoring the reconstitution of the blood system by the single HSC. Self renewal can also be assessed using an *in vitro* colony assay. Cells can be seeded into semi-solid media, which when cultured, will allow self renewing cells to develop colonies. Only cells that possess self renewal ability will generate colonies, allowing the frequency of self renewing cells to estimated (Ema et al, 2006).

1.1.4 Generation of mature blood cells

HSCs are the most primitive cell population within the haemopoietic system and are responsible for the generation of multipotent progenitor cells. Progenitor cells become committed towards myeloid or lymphoid lineages, forming either common

myeloid progenitors (CMP), or common lymphoid progenitors (CLP). These immature progenitor cells will continue to differentiate, eventually leading to the generation of mature red and white blood cells. The mature cells of the haemopoietic system consists of red blood cells (erythrocytes) and multiple lineages of white blood cells (leukocytes), which comprise the immune system, all of which must be tightly regulated in order to ensure tissue homeostasis and maximum immune protection.



(Figure taken from Wang & Wagers, 2011)

Figure 1.3. Production of multilineage mature blood cells.

HSCs are responsible for the generation of mature blood cells that are required in tissue homeostasis and effective immune responses. Multiple lineages of blood cells exist and each play distinct role. The regulation of blood cell differentiation is critical in order to ensure adequate and efficient production of all lineage and many factors are involved in regulating this process.

1.1.5 Effector cells of the haemopoietic system

The delivery of oxygen to hypoxic tissues and our immune systems response to potentially harmful pathogens are critical processes in maintaining the health and survival

of our body. Blood cells are required in high quantities and these short lived cells need to be constantly replenished so as not to diminish the blood cell pool and limit the function of the blood system.

1.1.5.1 Red blood cells

Red blood cells or erythrocytes are responsible for the delivery of oxygen around the body. Erythrocytes are rich in haemoglobin, an iron containing molecule that is capable of binding oxygen molecules and which is responsible for the red colour of the cells (Testa, 2004). Erythrocytes circulate the body through the circulatory system where they bind oxygen in the lungs and deliver it to other tissues throughout the body (Hattangadi et al, 2011). Red blood cells circulate for around 120 days before they have to be replaced (Hattangadi et al, 2011). Low oxygen concentration or hypoxia, is an important factor in regulating the production of red blood cells and is considered a signal that an increase in the delivery of oxygen molecules is needed to areas where oxygen is low. Erythrocyte production or erythropoiesis is stimulated in response to Erythropoietin (EPO), a hormone which is released by the kidneys in response to hypoxia (Gregoli et al, 1997; Testa, 2004). The production of red blood cells is critical for the maintenance of oxygen homeostasis within tissues and a number of medical conditions including polycythaemia and anaemia may arise if this process becomes dysregulated (Minamishima & Kaelin, 2010).

1.1.5.2 White blood cells

White blood cells or leukocytes consist of multiple lineages of cells that make up our immune system. Our immune system acts as a defensive barrier that fights against pathogens and infectious agents that invade our body. Immune cells play multiple roles in the prevention of infection and disease with different cell lineages being responsible for different roles. For example, dendritic cells are one of our first lines of defence by circulating and monitoring our blood and tissues for invading pathogens (Fong & Engleman, 2000). They are responsible for recognising and presenting foreign antigens to

other immune cells and initiating an immune response. Macrophages are large phagocytic cells that engulf invading microorganisms and digest them to prevent further infection (Wynn et al, 2013). These are examples of myeloid lineage cells that form our early responses and help initiate a more specific response. Lymphocytes play more specialised roles and can adapt to protect the body against specific pathogens and viruses. Different blood cells are identified by the specific pattern of cell surface markers that they express. The immune system is a large network of cells and every immunological threat requires a different response and the production of specific cells. The production of white blood cells is tightly regulated by a vast network of cytokines and growth factors such as Stem Cell Factor (SCF) and Interleukin-3 (IL-3), which stimulate haemopoiesis and the production of myeloid cells (Robin & Durand, 2010; Runnstrand, 2004). Cytokines are often secreted by circulating immune cells that detect the threat and dictate what type of response is needed.

1.1.6 Control of stem cell fates

Throughout adult life, HSCs are responsible for the maintenance and reconstitution of all the cells of the haemopoietic system (Benveniste et al, 2009; Challen et al, 2009; Wang et al, 2012). They must be able to differentiate into mature blood cells as well continuing to maintain the stem cell pool. Excessive proliferation and differentiation would diminish the stem cell pool and limit the function of the haemopoietic system by preventing the generation of new cells. The HSC stem cell pool is maintained through the tight regulation of several stem cell fates. HSC fates include self renewal, remaining quiescent, differentiating to CLP or CMP lineages and undergoing apoptosis. Stem cell fates are controlled by various factors including intrinsic and extrinsic signals which alter the genetic signature of HSCs and direct them towards the appropriate cell fate.

1.1.7 Hox genes in haemopoiesis

Hox genes are a family of conserved genes, well known for encoding homeodomain-containing transcription factors, which play a critical role in establishing

axial patterning and tissue fate during embryogenesis (Brunschwig et al, 1999). However, Hox genes are also believed to have a role in the regulation of HSCs and their differentiation (Lawrence et al, 1996). In mammals, there are 39 known Hox genes which are arranged into 4 clusters (A, B, C, D) based on sequence homology, and are found on 4 different chromosomes (Argiropoulos & Humphries, 2007). A large majority of Hox genes of clusters A, B and C are known to be expressed in haemopoietic cells (Argiropoulos & Humphries, 2007). The observation that different Hox family members were seen to be expressed in different lineages of haemopoietic cells, led to the implication that Hox may be involved in establishing the lineages of blood cells, much in the same way that they determine tissue fates during development (Lawrence et al, 1996). In addition, studies of Hox gene expression in haemopoietic cells have reportedly shown that the expression of individual Hox genes changes throughout the cell hierarchy. For example, Sauvageau et al found that HOXA10 gene is highly expressed throughout human $CD34^{+}$ cells, but is down regulated in more mature populations. Similarly, HOXB3 was only seen to be expressed in early progenitors (Sauvageau et al, 1994). Since then, several studies have been conducted using retroviral constructs overexpressing Hox genes and transgenic knock out models, aiming to further clarify the role of Hox, and numerous reviews are available detailing the phenotypes of these studies. Hoxa9 is known to be essential for the regulation of haemopoiesis and Hoxa9-defiecient mice show defects in B and T-cell lymphopoiesis and myelopoiesis (Huang et al, 2012). Although the role of Hox genes in haemopoiesis has been intensively studied, the mechanism by which they regulate this process remains largely unknown.

Meis1 is thought to interact with Hox proteins and is frequently linked to haemopoiesis. Like many Hox genes, Meis1 is also known to be expressed in the most primitive cells, but is down regulated in differentiated cells. Although the precise role played by Meis1 is unclear, is it thought to be important in the regulation of haemopoiesis (Argiropoulos et al, 2007). $Meis1^{-/-}$ mice are known to die at day 14.5 of embryonic development due to multiple haemopoietic and vascular defects (Simsek et al, 2010). Many Hox proteins are known to contain binding regions, allowing them to form complexes with several partner proteins including Meis1 (Shen et al, 1997). Studies by Hu

et al suggested that Hoxa9 may play a role in upregulating Meis1. The study showed that overexpression of Hoxa9 in mouse bone marrow cells resulted in the up regulation of Meis1 mRNA and protein. Furthermore, in cells which were lacking Hoxa9 (Hoxa9$^{-/-}$), Meis1 expression was significantly reduced. These studies show a close connection between Meis1 and Hoxa9 expression in the bone marrow and may indicate that expression of both proteins is required for the regulation of haemopoiesis (Hu et al, 2009).

1.2 The Bone Marrow Microenvironment

HSCs reside within the bone marrow, which contains specialized microenvironments that can affect cellular function, referred to as a niche (Kiel et al, 2005). The bone marrow niche was first proposed in 1978 by Schofield (Schofield, 1978) and has since then been the subject of many studies attempting to unravel the controlling factors in HSC function (Lo Celso & Scadden, 2011). Various studies have shown that the endosteal surface of the bone marrow along with osteoblastic cells found in the HSC niche play a role in maintaining HSC and influencing fate decisions (Calvi et al, 2003; Grassinger et al, 2010; Zhang et al, 2003) The niche environment is thought to interact with residing cells, altering their function through the secretion of cytokines and other growth factors, which help the cell to balance between self-renewal, differentiation and expansion (Kiel et al, 2005).

1.2.1 The osteoblastic niche

Different microenvironments within the bone marrow are thought to contribute to different aspects of HSC function. For example, the oesteoblastic niche is associated with LT-HSCs and is thought to regulate quiescence (Eliasson et al, 2010; Forristal et al, 2013). The oestoblastic niche is thought to be located close to the endosteal surface where slow-cycling or quiescent HSCs have been found (Eliasson et al, 2010; Forristal et al, 2013). Studies by Lo Celso et al showed that following transplantation of HSCs

extracted from donor mice to recipient mice, HSCs homed to a region close to the endosteum, with more mature subsets located progressively further from the endosteal region. Furthermore, they found that cells which were quiescent where localised within close proximity to osteoblasts (Lo Celso et al, 2009). Bone-forming osteoblasts that reside within this region have also been linked to HSC maintenance. Studies have shown that an expansion of osteoblasts in mice yielded an increase in HSCs, suggesting that osteoblasts play a role in HSC maintenance (Taichman & Emerson, 1994). Osteoblasts have also been linked to differentiation and are suspected to regulate myeloid differentiation through the secretion of granulocyte-macrophage colony stimulating factor (GM-CSF), a cytokine that is well known to regulate myeloid maturation (Taichman & Emerson, 1994).

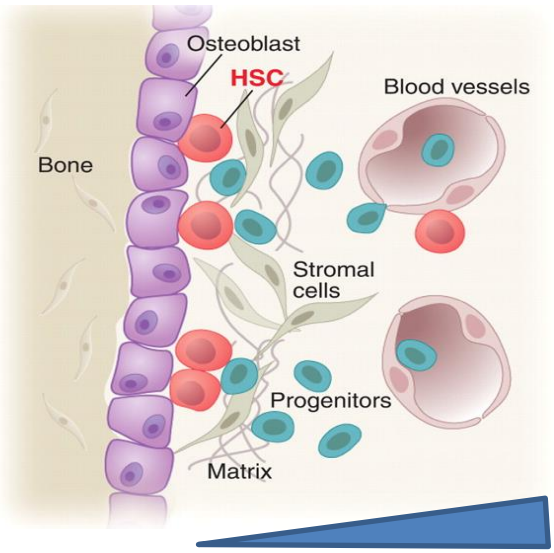
1.2.2 The vascular niche

The vascular niche is more frequently linked to ST-HSCs and proliferation (Forristal et al, 2013). Following injury, HSCs have been seen to mobilize into the vascular regions, where they associate with endothelial cells (ECs). ECs help to create a niche that promotes HSCs expansion (Guerrouahen et al, 2011). HSC expansion is particularly vital following transplantation or injury when there is a risk of the stem cell pool collapsing. Following transplantation, HSCs home to the bone marrow through the recognition and binding of cell surface molecules with their corresponding partners. HSCs express a number of adhesion molecules, which are known to bind with receptors expressed on ECs, allowing HSCs to migrate into the vascular region following transplantation (Guerrouahen et al, 2011). Studies have shown that the co-culture of HSCs with ECs can increase expansion of HSCs through the secretion of various angiocrine factors that promote haemopoiesis (Butler et al, 2010; Kobayashi et al, 2010).

1.2.3 The hypoxic niche

The bone marrow is believed to contain regions of low oxygen concentration that are referred to as hypoxic. The physiological oxygen concentrations (pO2) of the bone marrow can be evaluated using the Kroghian model, a 2D, multilayer mathematical

model. Chow et al devised the model which measures oxygen tensions within the heterogeneous environment of the bone marrow (Chow et al, 2001). Results of this study showed that oxygen tensions within the bone marrow are variable and that most primitive HSCs were located in regions with low oxygen concentration (Chow et al, 2001). Hypoxic regions in the bone marrow are long believed to influence haemopoiesis and low oxygen concentration is well known to regulate the balance amongst differentiation, expansion and quiescence (Pollard et al, 2010).



(Adapted figure taken from Moore & Lemischka, 2006)

Figure 1.4. Architecture of the bone marrow.

HSCs within the bone marrow are suspected to reside within specific niches that help to regulate their functions. HSCs are believed to interact with the niche environment and the cells within them to regulate stem cell fate decisions. HSCs with different proliferation potential are thought to reside in distinct niches. Quiescent, self-renewing LT-HSCs are believed to reside within areas of low oxygen known as the hypoxic niche. Hypoxia and hypoxia signalling is believed to be essential for the maintenance of HSCs.

HSCs are suspected to interact with the hypoxic environment, undergoing changes in their gene expression in order to adapt to this environment. HSCs display a hypoxic profile within the bone marrow which is believed to be essential for their maintenance (Simsek et al, 2010; Nombela-Arrieta et al, 2013). HSCs are known to express Hypoxia

Inducible Factor (HIF-1α), which is thought to be essential for HSC maintenance (Takubo et al, 2010). The hypoxic environment is also considered to be important in the regulation of quiescence in HSCs (Eliasson et al, 2010; Guitart et al, 2010; Forristal et al, 2013).

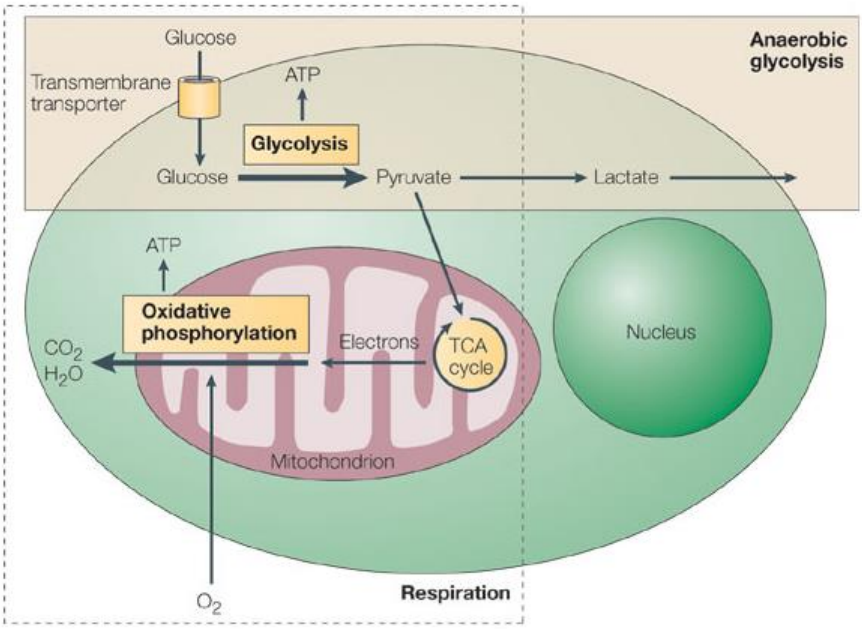
1.2.4 HSCs metabolic response to hypoxia

HSCs are shown to have a slower metabolism and proliferate at a slower rate than more mature cells. The quiescent state of HSCs has been linked to the availability of oxygen and the cells adaptation to the hypoxic environment (Forristal et al, 2011; Eliasson et al, 2010; Guitart et al, 2010).

The unique metabolic profile of HSCs and the reduced metabolic rate is considered to be a response to the hypoxic conditions within the bone marrow. Hypoxic adaption includes the differential regulation of metabolism, which helps the cell to survive without oxygen (Simsek et al, 2010; Suda et al, 2011). Cellular respiration requires oxygen in order to produce sufficient levels of ATP, which is used as an energy source to allow the cell to progress through the cell cycle. However, in hypoxic conditions where the oxygen concentration is low, the cell must adapt by slowing down proliferation or becoming quiescent, in an attempt to reduce its oxygen requirement. The mechanism that allows the cell to adapt to low oxygen is its ability to shift from a conventional method of energy production, the Kreb cycle, to glycolysis.

Complete cellular respiration requires oxygen and therefore, cannot be completed in hypoxic conditions. Energy production involves several cycles of chemical reactions, some of which require oxygen (aerobic) and some of which that do not (anaerobic). The first cycle of respiration is glycolysis. Glycolysis does not require oxygen and so this cycle can be utilised during hypoxia for energy production. This process requires glucose which is broken down to produce two molecules of ATP, two molecules of NADH and two molecules of pyruvic acid or pyruvate (Suda et al, 2011). This stage of energy production yields only small amounts of ATP but does not require oxygen and so can still be utilised in hypoxia. The genetic profile of HSCs that is expressed in hypoxic conditions includes

many of the genes required by the cell to switch to anaerobic respiration (Suda et al, 2011).



(Figure taken from Sitkovsky & Lukashev, 2005)

Figure 1.5. Cellular respiration by glycolysis and the TCA cycle.

Under anaerobic conditions when oxygen is not freely available, glucose is utilised in the production of small amounts of ATP in the process of glycolysis. When oxygen is available, full cellular respiration is possible and the tricarboxylic acid (TCA) cycle and oxidative phosphorylation takes places, allowing larger amounts of ATP to released, providing the cell with the energy source needed for proliferation.

When oxygen is available, the cell is able to complete full aerobic respiration, yielding much greater amounts of ATP. The aerobic stages of respiration are pyruvate oxidation and the TCA cycle or Krebs cycle, which takes place following glycolysis. In the presence of oxygen, pyruvate, produced during glycolysis is oxidised to acetate and subsequently converted to acetyl CoA in the first step in the TCA cycle (Jin et al, 2013). The TCA cycle is a series of chemical reactions which involves both the oxidation and

reduction of molecules. This series of reactions results in the release of free energy which is stored in oxidised electron carriers NAD+ and FAD (Sharma et al, 2005). These molecules go through a process called oxidative phosphorylation in which, electron carriers are oxidized and ATP is formed (Sharma et al, 2005). The TCA cycle is a continual process and products are constantly re-entered into the cycle. The cycle is regulated by the concentrations of starting materials, for example, oxygen. The TCA cycle is commonly linked to hypoxic responses and some of its products are thought to regulate expression of hypoxia signalling factors (Suda et al, 2011). Considering that a metabolic switch to glycolysis is a major response to hypoxia, it is logical that alternative metabolic processes would be involved in controlling this adaptation.

A recent study conducted by Simsek et al looked at the metabolic profile of stem cells and found that HSCs could be characterised by this distinctive profile (Simsek et al, 2010). Furthermore, HSC populations can be enriched based on their metabolic status. The study showed that LT-HSCs have lower levels of mitochondrial respiration, which is likely attributed to the utilisation of glycolysis and the lower energy requirements of the cell. This study also showed that LT-HSCs had a higher rate of glucose-derived ^{13}C lactate, a product of glycolysis, which is further evidence of a metabolic shift to glycolysis. Furthermore, HSCs showed a significant up regulation of numerous hypoxia related genes including Bax, Tgfb1, Notch1 and Epo (Simsek et al, 2010). This suggests that the induction of hypoxia related genes may be responsible for the metabolic shift of HSCs and that these are likely to be essential for the maintenance of HSCs.

1.2.5 Studying HSCs and the hypoxic niche

Although HSCs may appear to be hypoxic, their residence within the hypoxic niche has been challenging to explore and is not confirmed. This is due to a lack of techniques available, capable of achieving live imaging of the bone marrow cavity and the cells within it. The expression of HIF-1α is commonly used as to indicate hypoxia conditions as it is a vital component in hypoxic adaptation. However, a recent study conducted by Nombela-Arrieta et al has highlighted the controversies surrounding the hypoxic nature of HSCs and

how HIF-1α expression may not be an accurate indication of hypoxia (Nombela-Arrieta et al, 2013). In this study, Nombela-Arrieta et al utilized hypoxic marker pimonidazole (PIMO), which forms adducts under hypoxic conditions. Detection of hypoxia by PIMO staining was combined with real time imaging of HSCs within the bone marrow. Results showed that HSCs are not always in hypoxic regions as was originally proposed and that their distribution throughout the bone marrow cavity was random. They also stained for HIF-1α and showed that HSCs expressed HIF1-α regardless of their proximity to hypoxic regions (Nombela-Arrieta et al, 2013). However, regardless of the requirement for hypoxic conditions, the ability of HSCs to adapt to hypoxic conditions is essential for their survival (Takubo et al, 2012).

1.3 The Hypoxia Signalling Network

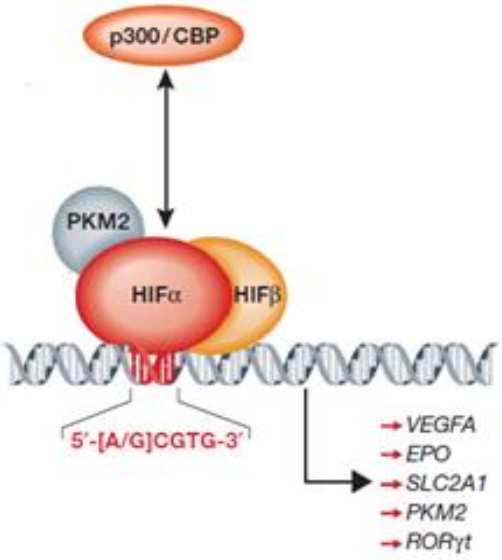
Hypoxia can affect cellular function and behaviour in a number of ways; however, it is needless to say that cells require oxygen to survive and that cells that reside within hypoxia must have the ability to adapt to low oxygen environments. The hypoxia signalling pathway contains several components that are thought to be essential for normal cellular function and survival in hypoxic environments.

1.3.1 Hypoxia signalling

Hypoxic adaption is mediated by the HIF, which facilitates transcription of hypoxia response genes. HIF is known to be responsible for the transcriptional activation of approximately 100-200 genes that aid hypoxic adaptation including genes involved in erythropoiesis, angiogenesis, autophagy and glycolysis (Kaelin & Ratcliffe, 2008).

HIF proteins exist as an unstable alpha subunit (HIF-1α or HIF-2α) located in the cytoplasm, and a stable beta subunit (HIF-1β or HIF-2β), located within the nucleus. In environments of low oxygen or hypoxia, HIF-1α and HIF-2α are stabilised, allowing them to translocate to the nucleus, where they bind to HIF-1β and HIF-2β respectively. The dimerization of HIF subunits allows HIF to recruit transcriptional coactivators p300/CBP and PKM2, which interact with two transactivation domains N-terminal transactivation domain (NTAD) or, and or C-terminal transactivation domain (CTAD) (Kaelin, 2005;

Schofield & Ratcliffe, 2004; Greer et al, 2012). This interaction allows HIF to bind conserved DNA sequences known as hypoxia response elements (HREs), inducing the transcription of the genes needed to facilitate hypoxic adaptation such as VEGF and EPO (Metzen et al, 2002).



(Figure adapted from Greer et al, 2012)

Figure 1.6. Hypoxia signalling by hypoxia inducible factors (HIFs).

Cellular adaption to hypoxic conditions is mediated by the hypoxia signalling pathway. Hypoxia inducible factors (HIFs), are transcription factors that bind to hypoxia response elements (HREs) to induce the transcription of hypoxia response genes. HIF proteins exist as an unstable alpha subunit (HIF-α) and stable beta subunit (HIF-β). Under hypoxic conditions, HIF-α is stabilised, allowing it translocate to the nucleus and dimerise with HIF-β. This dimerisation allows HIF to bind HRE regions as well as co-factors, in order to facilitate transcription of the genes required for hypoxic adaptation.

When oxygen is freely available (under normoxic conditions), hypoxia signalling and therefore HIF expression, is not required and must be repressed. A family of prolyl hydroxylase domain (PHD) proteins can post translationally modify HIF-α, affecting the stability of the alpha subunit and preventing its dimerisation with the beta subunit.

PHDs (also called EglN or HPH family), exist as a family of oxygen-, Fe^{2+}- and α-ketoglutarate (also called 2-oxoglutarate), -dependent family of dioxygenases that can split molecular oxygen and utilize it in the addition of oxygen molecules to target substrates, marking them for recognition by other components (Schofield & Zhang, 1999; Takubo et al, 2010). PHD can hydroxylate specific, highly conserved proline residues in the oxygen dependant domain (ODD) of the HIF alpha subunits, marking HIF for degradation (Jaakkola et al, 2001; Ivan et al, 2001; Yu et al, 2001).

The protein structure of PHD contains a two-histidine-one-carboxylate facial triad which facilitates a catalytic iron centre. This provides two free positions that can bind both 2-oxoglutarate and molecular oxygen simultaneously. Oxygen molecules are split from the co-substrates, one of which is used in the oxidative decarboxylation of 2-oxoglutarate forming succinate and CO_2. The other oxygen atom forms highly reactive iron intermediate Ferryl (Fe^{iv}=O), which binds and oxidises proline residues on HIF-α. PHD also requires ascorbate to complete hydroxylation, which is thought to reduce the levels of catalytic iron that are left uncoupled if HIF-α is not oxidised. This allows PHDs iron centre to remain oxidised and inactive (Kaelin et al, 2002; Schofield & Zhang, 1999). Hydroxylation of Pro-402 and Pro-564 enables von-Hippel Lindeu protein (pVHL) to form two hydrogen bonds with HIF-α, allowing it to bind strongly (Hon et al, 2002; Min et al, 2002). pVHL is an E3 ubiquitin ligase, which catalyses the polyubiquitination and proteasomal degradation of HIF-α.

1.3.2 Regulation of hypoxia signalling

PHDs are often considered to be oxygen sensors and are key factors in the induction of hypoxia signalling and the cells tolerance to hypoxic conditions (Safran & Kaelin, 2003; Schofield & Ratcliffe, 2004). Therefore, the regulation of the hypoxia signalling pathway, often involves the regulation of PHD. PHDs dependence on co-factors helps to regulate the hydroxylation activity of PHD and therefore, the activation of the hypoxia signalling pathway. PHDs are active in normoxic conditions due to the availability of oxygen, which is an essential substrate for PHD activity (Kaelin, 2008). Several

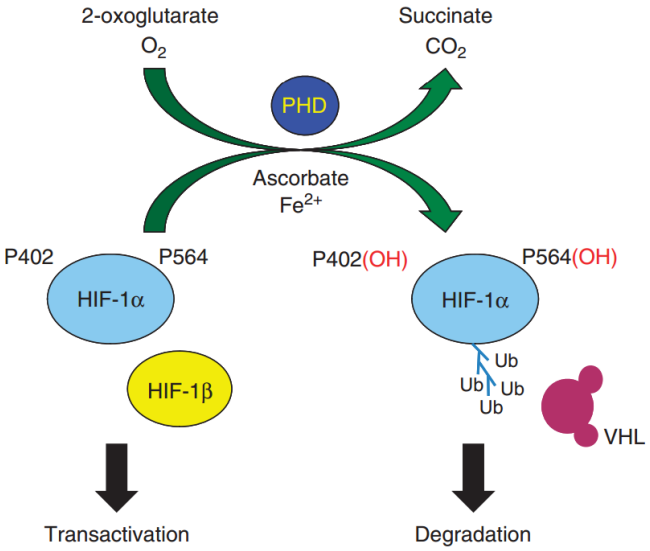
intermediates of the Kreb cycle are also thought to regulate the activity of PHD including citrate, succinate, fumarate, malate, oxaloacetate and pyruvate (Kaelin & Ratcliffe, 2008). These metabolic products are only produced when oxygen is available and when PHD activity is required to prevent activation of the hypoxic pathway and so their concentrations are closely linked to PHD activity. α-Ketoglutarate, also named 2-oxogluterate, is an intermediate product of the Kreb cycle and is also an essential substrate in the hydroxylation reaction. In hypoxia, the Kreb cycle does not take place and α-ketoglutarate is not produced. The absence of α-ketoglutarate inhibits PHD activity and allows HIF-α to stabilise, activating the hypoxic response and facilitating a metabolic switch to glycolysis (MacKenzie et al, 2007).

Fumarate is well known to inhibit PHD activity and is thought to outcompete α-ketoglutarate binding, preventing the hydroxylation reaction. Fumarate hydratase (FH) is the enzyme responsible for the production of fumarate. FH mutations are frequently linked to cancer. (Sudarshan et al, 2011). In cells carrying FH mutations, fumarate accumulates in the cell and inhibits PHD. This leads to the stabilisation of HIF which is commonly associated with poor prognosis (Sudarshan et al, 2011). Succinate is also an inhibitory factor of PHD activity. Succinate is a product of the hydroxylation reaction and is produced by PHD in a negative feedback loop in which PHD can regulate its own activity (Kaelin & Ratcliffe, 2008). Defects in the enzyme succinate dehydrogenase (SDH) have also been linked to cancers, with an accumulation of succinate leading to over stabilisation of HIF (Mackenzie et al, 2007; Pollard et al, 2005; Selak et al, 2005).

Reactive oxygen species (ROS) can inhibit PHD, although the mechanism through how ROS acts is unclear. It is suspected that this is through direct inhibition of PHD or through the up regulation of Kreb cycle intermediates (Kaelin & Ratcliffe, 2008). Nitric oxide (NO) is known to stabilise HIF through direct inhibition of PHD. Interestingly, inducible nitric oxide synthase (iNOS) is a HIF target gene, creating a negative feedback loop and helping to provide tight regulation of HIF expression (Kaelin & Ratcliffe, 2008).

Meis1 has also been linked to the regulation of the hypoxia signalling pathway. Simsek et al provided several lines of evidence which suggested that Meis1 may regulate the expression of HIF-1 (Simsek et al, 2010). This study identified an evolutionary

conserved Meis1 binding site with the sequence of HIF-1. Furthermore, when siRNA was used to knock down Meis1 expression, HIF-1 expression also showed a significant down regulation (Simsek et al, 2010). This suggests that hypoxia signalling may also be regulated by Meis1, a factor which is known to have a role in regulating normal haemopoiesis.



(Figure taken from Chan & Giaccia, 2010)

Figure 1.7. Regulation of hypoxia signalling by PHD.

Prolyl hydroxylases (PHDs) can hydroxylate 2 conserved proline resides on the HIF-α subunit. Hydroxylated proline resides can then be recognised by E3 ubiquitin ligase VHL, resulting in HIF-α becoming polyubiquitinated and marking it for proteasomal degradation. PHD activity is known to be dependent on oxygen, $Fe^{2+,}$ ascorbate and 2-oxoglutarate which are cofactors in the hydroxylation reaction which also results in the formation of CO_2 and succinate.

1.3.3 Multiple isoforms of HIF

HIF proteins exist as heterodimers composed of an α and β subunit. These subunits are part of a basic Helix-Loop-Helix PER-ARNT-SIM (bHLH-PAS) family of transcription factors. In lower animals such as C.elegans and drosophila, only one isoform of HIF exists. (Greer et al, 2012) However, in higher animals, there are three isoforms of HIF proteins, HIF-1, HIF-2 and HIF-3. In humans, HIF proteins are encoded for by

conserved genes namely HIF1A (encodes HIF-1), EPAS1 (encodes HIF-2) and HIF3A, which encodes multiple splice variants of HIF-3 (Xia et al, 2012).

The existence of three conserved HIF genes, suggests that the translated proteins have distinct, non-overlapping functions. HIF-1 and HIF-2 have been extensively studied and there is a lot a literature available concerning the individual properties and functions of these proteins (Holmquist-mengelbier et al, 2006; Hu et al, 2006; Ravel et al, 2005).

Specific HIF-1 target genes, as well as exclusive HIF-2 target genes have been identified, suggesting distinct functions between HIF-1 and HIF-2. However, HIF-1 and HIF-2 have also been shown to have some overlapping targets. The selectivity of HIF target genes is thought to involve the HRE sequences in the CTAD to which HIF proteins bind (Holmquist-mengelbier et al, 2006). Differences in HIF induced gene transcription may also be related to cell type, as well as hypoxic conditions. HIF-1 is seen to be ubiquitously expressed, however HIF-2 transcripts are seen to be enriched in particular cell types such as vascular endothelial cells, kidney fibroblasts, hepatocytes and glial cells (Hu et al, 2006; Park et al, 2003).

Studies by Stroka et al detected the stabilisation of HIF-1α detected in various tissues of mice which were exposed to moderate levels of hypoxia. Immunohistochemical analysis of brain, kidney, liver, heart, and skeletal muscle showed that HIF-1α stabilisation differed between the tissues and was dependant on the length of exposure and the oxygen concentration (Stroka et al, 2001). Bracken et al preformed studies using a variety of non-haemopoietic cell lines showing that HIF1-α was stabilised in the cell lines between 4 and 24 hours following exposure to hypoxia. However, Bracken et al also observed that HIF-1α induction varied between cell lines (Bracken et al, 2006). This study showed that HIF-1α was highly expressed in 293T cells following 2% oxygen exposure, although HIF-1α expression was still detected at 5%. These studies suggest that HIF protein expression and hypoxic response is cell specific and may relate to differences in the cells' sensitivity to hypoxia.

Bracken et al also investigated the stabilisation of HIF-2α protein, which was also observed in all cell lines tested (Bracken et al, 2006). Maximal expression of HIF-2α was

detected following exposure to 1% oxygen and similar cell-specific differences were seen as with HIF-1α stabilisation. However, although a 4 hour hypoxia treatment was sufficient for HIF-1α detection, this was insufficient to stabilise HIF-2α and a prolonged exposure of 16 hours was required (Bracken et al, 2006). This suggests that although HIF-1α and HIF-2α are stabilised under similar conditions, the kinetics of their response differs.

Little is known regarding the functions of lesser known HIF-3 although several splice variants have been described. HIF-1 and HIF-2 both contain two transactivation domains (NTAD and CTAD, as described earlier) (Kaelin & Ratcliffe, 2008). However, some splice variants of HIF-3 are lacking transactivation domains are and thought to act as dominant-inhibitory factors in the transcriptional activity of HIF-1 and HIF-2 (Kaelin & Ratcliffe, 2008). An alternative splice product of HIF-3 termed iPAS, has been seen to bind and inactivate HIF-1 (Makino et al, 2002). iPAS is a target of HIF-1, suggesting a negative feedback loop that helps regulate HIF responses (Makino et al, 2002).

1.3.4 Non-hypoxic roles of HIF

Although HIF is best known for its role in hypoxic adaptation, it has been associated with the activation of several major signalling pathways including the Notch (Gustafsson et al, 2005), Wnt (Kaidi et al, 2007) and Myc pathways (Koshiji et al, 2004). Studies have shown that HREs can interact with Myc binding sites. HIF-1, but not HIF-2, has also been to shown to bind Myc directly, preventing it from binding certain promoters (Gordan et al, 2007; Koshiji et al, 2004). This leads to Myc-dependent genes such as those encoding cyclin D2 and E2F being down regulated, and Myc-repressed genes p21 and p27 being upregulated. These genes are important factors in the regulation of the cell cycle, which implies a regulatory role for HIF in the cell cycle. Non-hypoxic roles of HIF-2 have also been identified. HIF-2 has been shown to repress transcription of cyclin D1 and TGFα, which are essential for cell growth (Kim & Kaelin, 2006). HIF may also have a role in differentiation. HIF-1 is shown to interact with Notch in hypoxic muscle cells to help maintain an undifferentiated phenotype (Gustafsson et al, 2005). HIF-2 is shown to repress Oct4, an essential gene in cell differentiation (Covello et al, 2006). Oct4 is one of

four factors used in the generation of induced pluripotent stem (iPS) cells. The role of HIF in the differentiation of stem cells and the reversion of mature cells to a more stem-like state is currently being investigated.

The requirement for HIF during development has been investigated in studies using C.elegans and drosophila as model organisms (Centanin et al, 2005; Jiang et al, 2001). Inactivation of HIF from these systems allowed the development to adult life, but with only a partially functional oxygen deliver system. However in mammalian development, hypoxia and hypoxia signalling is considered a signal in the developmental process and the deletion of HIF can cause severe developmental abnormalities (Iyer et al, 1998; Ryan et al, 1998). Mouse embryos are known to contain regions of severe hypoxia (Lee et al, 2001). Differences in HIF-2 activity are seen during development. In studies using mouse embryonic stem cells, HIF-2 appeared to bind HREs but did not transactivate them (Hu et al, 2006). Interestingly, when HIF-2 was overexpressed in these cells, transcription of target genes was achieved. This suggests that HIF-2 is being repressed, where HIF-1 transactivation of target genes is preferential (Hu et al, 2006).

1.3.5 Multiple isoforms of PHD

PHD was first discovered as a single isoform that was identified in drosophila and C.elegans, namely dHPH in drosophila and EGLN or EGLN-9 in C.elegans (Fong & Takeda, 2008). However, in mammalian cells, four isoforms of the PHD has been discovered - PHD1, PHD2, PHD3 and PHD4. Belonging to the same gene family as EGLN-9, these mammalian isoforms are also sometimes referred to as EGLN1, EGLN2, EGLN3 and HPH-4 (Metzen et al, 2002). Few studies have been published regarding the specific functions of PHD4, however it is reported to play a role in the regulation of HIF (Bruik et al, 2001). However, PHD1, PHD2 and PHD3 have been more fully characterised and many studies have been concluded, reporting multiple biological roles of each isoform, each with distinct functions and specificities for HIF, although all three isoforms are reported to play a role in hypoxia signalling regulation (Takeda et al, 2008; Nytko et al, 2011). PHD1 and PHD3 are known to be more active in the regulation of HIF-2α, whereas PHD2 is best

known for its role in the regulation of HIF-1α and is often considered to be the most critical regulator of hypoxia signalling (Appelhoff et al, 2004). In cell based studies by Takeda et al, it was seen that PHD2 deficiency in the liver and kidney of mice, resulted in the accumulation of HIF-1α but not HIF-2α (Takeda et al, 2007). Further studies by this group also showed that an accumulation of HIF-2α was seen in the kidneys of mice that were deficient in both PHD1 and PHD3 (Takeda et al, 2008). However, it is unclear as to whether PHD isoforms can compensate for each other. A study by Berra et al recently gave some insight into compensatory functions of PHD in hypoxic regulation. This study used siRNA to ablate each PHD isoform individually, in order to assess the effect of HIF expression and function, based on luciferase activity. The study showed that ablation of PHD2 allowed the accumulation of HIF-1α, which remained fully functional, suggesting that PHD1 and PHD3 were not able to compensate for the hypoxic regulation of PHD2 (Berra et al, 2003). Furthermore, ablation of PHD1 and PHD3 did not affect the degradation of HIF-1α or the mRNA levels of PHD2, further suggesting that only PHD2 is essential for hypoxic regulation of HIF-1α (Berra et al, 2003). However, this study did not investigate the effect of PHD ablation on HIF-2α, which is thought to be preferentially regulated by PHD1 and PHD3. Differential regulation of specific HIF isoforms may also be linked to the sensitivity of PHDs to oxygen levels. As previously discussed, many of the factors regulating the activity of PHD are related to the availability of oxygen and the induction of hypoxia signalling. However, PHD isoforms have been shown to have different tolerances to hypoxia and may even have some activity under hypoxic conditions. In a study by Metzen et al, PHD2 and PHD3 were both seen to be induced following exposure to hypoxia. The mRNA levels of PHD isoforms were measured following exposure to hypoxia, suggesting that both PHD2 and PHD3 may limit the activity of HIF in both normoxic and hypoxic conditions (Metzen et al 2002).

PHD isoforms are also seen to have differences in their expression patterns of the three isoforms. The study conducted by Metzen et al also identified the localization of PHD isoforms by fusing GFP tags to PHD proteins. PHD2 was seen to be mainly localised in the cytoplasm, whereas PHD1 was seen to be exclusively nuclear. PHD3 was found to be expressed in both the cytoplasm and the nucleus but at very low levels (Metzen et al

2002). However, in a separate study by Huang et al, PHD3 was seen to localise predominantly in the cytoplasm (Huang et al, 2012). The disagreement between these studies may be reflected in the observation that the expression levels of PHD are also known to differ between cell types. Although PHDs are known to be widely expressed, some cell types have higher levels of expression and may produce different results in cell based experiments. For example, the study by Metzen showed that expression levels of PHD3 were barely detectable in HeLa cells by qPCR, whereas, PHD3 is known to be expressed at high levels in the heart (Kaelin & Ratcliffe, 2008). PHD1 mRNA levels are also reported to be more highly expressed in the testes. It is likely that the higher expression seen in these cells types may be related to the non-hypoxic functions of these proteins.

1.3.6 Non-hypoxic roles of PHD

PHDs are also known to function in a HIF-independent manner, where they can regulate the expression of target proteins, with or without hydroxylation (Hiwatashi et al, 2011). As well as hypoxia signalling, PHDs have been linked to several cellular functions including proliferation, metabolic regulation and angiogenesis (Hiwatashi et al, 2011), although much more work needs to be done in this area to define the functions of these proteins.

PHD1 and PHD3 have been seen to regulate ATF-4 (Koditz et al, 2007). ATF-4 is known to be induced following ER stress, oxidative stress and amino acid deprivation (Hiwatashi et al, 2011). ATF-4 induces transcription of many genes involved in apoptosis, mitochondrial function and redox balance and its regulation by PHD creates a possible role for PHD in these processes. Studies conducted by Koditz et al utilized a yeast-2-hybrid system to identify novel interactions of PHD proteins. Their results showed an interaction between PHD1 and ATF-4 and PHD3 and ATF-4, a member of the ATF/CREB family of transcription factors which are known to induce transcription of stress response genes (Koditz et al, 2007). Koditz et al also reported that HeLa cells which were treated with dimethyloxaloylglycine or DMOG, a PHD inhibitor, or exposed to hypoxia, showed an increase in ATF-4 activity, suggesting that PHD negatively regulates ATF-4. In support of

this finding, they also showed that when cells exposed to hypoxia were reoxygenated, ATF-4 was quickly degraded. Hiwatashi et al confirmed the interaction between PHD3 and ATF-4 by performing an immunoprecipitation assay using tagged AFT-4. They showed that PHD3 co-precipitated with ATF-4, suggesting a direct interaction between the two proteins (Hiwatshi et al, 2011). Furthermore, they also showed an interaction between PHD1 and ATF-4 through co-precipitation. However, this study showed that ATF-4 did not act as a substrate for PHD. They proposed that ATF-4 regulation did not involve direct hydroxylation and that it was possible that another unidentified molecule was involved (Hiwatshi et al, 2011). It has recently been suggested that ATF-4 may play a role in cancer progression. A recent study showed that blocking ATF-4 expression blocked tumour growth, suggesting a possible role for PHD in cancer (Kumanoua et al, 2010).

PHD has also been associated with the NF-κβ pathway which is known to play a role many biological processes, proposing a possible role for PHD in apoptosis, differentiation, proliferation and immune responses (Fu & Taubman, 2010). PHD3 is seen to be upregulated during myogenic differentiation and its expression is thought to promote skeletal muscle differentiation by negatively regulating NF-κβ (Fu & Taubman, 2010). Studies by Fu et al have shown that cells treated with DMOG PHD inhibitor had enhanced activation of the NF-κβ signalling pathway. The induced expression of NF-κβ target genes YY1 and Cyclin D1 was also seen (Fu et al, 2007). The induction of Cyclin D1 has been proposed as a possible mechanism by which NF-κβ negatively regulates myogenesis. PHD1 has also been reported to bind and inhibit Iκκβ, which is known to regulate NF-κβ activity (Cummins et al, 2006).PHD3 has been seen to be a negative regulator of NF-κβ in colon adenocarcinoma cells. It is suspected that PHD3 mediates apoptosis in neutrophils at least partially through the NF-κβ signalling pathway (Fu et al, 2007; Fu & Taubman, 2010).

PHD3 is well known to play a role in the regulation of cell death (Lee et al, 2005). In studies using synthetic neurons, PHD3 expression is seen to be upregulated during the stress-induced response of cells, following the withdrawal of nerve growth factor (NGF) (Lipscomb et al, 1999). Lipscomb et al also showed that PHD3 is required for neural apoptosis during development. Studies using a catalytically inactive mutant of PHD3 also

showed that cell death is dependent on the hydroxylation activity of PHD (Lipscomb et al, 1999). The mechanism by which PHD3 regulates apoptosis is not fully understood. Although, it has been proposed that this may involve the regulation of kinesin family member 1B-β (KIF1B-β), which is involved in apoptosis (Schlisio et al, 2008). However, under hypoxic conditions PHD3 may play a contradictory role in regulating apoptosis in other cell types. Walmsley et al showed that apoptosis was increased in neutrophils under hypoxic conditions in PHD3$^{-/-}$ mice. An increase in pro-apoptotic factor Siva-1 and a down regulation of anti-apoptotic factor Bcl-xl was also seen (Walmsley et al, 2011).

PHD2 has also been linked to the regulation of cell death in cardiac cells. Natarajan et al performed ischemia-reperfusion in murine hearts, which resulted in large infarcts forming due to extensive cell death. The study showed that PHD2 knockdown in cardiac cells reduced infarct sizes, suggesting that PHD2 may promote cell death in the heart (Natarajan et al, 2006).

A role for PHD3 in proliferation has also been proposed. Hogel et al showed that PHD3 deficiency in several different cell lines including HeLa cells and primary squamous carcinoma cell lines, resulted in the inhibition of cell growth under hypoxic conditions. Cell cycle arrest at G1 was seen in cells, as well as an upregulation of negative cell cycle regulator p27/Kip (CDKN1B) (Hogel et al, 2011). Interestingly, PHD3 ablation did not appear to affect cell growth under normoxic conditions.

1.3.7 The requirement of hypoxia signalling in HSCs

It has long been believed that HSCs reside in hypoxia and therefore, hypoxia adaptation and HIF expression are essential. However, recent studies by Nombela-Arrieta et al showed that HSCs do not always reside in hypoxic regions but do express HIF-1α (Nombela-Arrieta et al, 2013). This suggests that although the role of hypoxia in HSC maintenance has been challenged, the expression of HIF-1α in HSCs throughout the bone marrow does suggest an important role for HIF-1α in HSC maintenance. In accordance with this, a study by Takubo et al provided evidence that HIF-1α is essential for the maintenance of HSCs (Takubo et al, 2012). This study analysed the mRNA expression of

HIF-1α, HIF-2α and HIF-3α in different populations within the stem cell compartment. They found that all 3 isoforms of HIF-α were highly expressed in LT-HSCs compared to other haemopoietic populations. Next the study aimed to identify the role of HIF-1α in the maintenance of HSCs through the conditional deletion of HIF-1α in the haemopoietic system. A bone marrow reconstitution assay was used to determine the effect of HIF-1α deletion on reconstitution ability. Upon initial analysis, HIF-$1\alpha^{-/-}$ cells appeared to have improved chimerism, as determined by the frequency of donor derived cells in the peripheral blood, compared to host cells. A significant increase in the transcription of *Ink4a*, a marker for senescent stem cells, indicated that this may be attributed to increased proliferation although a correlation between these factors was not examined. Analysis at 4 months post transplantation indicated that HSC levels had decreased, suggesting that HSCs are not able to sustain long-term maintenance in the absence of HIF-1α due to senescence-induced exhaustion. Furthermore, upon secondary transplantation of LSK cells, HIF-$1\alpha^{-/-}$ cells were unable to reconstitute the haemopoietic system. To analyse the impact of Ink4a upregulation on HSC maintenance, cells were transduced with Bmi retrovirus which causes repression of Ink4a. This restored the ability of HIF-$1\alpha^{-/-}$ cells to reconstitute the bone marrow, suggesting that HIF-1α plays a role in protecting HSCs from exhaustion by mediating Ink4a gene products. Interestingly, Takubo et al also showed that HIF-1α overexpression, through the deletion of negative regulator VHL, caused a loss of stem cell capacity *in vivo*, suggesting that a tight regulation of HIF-1α is required for the maintenance of HSCs.

However, recent studies by Guitart et al have suggested that HIF-2α may not be required for HSC maintenance. Mice lacking expression of HIF-2α in the haemopoietic system were used to determine if HIF ablation affects the HSCs ability to maintain multi-lineage haemopoiesis. Bone marrow was analysed by flow cytometry to identify the frequency of mature lineage cells such as T cells, B cells and erythroctyes. Results showed that ablation of HIF-2α or both HIF-1α and HIF-2α had no effect on the generation of progenitor cells. Furthermore, frequencies of LT-HSCs and ST-HSCs were unaffected, suggesting that HIF-2α is not required for HSC maintenance or function (Guitart et al, 2013). However, in this model, HIF-2α deletion was only in haemopoietic cells and

remained functional within the bone marrow niche, posing the question if HIF-2α expression within the niche is required for HSC maintenance. In another mouse model, HIF-2α is conditionally deleted in all cells following injection with Poly(I) Poly(C) which induces deletion of the target gene. Bone marrow from HIF-$2\alpha^{fl/fl}$ mice was transplanted to recipient mice to assess the ability of HSCs to reconstitute the haemopoietic system. Results showed that mice injected with bone marrow lacking HIF-2α expression in the entire bone marrow had a significant reduction of LT-HSCs, suggesting that HIF-2α is required for long term maintenance of HSCs in a non-cell autonomous manner (Unpublished by Guitart et al).

The requirement of PHD in HSC maintenance is unclear. It has recently been published that PHD2 has an essential role in the maintenance of HSCs (Singh et al, 2013). Singh et al utilized a mouse model in which PHD2 was conditionally deleted throughout the haemopoietic system. They reported that conditional knockout (cKO) mice had almost 50% more LSK cells in the bone marrow compared to WT mice. Similarly, they found a significant increase in the frequencies of MPPs. Singh et al also investigated the proliferation rate of LSKs and found that cKO LSKs showed a higher rate of actively cycling cells compared to WT LSKs. Following analysis of apoptotic frequencies, no differences were seen between the genotypes. They also found a significant induction of SMAD7, which promotes cell cycle progression through inhibition of TGF-β. Next the group examined if this phenotype was dependent of HIF by generating a mouse model deficient for both PHD2 and HIF-1α. They found that the ablation of HIF-1α abolished the phenotype seen in cKO mice and LSK and MPP frequencies were similar to WT counterparts. This study also examined the effect of PHD2 ablation under stressful conditions. Competitive bone marrow transplantation was performed in which, cKO or WT LSK cells (CD45.2) are transplanted alongside CD45.1 competitor cells, to lethally irradiated mice. Recipient bone marrow was analysed 4 weeks after transplantation. cKO donor cells showed significantly lower chimerism compared to WT cells, showing that PHD2 deficient LSKs had a diminished ability to repopulate. These data show that PHD ablation leads to increased proliferation under steady state conditions and a diminished

reconstitution ability under stressful conditions. This suggests that PHD2 does play a role in maintaining the stem cell compartment in a HIF-1α dependent manner.

However, work conducted by Guitart et al have not been able to confirm these results, casting uncertainty on the reliability of this study and on the requirement of PHD in HSC maintenance. Unpublished studies by Guitart et al analysed the stem cell compartment of mice lacking PHD2 within the haemopoietic system to identify if PHD2 had a role in haemopoiesis. Results showed that mice lacking PHD2 had frequencies of LT-HSCs, ST-HSCs and MPPs which were comparable to WT mice, suggesting that PHD2 is not required for the maintenance of the stem cell compartment. Competitive bone marrow transplantation assays were also conducted where $PHD2^{fl/fl}$ bone marrow was transplanted to recipient mice. $PHD2^{fl/fl}$ bone marrow was seen to successfully reconstitute the bone marrow of the recipient mice suggesting that the reconstitution ability of HSCs is unaffected by PHD2 ablation (Unpublished by Guitart et al). The contradictory results seen in these 2 studies means that the role of PHD2 in HSC maintenance is still unclear and that more work needs to be undertaken in order to confirm this.

1.3.8 Hypoxia signalling in disease

The mammalian system must have the ability to adapt to hypoxic conditions by increasing the efficiency of oxygen delivery and reducing the consumption of oxygen, in order for cells to survive in low oxygen conditions. Hypoxic adaptation induces various biological processes that help cells to adapt including angiogenesis, glycolysis and erythropoiesis. These are tightly regulated processes and it stands to reason that a defect in the regulation of hypoxia signalling, such as up or down regulation of PHD, could have effects on these hypoxia-sensitive functions. This is particularly crucial when there has been significant tissue damage, or in organs where large, constant supplies of oxygen as required, for example, the heart. It is for this reason that PHD and HIF isoforms have received much attention for their potential as therapeutic targets.

As previously discussed, erythropoiesis is a tightly regulated process which produces red blood cells, through stimulation by EPO (Rankin et al, 2007). EPO is a well-known HIF target gene suggesting that the regulation of erythropoiesis is closely linked to the regulation of hypoxia signalling. A defect in the regulation of erythropoiesis can cause a decrease in the production of red blood cells in conditions such as anaemia. Overexpression of PHD has also been reported in anaemia, likely caused by over-degradation of HIF and a decreased production of EPO (Minamishima & Kaelin, 2010).

Polycythaemia is a condition that results in the overproduction of red blood cells and which has been associated with the overexpression of HIF, resulting from the ablation of PHD genes. Studies by Takeda et al investigated the role of PHD1, PHD2 and PHD3 individually in the regulation of erythrocytosis, an increase in red cell mass. They found that mice lacking PHD1 and PHD3 together had significantly increased numbers of RBCs. Interestingly, mice lacking single isoforms of either PHD1 or PHD3 had no apparent differences in blood production. However, mice lacking PHD2 (PHD2 cKO) showed signs of severe polycythaemia with increased blood counts and dramatically increased levels of EPO and HIF-1α (Takeda et al, 2008). In a separate study conducted by Rankin et al, mice were treated with PHD inhibitor DMOG to assess the effect on erythropoiesis. The study also showed that HIF was stabilized following treatment with DMOG and EPO production was increased. However, this same result was seen in mice lacking VHL, suggesting that HIF stabilisation, rather than PHD ablation, is responsible for increased EPO production (Rankin et al 2012). It has also been reported that PHD2 mutations have been identified in patients with increased erythropoiesis and polycythaemia (Percy et al, 2007). This was also confirmed by studies using mice. However, in a study by Minamishima et al, they found that the mice that had increased red blood cell production due to inactivation of PHD2, died prematurely following the development of cardiomyopathy (Minamishima et al, 2008).

1.4 Leukaemic Development

1.4.1 Acute myeloid leukaemia

AML (Acute Myeloid Leukaemia) is a heterogeneous disease with over 100 genetic aberrations being linked to its development (Zuber et al, 2009). AML can be separated into subtypes which are based on morphological features of the disease as determined by the French-British classification method which was proposed by Bennett et al in 1976 (See Table 1). However, a more recent model proposed by the World Health Organisation uses more up to date techniques such as flow cytometry and molecular analysis to classify AML based on the identification of genetic aberrations through (See Table 2).

AML emerges through the overgrowth of immature progenitors and haemopoietic cells in the bone marrow and peripheral blood known as blast cells. Current treatment for AML patients involves induction chemotherapy followed by further cycles of chemotherapy and stem cell transplantation (Zuber et al, 2009; Wunderlich et al, 2013; Pardee, 2012). However, patients show varied responses to treatment and in some subsets of AML, relapse is more common. Many AML patients achieve complete remission but relapse in this disease reduces mortality to a five year survival in just 50% of patients (Guinn et al, 2007). The median age of AML patients is 63 years old. This creates a further complication of many patients being ineligible for stem cell transplantation due to their age and the risks associated with surgery, reducing the rate of remission to just 11% (Guinn et al, 2007).

FAB classification	Morphological features
M1	Predominance of myeloblasts with <10% granulocytic differentiation
M2	More than 30% myeloblasts with >10% differentiating granulocytes, NSE <20%
M3a	Hypergranular promyelocytes with numerous Auer rods on Wright-stain or CAE
M3b	A variant form showing cells with bilobed, multibed or reniform nuclei (NSE-negative) and relative scarcity of hypergranular promyelocytes or of primitive cells with multiple Auer rods
M4	Monocytic cells with >20% but <80% NSE-butyrate positivity
M5	Monocytic cells with >80% NSE positivity
M5a	Monoblastic, poorly differentiated
M5b	Monoblastic, differentiated
M6	More than 50% erythroblasts with >30% myeloblasts excluding the erythrothroid cells

(Table adapted from Neame et al,1986)

Table 1.1. French-American-British classification of AML subtypes.

The French-American-British (FAB) classification model was proposed in order to standardise the way that AML is classified to make it easier for results in different institutes to be compared. The FAB system classifies AML into subtypes based on morphological and cytochemical features of the disease.

WHO classification of AML
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22), *(AML1/ETO)*
AML with abnormal bone marrow eosinophils and inv(16)(p13.1q22) or t(16;16)(p13.1;q22), *(CBFβ-MYH11)*
Acute promyelocytic leukaemia with t(15;17)(q22;q12), *(PML/RARα)* and varients
AML with 11q23 (MLL) abnormalities
AML with with multilineage dysplasia
Following MDS or MDS/MPD
Without antecedent MDS or MDS/MPD, but with dysplasia in at least 50% of cells in 2 or more myeloid lineages
Acute myeloid leukaemia and myelodysplastic syndromes, therapy related
Alkylating agent/radiation–related type
Topoisomerase II inhibitor–related type (some may be lymphoid)
Others
Acute myeloid leukaemia, not otherwise categorized
Classify as:
Acute myeloid leukaemia, minimally differentiated
Acute myeloid leukaemia without maturation
Acute myeloid leukaemia with maturation
Acute myelomonocytic leukaemia
Acute monoblastic/acute monocytic leukaemia
Acute erythroid leukaemia (erythroid/myeloid and pure erythroleukaemia)
Acute megakaryoblastic leukaemia
Acute basophilic leukaemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma

(Table adapted from Vardiman et al, 2002)

Table 1.2. World Health Organisation classification of AML subtypes.

The World Health Organisation (WHO) classification method was proposed to categorise AML based on the genetic abnormalities found in various subgroups. Many of the criteria used in the FAB classification system is incorporated into the WHO method. However, this system also considers more recent advances in the identification of genetic changes through advancement of medical diagnostic techniques.

1.4.2 Cancer stem cells

The existence of cancer stem cells (CSCs) is constantly debated but is well documented in AML. The cancer stem cell theory encompasses the idea that not all tumour cells are capable of initiating disease when transplanted. Rather, that there is a rare population of self renewing stem cell-like cells which are responsible for initiating disease. This was first seen when a single human AML cells was transplanted into SCID mice. Mice developed leukaemic disease that recapitulated human AML. The primitive cell that was responsible for initiating disease was termed the SCID leukaemia-initiating cell (SL-IC). Purification of these cells confirmed that SL-ICs were exclusively $CD34^{+}CD38^{-}$. (Lapidot et al, 1994). Further studies by this group also showed that SL-ICs are capable of differentiating *in vivo* to recapitulate the original disease, whilst maintaining self renewal capacity (Dick & Bonnet, 1997). Histochemical and flow cytometric analysis was performed to determine the differentiation of transplanted cells. NOD/SCID mice were transplanted with either sorted $CD34^{+}CD38^{-}$ SL-ICs or unfractionated samples from AML patients. Bone marrow of mice transplanted with unfractionated cells showed undifferentiated leukaemic cells. However, bone marrow transplanted with $CD34^{+}CD38^{-}$ SL-IC showed myeloblastic and monoblastic differentiation (Dick & Bonnet, 1997). Furthermore, upon serial transplantation to secondary recipients, $CD34^{+}CD38^{-}$ SL-ICs successfully engrafted recipient bone marrow showing that cells had retained their self renewal capacity.

1.4.3 Leukaemic stem cells

As previously discussed, LSCs are responsible for initiating leukaemia. The self renewal capacity of LSCs, as well as similarities in phenotype, suggest that early leukemic mutations may arise in self renewing HSCs (Al-Hajj et al, 2004). The similar properties between HSCs and LSCs would mean that fewer mutations would be needed for the cell to achieve LSC status and leukaemic potential. The self renewal capacity of LSCs is an important characteristic of these cells which is not seen in non-LSCs. Much like the haemopoietic stem cell compartment, leukaemia is also considered to be organised into a hierarchy with LSCs having the potential to self renew and to generate leukaemic cells

(Bonnet & Dick, 1997). This difference in properties between LSCs and leukaemic blast cells has led to further studies, investigating if these differing properties have clinical relevance in diagnosis and may be potential therapeutic targets (Eppert et al, 2011). Studies by Eppert et al have identified an LSC-specific genetic signature that has been shown to be negatively correlated to patient outcome. This LSC-signature will be important in future work to identify novel therapies that can target LSC-specific genes, many of which are involved in the regulation of self renewal (Eppert et al, 2011). Furthermore, many mutations implicated in carcinogenesis have also been seen to be involved in the regulation of self-renewal including Wnt, Bmi-1, Notch and Sonic hedgehog signalling pathways (Al-Hajj et al, 2004). However, the HSC origin of LSCs has been challenged by more recent genetic profiling of LSCs which has suggested that the transcriptional program expressed by LSCs is closer to that of embryonic stem cells (ESCs) rather than HSCs (Somervaille et al, 2009). The expression of ESC programs is seen in more mature progenitor cells suggesting that LSCs may arise from progenitors, rather than HSCs, (Somervaille et al, 2009). Furthermore, the immunophenotypic analysis of LSCs has also shown that this may be closer to the immunophenotype of granulocyte-macrophage progenitors (GMP) rather than that of stem cells, leaving the cell of origin for LSCs widely debated (Goardon et al, 2011). It is possible, and generally accepted that pre-LSCs may arise from haemopoietic stem and progenitor cells residing within the hypoxic niche in the bone marrow. Studies have indicated that LSCs may also interact with the same niche environment adopted by HSCs, in order to sustain their ability to self-renew (Lane et al, 2011). Studies following the localisation of transplanted human AML LSCs ($CD34^{+}CD38^{-}$) showed that LSCs preferentially home to the endosteal region, which is commonly thought of as hypoxic and is considered the prefer site for normal HSCs (Zhang et all, 2003; Calvi et al, 2003). Furthermore, LSCs were seen to continue residence within this region for up to 4 months following transplantation and during AML development within the bone marrow (Ishikawa et al, 2007).

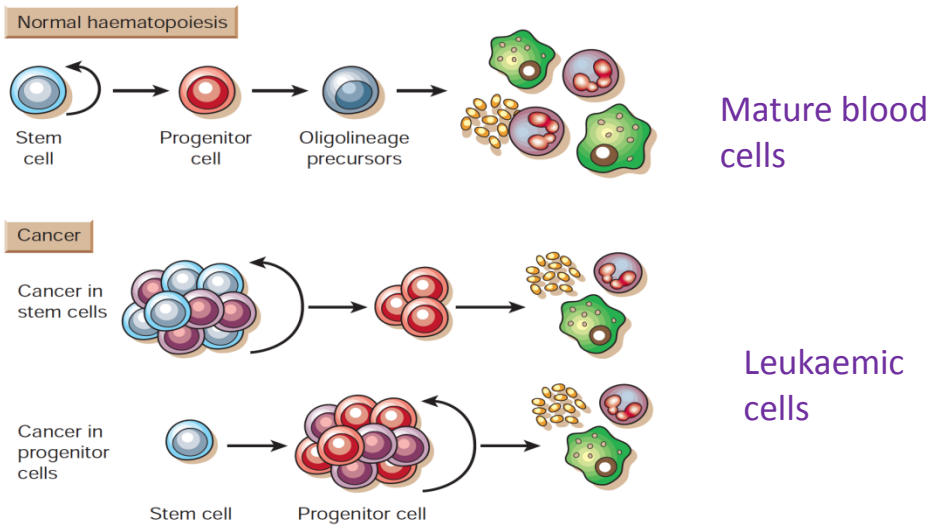


Figure adapted from Reya et al, 2001.

Figure 1.8. The origin of leukaemic stem cells from haemopoietic stem cells.

Leukaemic stem cells (LSCs) are capable of initiating leukaemia. LSCs share many functional and immunophenotypical properties with HSCs. It has also been proposed that LSCs share properties with early progenitor cells and they are believed to originate from either stem cells or early progenitor cells. LSCs are responsible for the generation of leukaemic blast cells in the same way that HSCs are responsible for the generation of blood cells.

1.4.4 Genetic factors in the development of AML

Mixed lineage leukaemia (MLL) gene encodes a histone methyltransferase which is essential in embryonic development and haemopoiesis (Wang et al, 2011). Human AML is often associated with chromosomal translocations of MLL, which results in various MLL fusions that are often associated with poor survival rates and a higher risk of patient relapse following treatment (Glaser et al, 2011; Wang et al, 2011). Previous studies have identified 64 translocation partner genes, with which, MLL can fuse with to form fusion products such as MLLT3/AF9, MLLT1/ENL and MLLT10/AF10, all of which have been identified in human samples (Meyer et al, 2009). The fusion proteins that result from MLL translocations are known to induce AML development by acquiring novel biological functions. This often involves the constitutive activation of target genes, including

transcription factors, leading to dysregulation of gene expression. MLL fusions can result in the overexpression of multiple genes that are associated with AML including Meis1 and Hoxa9.

1.4.5 Hoxa9 and Meis1 in AML development

Hox genes are well known for the role they play in the development of AML and are reportedly upregulated in human AML (Eklund, 2011). Hoxa9 has been described as 1 of 20 genes that distinguish AML from acute lymphoid leukaemia (ALL) (Dorsam et al, 2004). Several Hox genes, including Hoxa9, are known to be target genes of MLL and the induction of Hoxa9 is common in MLL-induced leukaemia (Dorsam et al, 2004). In addition, MLL has been seen to bind to the promoter region of multiple Hox proteins (Eklund, 2011). The expression of Hoxa9 has been shown to correlate closely with treatment outcome and is associated with poor prognosis in AML patients (Hu et al, 2009; Dorsam et al, 2004). Hox genes are frequently overexpressed in MLL-leukaemias and are also involved in the self renewal of normal stem cells, making their dysregulation an advantageous target for leukaemic development (Wong et al, 2007). Numerous studies have shown that several Hox family members are dysregulated in cancers including lung, breast, colon, prostate and ovarian cancer (Kelly et al, 2011).

The mechanism through which Meis1 and Hoxa9 induce leukaemia is unclear. However, studies have indicated that Meis1 may form complexes with other leukaemic factors such as Pbx1/2 (Wang et al, 2006). Meis1 and Pbx1/2 are also known to form complexes that help regulate the expansion of progenitors in normal haemopoiesis. These leukaemia-inducing complexes are thought to bind and activate promoters that are required for leukaemogenesis. The exact nature by which Meis1 and Hoxa9 interact is also unclear. However, Hox proteins are known to induce transcription of genes that are also regulated by Meis1. It is believed that Hoxa9 directly interacts with Meis1, forming complexes that regulate normal haemopoiesis and possibly leukaemogenesis (Wang et al, 2006).

Meis1 and Hoxa9 are often used as tools to study the development of AML in mice. Bone marrow cells collected from mice can be transduced with Meis1 and Hoxa9 oncogenic viruses, resulting in the over expression of these genes in bone marrow cells (Kroon et al, 1998; Wang et al, 2006). Bone marrow cells over expressing Meis1 and Hoxa9 can then be transplanted to lethally irradiated recipient mice. Transplanted recipient mice are known to develop AML that is similar to human AML within 2-6 months (Kroon et al, 1998; Wang et al, 2006; Gibbs et al, 2012). This allows a fast and efficient system in which to study the development of AML as well as the role of Meis1 and Hoxa9 in leukaemogenesis.

However in previous models, Hox dysregulation alone does not recapitulate MLL leukaemia (Wong et al, 2007). Studies by Wong et al showed that co-expression of Hoxa9 with a known binding partner Meis1, significantly shortens the latency period of the disease as well as recapitulating many of the same features seen in MLL-AML (Wong et al, 2007). Similarly, Thorsteinsdottir et al showed corresponding results that show that Meis1 and Hoxa9 cooperate to induce disease similar to human MLL-AML and with a short latency (Thorsteinsdottir et al, 2001). Meis1 and Hoxa9 are frequently shown to be expressed together in patients with MLL-induced (Imamura et al, 2002; Huang et al, 2012). Studies by Hu et al examined human leukaemia bone marrow samples. They found correlated expression of Meis1 and Hoxa9 in all 21 leukaemic samples. These studies have shown that overexpression of Hoxa9 and Meis1 may produce a useful system for studying MLL-AML.

1.5 The Role of Hypoxia Signalling in Cancer

1.5.1 HIF proteins in cancer development

Solid tumours are well known to contain hypoxic regions which are thought to influence the survival of cancer cells. In response to hypoxic conditions, tumour cells have been seen to adapt pro-survival changes in gene expression, allowing tumour cells to

grow within the hypoxic environment. These pro-survival changes have led to them being considered hypoxic tumour cells which are often considered to have a more aggressive phenotype and are often associated with metastasis and resistance to conventional chemotherapy (Poon et al, 2009). Hypoxia in tumours has been seen to enhance tumour angiogenesis, vasculogenesis and metastasis whilst repressing apoptosis and immune response (Wilson & Hay, 2011). Hypoxia signalling is also frequently linked to cancer and both HIF and PHD have been implicated in various forms of cancer. However, much like their normal biological functions, the exact role of each isoform appears to be cancer-specific.

HIF proteins have been implicated in melanoma, lung cancer, breast cancer, colorectal cancer, neuroblastoma and leukaemia (Noguera et al, 2009; Ryan et al, 2000; Acker et al, 2005; Carmeliet et al, 1998; Koh et al, 2011). Studies by Ryan et al adopted the cre/loxP system to create HIF-1α null cell lines, which were used to investigate the role of HIF-1α in tumours derived from ES cells. HIF-1α null cell lines were transformed and transplanted in to immunocompromised mice which developed solid tumours. Results showed that the absence of HIF-1α resulted in a significant decrease in tumour mass, suggesting a role for HIF-1α in regulating tumour growth and implicating HIF-1α as a tumour promoter in solid tumours (Ryan et al, 2000). However, a separate study by Carmeliet et al showed contradictory results (Carmeliet et al, 1998). Carmeliet et al showed that HIF-1α-deficient tumours had impaired vascular function with a decreased ability to form new blood vessels, resulting in tumours with large areas of hypoxia (Carmeliet et al, 1998). This study also showed that tumours lacking HIF-1α showed accelerated growth which was attributed to a decrease in hypoxia-induced apoptosis and an increase in stress-induced proliferation. These findings imply that HIF-1α may play a negative role in tumour development acting as tumour suppressor.

1.5.2 HIF in leukaemic development

HIF expression has also been implicated in leukaemia (Martin et al, 2011; Wang et al, 2011; Matsunaga et al, 2012; Zhang et al, 2012; He et al, 2013). The factors involved in

LSC maintenance and leukaemogenesis are largely unknown. However, the requirement of HIF and hypoxia signalling in HSC maintenance provides a possible link between HIF and leukaemogenesis. Several studies have reported a role for HIF in the development of various subsets of leukaemia.

A possible role for HIF-1α in the development of chronic myeloid leukaemia (CML) has also been identified (Zhang et al, 2012). Zhang et al showed that HIF-1α target genes were highly upregulated in CML LSCs carrying the BCR-ABL mutation, a product of the causative chromosomal translocation of CML. HIF-1α target genes were upregulated in HSCs transformed with BCR-ABL when compared to normal HSCs, suggesting that the presence of BCR-ABL may upregulate HIF-1α. This study also aimed to identify the requirement of HIF-1α in CML by using HIF-$1\alpha^{fl/fl}$ mice, where HIF-1α is deleted in the haemopoietic system. HIF-$1\alpha^{fl/fl}$ BM cells were transformed using BCR-ABL retrovirus, before being transplanted to lethally irradiated mice, in order to develop CML. Following development of CML, leukaemic cells were used to conduct a secondary transplantation. Following the secondary transplant, HIF-$1\alpha^{fl/fl}$ CML cells failed to induce CML in recipient mice, although WT CML cells did induce disease. This suggests that HIF-1α may be required for the development of CML (Zhang et al, 2011).

Matsunaga et al investigated the role of HIF-1α in resistance to chemotherapy (Matsunaga et al, 2012). AML LSCs have been reported to localise within hypoxic regions in the bone marrow. Hypoxic treatment of solid cancers has been seen to induce cell cycle arrest and it is this mechanism that is suspected to convey chemoresistance in hypoxic AML LSCs. Considering this, AML cell lines were subjected to hypoxic conditions. Cells were then treated with common leukaemia agent Ara-C. Cells subjected to hypoxia were seen to express high levels of HIF-1α and were seen to be significantly less sensitive to treatment compared to normoxic LSCs. This suggests that the hypoxic induction of HIF-1α in AML may contribute to chemoresistance that is often seen in AML (Matsunaga et al, 2012).

Recently, Wang et al conducted a study providing evidence that HIF-1α expression is required for the maintenance of CSCs in haematological malignancy (Wang et al, 2011). Cultured lymphoma cells were transplanted to immunocompromised mice in order for

lymphoma tumours to develop in recipient mice. Selected tumour-bearing mice were then treated with HIF-1α inhibitor echinomycin. Untreated mice all developed disease and survived just 6-10 weeks. Mice treated with echinomycin were euthanized at 134 or 252 days with no sign of tumour development. A significant upregulation of Notch1 target Hes1 was also seen, suggesting that HIF-1α may be involved in the development of lymphoma by mediating Notch1 signalling.

1.5.3 PHD in cancer development

PHD2 is frequently associated with cancer and a high incidence of PHD2 mutations are reported in endometrial cancers (Kato et al, 2006). Chan et al performed studies to determine the role of PHD in colorectal cancer and whether this role is dependant of HIF (Chan et al, 2009). Studies show that PHD2 expression was decreased in colorectal tumours compared to surrounding colon tissue, suggesting that PHD2 may regulate tumour development. Chan et al used colorectal carcinoma cells in which, HIF-1α, PHD2 or both HIF-1α and PHD2 were silenced. Transplantation of cells to SCID mice resulted in a significant increase in tumour growth in absence of PHD2. Tumours lacking PHD2 also showed increased blood vessel formation, suggesting that PHD2 functions as a tumour suppressor by negatively regulating angiogenesis. Tumours lacking both HIF-1α and PHD2 grew faster than tumour lacking only HIF-1α. This suggests that the mechanism by which PHD2 influences tumour growth is not dependent on HIF expression (Chan et al, 2009).

Studies by Lee et al also suggest a role for PHD2 as a tumour suppressor (Lee et al, 2008). Human fibroblast cell lines that have acquired malignant characteristics and become fully transformed following the acquisition of a further random genetic mutation, were used to assess the level of transformation in cells, based on the production of colonies in an in vitro colony formation assay. This study showed that an overexpression of PHD2 altered the transformed phenotype of the cells and several samples lost the ability to form colonies altogether. Furthermore, PHD2 expression was reported to

negatively correlate with transformation of cell lines, suggesting a tumour suppressor role for PHD2 (Lee et al, 2008).

PHD2 has also been suggested to play a role in the response to chemotherapy. Leite de Oliveira et al investigated the role of PHD2 by transplanting B16 melanoma cells to WT and $PHD2^{+/-}$ mice, which developed melanoma tumours. Mice were then treated with chemotherapeutic agent cisplatin. Platinum accumulation was measured to assess the effect of treatment. Intratumoural platinum levels and drug diffusion within the tumour was higher in $PHD2^{+/-}$ mice, suggesting that PHD2 deletion increases the efficiency of drug delivery. Furthermore, following treatment with suboptimal doses of cisplatin, WT tumours were unaffected. However, $PHD2^{+/-}$ tumours were reduced by more than 70%, suggesting that PHD2 ablation increased the response to chemotherapy. Interestingly, PHD2 deletion also conveyed a protective role in organs that are often affected by the side effects of chemotherapy. The level of apoptotic cells in the kidneys of treated mice were high in WT mice but were barely detectable in $PHD2^{+/-}$ mice. The effect of cardiac output was also assessed and was seen to be reduced by 22.3% in WT mice but was unaffected in $PHD2^{+/-}$ mice. These interesting results not only support a role for PHD2 in tumour response to chemotherapy but also the response of distant organs to the side effects of chemotherapy (Leite de Oliveira et al, 2012).

Several studies have also indicated a role for PHD3 as a tumour suppressor. PHD3 expression has been seen in a variety of human cancers such as pancreatic cancer, lung cancer, colorectal cancer, breast cancer and gastric cancer (Su et al, 2012; Anderson et al, 2011). The expression of PHD3 has been associated with well differentiated tumours and is considered a prognosticator for good survival rates in gastric cancer (Su et al, 2012). Tennant and Gottlieb investigated the role of PHD in tumour growth by treating tumours with α-ketoglutarate, which is known to activate PHD (Tennant & Gottlieb, 2010). Various cancer cell lines were used to develop xenograph tumours in mice. Following treatment with α-ketoglutarate and subsequent activation of PHDs, tumour growth was inhibited. Furthermore, increased apoptosis was seen, suggesting that the reduction in tumour growth was attributed to increased cell death. shRNAs targeting PHD1, PHD2 and PHD3 were used to determine which isoform was responsible for the reduced tumour growth.

Results showed that xenograph tumours lacking PHD3 expression showed no effect on growth, following treatment with α-ketoglutarate. These findings suggest that PHD3 plays a tumour suppressor role by influencing tumour growth through increased apoptosis (Tennant & Gottlieb, 2010).

The overexpression of PHD1, PHD2 and PHD3 has been seen in non-small cell lung cancer (NSCLC) and are associated with poor survival rates (Anderson et al, 2011). PHD1 and PHD2 expression is also seen in pancreatic cancer and are correlated to negative outcome. These studies implicate PHDs are tumour promoters. However, there are numerous studies indicating a tumour suppressor role for PHD. Much like their normal biological functions, the exact role of each isoform appears to be cancer specific, making it difficult to speculate their roles in cancers that have not been directly studied.

1.5.4 PHD in leukaemic development

To date, the role of PHD in leukaemia has not been identified. The cancer and cell specific functions of PHD, as well as the existence of both HIF-dependent and HIF-independent functions, make it difficult to speculate the role of PHD in leukaemia. However, several studies assessing the role of isocitrate dehydrogenases (IDH) mutations in leukaemia have provided some insight. IDH mutations are seen in 20% of AML (Xu et al, 2011). IDH1 and IDH2 are metabolic enzymes that convert isocitrate to α-ketoglutarate which is known to regulate PHD. Therefore, a dysregulation in IDH activity may disrupt the regulation of PHD. IDH mutations in cancer are seen to convert 2-OG to (R)-enantiomer of 2-hydroxyglutarate ((R)-2HG), which is known to stimulate PHD activity (Koivunen et al, 2012; Losman et al, 2013). Studies by Losman et al showed that cells passaged in the presence of (R)-2HG displayed two hallmarks of leukaemia; enhanced proliferation and impaired differentiation. Following the suggestion that this may be attributed to the inhibition of 2-OG-dependent enzymes, such as PHD, the study investigated the role of PHD in transformation. shRNA was used to knockdown PHD activity. PHD inhibition abrogated the transformed phenotype of the cells, suggesting that PHD may be required for leukaemic transformation (Losman et al, 2013). Other studies have shown that cells containing tumour-derived mutant IDH1 (IDH1 R132H), had

enhanced proliferation, along with increased levels of (R)-2HG (Koivunen et al, 2012). Koivunen et al showed that (R)-2HG expression promoted the activity of PHD1 and PHD2. These results suggest that PHD may influence transformation of IDH-mutated cells and it is possible that PHD may be involved in the transformation of IDH-associated leukaemia. However, this study also showed a downregulation of HIF-1α, which is seen to be required for leukaemic transformation, suggesting a more complicated role for hypoxia signalling in leukaemogenesis that requires much further investigation.

1.6 Thesis Aims

The overall aim of this study is to investigate the role of PHD isoforms in the transformation and function of leukaemic stem cells. This aim can be broken down as follows:

1. To optimise an in vitro assay which can be used to retrovirally transduce HSPCs in the generation of pre-LSCs, which can be used in further studies

A previously published methylcellulose-based in vitro assay will be adapted in order to achieve the generation of transformed pre-LSCs. HSPCs will be retrovirally transduced with Meis1 and Hoxa9 viruses and seeded into methylcellulose, to allow cells to become transformed over several passages. Transformed pre-LSCs developed in this assay can then be used to achieve the further objectives of this study.

2. To assess the self renewal capacity and transformation of HSPCs in the absence of PHD genes.

The in vitro assay utilized in the transformation of HSPCs can be used to assess the self renewal capacity of cells, and in addition, can give an indication of the transformed status of cells. Transduced HSPCs are seeded into methylcellulose cultures, in which, self renewing cells will generate colonies, which can be quantified in order to give a measure of self renewal. Comparison of colony production between WT HSPCs and HSPCs lacking PHD genes (PHD KO) can indicate if PHD plays a role in self renewal. Transformation is known to enhance self renewal capacity, therefore, measuring the self renewal of WT and PHD KO cells can also indicate is PHD ablation affects the transformation of HSPCs to pre-LSCs. In addition, the immunophenotype of pre-LSCs can be assessed in order to determine if the phenotype of transformed cells is affected in the absence of PHD.

3. To examine the role of PHD isoforms in the control of biological properties of pre-LSCs.

WT and PHD KO pre-LSCs generated in the in vitro transformation assay will be used to establish cell lines, which will then be used to perform various biochemical assays to assess the biological functions of pre-LSCs in the absence of PHD. Cell viability, cell cycle status, mitochondrial function and apoptosis will be examined and compared between WT and PHD KO cell lines to assess if PHD ablation affects the biological function of pre-LSCs.

4. To investigate the leukaemic potential of pre-LSCs lacking PHD isoforms.

Similar studies utilizing the in vitro transformation assay have reported that pre-LSCs have the ability to generate leukaemia when transplanted to immunocompromised mice. The role of PHD in leukaemia is unknown. WT and PHD KO pre-LSCs will be transplanted in to lethally irradiated mice in order to examine if PHD ablation affects the leukaemic potential of pre-LSCs *in vivo*.

5. To investigate the role of PHD in the resistance to chemotherapy.

Hypoxia signalling has been implicated in the response to chemotherapy in other cancers; therefore, we sought to investigate if PHD plays a role in the response of leukaemic cells to chemotherapy. Pre-LSC cell lines will be used and treated with common leukaemia agents Ara-C and doxorubicin. Cell viability, cell growth and apoptosis will be compared between WT and PHD KO cells in order to determine if cells respond differently to chemotherapy treatment in the absence of PHD.

2 Materials & Methods

2.1 Materials

2.1.1. Mice

Strain	Genotype	Sex	Genotype	Mouse ID
PHD1/2/3Vav	WT	M	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	1659
PHD1/2/3Vav	WT		Not known	1961
PHD1/2/3Vav	WT	M	$Phd1^{+/+}Phd2^{fl/fl}PHD3^{+/+}Vav^{-}$	2183
PHD1/2/3Vav	WT	M	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	2603
PHD1/2/3Vav	WT	F	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	2608
PHD1/2/3Vav	WT	F	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	2609
PHD1/2/3Vav	WT	M	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	2862
PHD1floxVav	WT	M	$Phd1^{+/+}Vav^{-}$	3389
PHD1floxVav	WT	M	$Phd1^{+/+}Vav^{+}$	3390
PHD1/2/3Vav	WT	M	$Phd1^{+/+}Phd2^{+/+}Phd3^{+/+}Vav^{-}$	3408
PHD1/2/3Vav	WT	M	$Phd1^{+/+}Phd2^{+/+}Phd3^{+/+}Vav^{-}$	3492
PHD1floxVav	WT	M	$Phd1^{+/fl}Vav^{-}$	3814
PHD1/2/3Vav	WT	M	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	3829
PHD1/2/3Vav	WT	M	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	3830
PHD1/2/3Vav	WT	F	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	4311
PHD1/2/3Vav	WT	M	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	4310
PHD1/2/3Vav	WT	F	$Phd1^{+/+}Phd2^{+/fl}Phd3^{+/+}Vav^{-}$	031380
PHD1floxVav	WT	M	Vav^{-}	4734
PHD1/2/3Vav	PHD1 KO	M	$Phd1^{-/-}Phd2^{+/+}Phd3^{+/+}Vav^{-}$	1666
PHD1/2/3Vav	PHD1 KO		Not known	2003
PHD1floxVav	PHD1 KO	M	$Phd1^{fl/fl}Vav^{+}$	3388
PHD1floxVav	PHD1 KO	F	$Phd1^{fl/fl}Vav^{+}$	3392
PHD1/2/3Vav	PHD1 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	3588
PHD1/2/3Vav	PHD1 KO	M	$Phd1^{-/-}Phd2^{+/fl}Phd3^{+/+}Vav^{-}$	3589
PHD1floxVav	PHD1 KO	F	$Phd1^{fl/fl}Vav^{+}$	3817
PHD1floxVav	PHD1 KO	F	$Phd1^{fl/fl}Vav^{+}$	3819
PHD1floxVav	PHD1 KO	F	$Phd1^{fl/fl}Vav^{+}$	3820
PHD1/2/3Vav	PHD1 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	4165
PHD1/2/3Vav	PHD1 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	4171
PHD1/2/3Vav	PHD1 KO	F	$Phd1^{-/-}Phd2^{+/+}Phd3^{+/+}Vav^{+}$	4175
PHD1/2/3Vav	PHD1 KO	M	$Phd1^{-/-}Phd2^{+/+}Phd3^{+/+}Vav^{+}$	4342
PHD1/2/3Vav	PHD1 KO	M	$Phd1^{-/-}Phd2^{+/+}Phd3^{+/+}Vav^{-}$	4619
PHD1/2/3Vav	PHD1 KO	M	$Phd1^{-/-}Phd2^{+/fl}Phd3^{+/+}Vav^{-}$	4620
PHD1/2/3Vav	PHD2 KO		Not known	1960
PHD1/2/3Vav	PHD2 KO	M	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2184
PHD1/2/3Vav	PHD2 KO	F	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2190
PHD1/2/3Vav	PHD2 KO	F	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2191
PHD1/2/3Vav	PHD2 KO	M	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2602
PHD1/2/3Vav	PHD2 KO	F	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2607

PHD1/2/3Vav	PHD2 KO	F	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2610
PHD1/2/3Vav	PHD2 KO	M	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2863
PHD1/2/3Vav	PHD3 KO	M	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{-/-}Vav^{-}$	3989
PHD3floxVav	PHD3 KO	F	$Phd3^{fl/fl}Vav^{+}$	4655
PHD3floxVav	PHD3 KO	F	$Phd3^{fl/fl}Vav^{+}$	4656
PHD1/2/3Vav	PHD3 KO	F	$Phd1^{+/+}Phd2^{+/+}Phd3^{-/-}Vav^{-}$	031385
PHD1/2/3Vav	PHD3 KO	F	$Phd1^{+/+}Phd2^{+/+}Phd3^{-/-}Vav^{-}$	031386
PHD1/2/3Vav	PHD3 KO	F	$Phd1^{+/+}Phd2^{+/+}Phd3^{-/-}Vav^{-}$	031387
PHD1/2/3Vav	PHD3 KO	F	$Phd1^{+/+}Phd2^{+/+}Phd3^{-/-}Vav^{-}$	031388
PHD1/2/3Vav	PHD3 KO	F	$Phd1^{+/+}Phd2^{+/+}Phd3^{-/-}Vav^{-}$	031389
PHD1/2/3Vav	PHD1/2 KO		Not known	2000
PHD1/2/3Vav	PHD1/2 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2871
PHD1/2/3Vav	PHD1/2 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2872
PHD1/2/3Vav	PHD1/2 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	4174
PHD1/2/3Vav	PHD1/2 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	4621
PHD1/2/3Vav	PHD1/3 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{-/-}Vav^{-}$	2874
PHD1/2/3Vav	PHD1/3 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{-/-}Vav^{-}$	2876
PHD1/2/3Vav	PHD1/3 KO	M	$Phd1^{+/+}Phd2^{+/fl}Phd3^{-/-}Vav^{-}$	3351
PHD1/2/3Vav	PHD1/3 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{-/-}Vav^{-}$	3352
PHD1/2/3Vav	PHD1/3 KO	F	$Phd1^{-/-}Phd2^{+/fl}Phd3^{-/-}Vav^{-}$	3354
PHD1/2/3Vav	PHD1/3 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{-/-}Vav^{-}$	3591
PHD1/2/3Vav	PHD1/3 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{-/-}Vav^{-}$	3594
PHD1/2/3Vav	PHD1/3 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{-/-}Vav^{-}$	3620
PHD1/2/3Vav	PHD1/3 KO	F	$Phd1^{-/-}Phd2^{+/fl}Phd3^{-/-}Vav^{-}$	3854
PHD1/2/3Vav	PHD1/3 KO	M	$Phd1^{-/-}Phd2^{+/+}Phd3^{-/-}Vav^{-}$	3826
PHD1/2/3Vav	PHD1/3 KO	M	$Phd1^{-/-}Phd2^{+/fl}Phd3^{-/-}Vav^{-}$	3984
PHD1/2/3Vav	PHD1/3 KO	M	$Phd1^{-/-}Phd2^{+/+}Phd3^{-/-}Vav^{+}$	4027
PHD1/2/3Vav	PHD1/3 KO	M	$Phd1^{-/-}Phd2^{+/fl}Phd3^{-/-}Vav^{-}$	4219
PHD1/2/3Vav	PHD1/3 KO	F	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{-/-}Vav^{-}$	4340
PHD1/2/3Vav	PHD1/3 KO	F	$Phd1^{-/-}Phd2^{+/fl}Phd3^{-/-}Vav^{-}$	4451
PHD1/2/3Vav	PHD1/3 KO	F	$Phd1^{-/-}Phd2^{+/fl}Phd2^{-/-}Vav^{-}$	4452
PHD1/2/3Vav	PHD1/2/3 KO	F	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{-/-}Vav^{+}$	3992
PHD1/2/3Vav	PHD1/2/3 KO	M	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{-/-}Vav^{+}$	4337

Table 2.1. Details of mice used for *in vitro* analysis

2.1.2 Cell lines

2.1.2.1 Commercial cell lines

Cell line	Origin
NIH3T3	Mouse Fibroblasts
Plat-E	Commercially generated, available from Cell Biolabs Inc.

Table 2.2. Details of commercial cell lines

Details of commercially available cell lines used in this study, along with their origin, are listed in table 2.2. NIH3T3 cells are commonly used and were available 'in house'. NIH3T3 cells were chosen to test efficiency of retroviral production and transfection due to their murine origin and accessibility. Commercially available Platinum-E (Plat-E) retroviral packaging cell line was chosen for the production of virus. Plat-E cells are specially designed for rapid production of high-titre ecotropic retrovirus.

2.1.1.2 Established murine cell lines

Cell line	Original Mouse Genotype	Mouse ID
WT	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	1659
WT	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	2609
WT	$Phd1^{+/+}Phd2^{+/+}Phd3^{+/+}Vav^{-}$	3408
WT	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	3829
WT	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{-}$	4310
PHD1 KO	$Phd1^{-/-}Phd2^{+/+}Phd3^{+/+}Vav^{-}$	1666
PHD1 KO	$Phd1^{fl/fl}Vav^{+}$	3817
PHD1 KO	$Phd1^{fl/fl}Vav^{+}$	3820
PHD1 KO	$Phd1^{fl/fl}Vav^{+}$	3819
PHD1 KO	$Phd1^{-/-}Phd2^{+/+}Phd3^{+/+}Vav^{-}$	4619
PHD1 KO	$Phd1^{-/-}Phd2^{+/fl}Phd3^{+/+}Vav^{-}$	4620
PHD2 KO	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2190
PHD2 KO	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2863
PHD2 KO	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2607
PHD2 KO	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2191
PHD2 KO	$Phd1^{+/+}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	2610
PHD12 KO	$Phd1^{-/-}Phd2^{fl/fl}Phd3^{+/+}Vav^{+}$	4621

Table 2.3. Details of mice used to establish cell lines for *in vitro* analysis.

Details of cell lines established during the study, along with details of mice used to collect cells, are listed in table 2.3. The methods by which cell lines were established are detailed in section 2.3.5.5.

2.1.3 Plasmids

Plasmids were used in this study to overexpress genes known to have a role in leukaemogenesis. Meis1 and Hoxa9 plasmids were constructed using an MSCV backbone and were a kind gift from Tim Somervaille, (Paterson Institute for Cancer Research, Manchester). Meis1 plasmid contained a puromycin resistance cassette allowing successfully transfected cells to be selected using puromycin antibiotic. Hoxa9 plasmid contained a neomycin resistance cassette and successfully transfected cells were selected using neomycin. A packaging plasmid VSV-G was also used to provide the necessary enveloping proteins. Addition of VSV-G plasmid is known to increase efficiency of retroviral transfection (Okimoto et al, 2001). VSV-G plasmid was available 'in house'. MSCVneo and MSCVpuro plasmids were used to generate control viruses lacking Meis1 and Hoxa9 inserts.

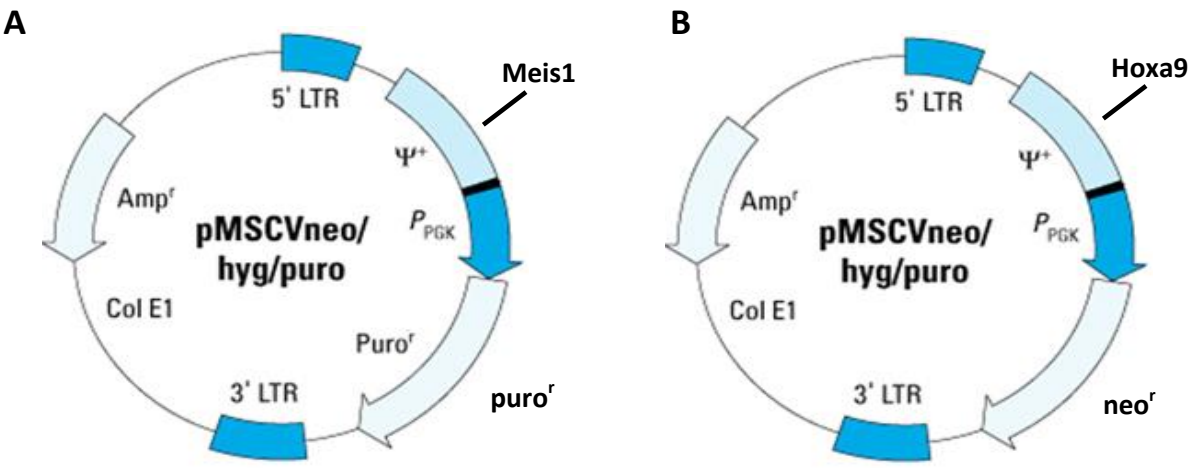


Figure 2.1. Restriction map of pMSCV plasmid.

MSCV based plasmid containing Meis1 gene and puromycin resistance cassette (A) or Hoxa9 gene and neomycin resistance cassette (B).

2.1.4 Tissue culture supplies

Supplier	Product
Baxter Healthcare, Nottingham, UK	Sterile water
Biolegend, London, UK	Mouse recombinant Interleukin-3 (IL-3)
	Mouse recombinant Interleukin-6 (IL-6)
	Mouse recombinant Stem Cell Factor (SCF)
	Mouse recombinant Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF)
Gilson, Bedfordshire, UK	P1000 & P200 plastic pipette tips
Greiner, Bio-One, Gloucestershire, UK	Cryotubes
	Pipettes (5mL, 10mL & 25mL)
	Tissue culture flasks ($25cm^2$, $75cm^2$, $175cm^2$)
	Tissue culture plates (6-well, 12-well, 24-well, 48-well & 96-well)
Invitrogen, Paisley, UK	2-mercaptoethanol (2-ME)
	Dulbecco's Modified Eagle Medium (DMEM)
	Iscove's Modifided Dulbecco Medium (IMDM)
	Dulbecco's Phosphate Buffered Saline (PBS)
	Foetal Calf Serum (FCS)
	L-glutamine (200mM)
Miltenyi Biotec, Bisley, UK	c-Kit microBead™ kit mouse
Nalgene Labware, Roskilde, Denmark	$25cm^2$ and $75cm^2$ tissue culture flasks
Sartorius, Hannover, Germany	Minisart 0.2µM sterile filters
	Minisart 0.45µM sterile filters
Sigma-Aldrich, Dorset, UK	Bovine serum albumin (BSA)
	DMSO
	Gelatin
	Hank's buffered salt solution (HBSS)
	Hydrochloric acid (HCl)
	Magnesium chloride ($MgCl_2$)
	Polybrene®
	Sodium azide
	Trypan blue
	Trypsin-EDTA
StemCell™ Technologies, Grenoble, France	Ammonium chloride solution (NH_4Cl)
	Methocult™ M3231
Sterilin Ltd, Hounslow, UK	Disposable pipettes (5mL, 10mL, 25mL)
	Pastettes
	Sterile plastic falcon tubes (15mL & 50mL)

Table 2.4. Details of tissue culture supplies.

2.1.5 Molecular biology supplies

Supplier	Product
Abcam, Cambridge, UK	Anti-GAPDH
Bioline, London, UK	Crystal 5X DNA loading buffer, blue
	HyperPAGE prestained protein marker
	ISOLATE II Genomic DNA kit
	MangoMix™
	α-select competent cells (silver efficiency)
Bio-Rad Laboratories Ltd, Sussex, UK	Gel combs
	Gel casting trays
	Immuno-Blot™ polyvinylidene fluoride (PVDF) membrane
Cell Signalling Technology, New England Biolabs, UK	1kiloBP DNA ladder
Chemical store, University of Glasgow	Ethanol
	Isopropanol
	Methanol
Eurofins MWG Operon, Wolverhampton, UK	PCR primers
Life Technologies Ltd, Paisley, UK	SYBR®safe
	Tris EDTA (TE) buffer
	XCell Surelock Mini-Cell system
	NuPAGE® LDS Sample Buffer (4X)
	NuPAGE® Novex® 4-12% Bis-Tris Protein Gels
Marvel Originals	Dried skimmed milk
Novus Biologicals, Cambridge, UK	Anti-HIF-1α
	Anti-HIF-2α
Qiagen, West Sussex, UK	HiSpeed ® Plasmid Maxi kit
Sigma-Aldrich, Dorset, UK	4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES)
	Agarose
	Calcium chloride ($CaCl_2$)
	EDTA
	Sodium docedyl sulphate (SDS)
	Tris base
	TWEEN 20 for electrophoresis
Thermo Scientific Inc. (Pierce), Hempstead, UK	Bicinchoninic acid (BCA)™ protein assay kit
	RIPA buffer
	DTT powder
	10X Tris-Glycine-SDS Buffer (transfer buffer)
	10X Tris-Glycine Buffer (running buffer)
	Supersignal West Dura chemiluminescent substrate
	CL-XPosure film
Roche Applied Sciences, West Sussex, UK	PhosSTOP; Phosphatase Inhibitor Cocktail Tablets
	cOmplete Protease Inhibitor Cocktail Tablet

Table 2.5. Details of molecular biology supplies.

2.1.6 Flow cytometry supplies

Supplier	Product
BD Biosciences, Oxford, UK	Annexin-V Allophycocyanin (APC)/Phycoerithrin (PE)
	Fluorescein isothiocyanate (FITC)Anti- Ki67 Kit
	Fluorescence Activated Cell Sorter (FACS)
	Flow/FACS Clean
	BD™ CompBeads Anti-mouse Ig κ
Biolegend UK, London, UK	Anti-mouse CD11b/Mac-1 (PE)
	Anti-mouse CD11b/Mac-1 (APC)
	Anti-mouse Gr-1 (PE)
	Anti-mouse Gr-1 (APC-Cy7)
	Anti-mouse c-Kit (APC)
	Anti-mouse c-Kit (APC-Cy7)
	Anti-mouse CD45.1 (FITC)
	Anti-mouse CD45.2 (Pacific Blue™)
	Purified Anti-H2A.X-Phosphorylated (Ser139)
Life Technologies Ltd, Paisley, UK	Alexa Fluor® 488 goat anti-rabbit IgG
	MitoTracker® Red CMXRos
	MitoTracker® Green FM
Sigma-Aldrich, Dorset UK	4'6-Diamidino-2-phenylindole dihydrochloride (DAPI)

Table 2.6. Details of flow cytometry supplies.

2.1.7 PCR primer sequences

Primer	Sequence
Mouse Vav iCre Forward	CCGAGGGGCCAAGTGAGAGG
Mouse Vav iCre Reverse	GGAGGGCAGGCAGGTTTTGGTTTTGGTG
Mouse Phd1 flox Forward	GAGGCTCCTTGAGTCTG
Mouse Phd1 flox Reverse	ATTTAGTTCAGTTCTCAG
Mouse Phd2 flox Forward	TTGCAGTGTGCAACAGTCAG
Mouse Phd2 flox Reverse	CACGGGGAACTCTGATTCAT
Mouse Phd2 flox (excised band)	CCAAAATCACCAATCTAGAATAACTTC
Mouse Phd3 flox Forward	AGTCTCAAAAGCATCAGG
Mouse Phd3 flox Reverse	ACTACATGCAATGGCACA
Mouse Phd1 Multiplex Forward (WT)	GAAGGAGGACAAAGGTCTCTTGG
Mouse Phd1 Multiplex Forward (KO)	CCTATATTCACGGGACAGATCCTG
Mouse Phd1 Multiplex Reverse (WT)	GGGCCCACAGTCAGCTAAAG
Mouse Phd1 Multiplex Reverse (KO)	AGAGGCCACTTGTGTAGCGC
Mouse Phd3 Multiplex Forward	GAGCACCCTTATAAAAAGCAAGTGA
Mouse Phd3 Multiplex Reverse (WT)	TGCAGAAACACCCCAGATGA
Mouse Phd3 Multiplex Reverse (KO)	GGAAAAGCGCCTCCCCTA

Table 2.7. Details of PCR primer sequences.

2.2 Media & Solutions

2.2.1. Tissue culture

2.2.1.1 DMEM

DMEM	440mL
FCS	50mL
L-glutamine (200mM)	5mL
Penicillin/Streptomycin ($10,000UmL^{-1}$/$10,000gmL^{-1}$)	5mL

2.2.1.2 IMDM base

IMDM	445mL
FCS	50mL
Penicillin/Streptomycin ($10,000UmL^{-1}$/$10,000gmL^{-1}$)	5mL

2.2.1.3 IMDM 40/20/20

IMDM base*	50mL
Mouse SCF (100µg/mL)	20µl (40ng/mL)
Mouse IL-3 (100µg/mL)	10µl (20ng/mL)
Mouse IL-6 (100µg/mL)	10µl (20ng/mL)

2.2.1.4 IMDM Intermediate

IMDM base*	50mL
Mouse SCF (100µg/mL)	10µl (20ng/mL)
Mouse IL-3 (100µg/mL)	5µl (10ng/mL)
Mouse IL-6 (100µg/mL)	5µl (10ng/mL)

2.2.1.5 IMDM IL-3

IMDM base*	50mL
Mouse IL-3 (100µg/mL)	5µl (10ng/mL)

**IMDM base prepared as detailed in section 2.2.1.2.*

2.2.1.6 Methylcellulose

Methocult™ 3231	80mL
IMDM	20mL
Mouse SCF (100µg/mL)	10µl (20ng/mL)
Mouse IL-3 (100µg/mL)	5µl (10ng/mL)
Mouse IL-6 (100µg/mL	5µl (10ng/mL)
Mouse GM-CSF (100µg/mL)	5µl (10ng/mL)

2.2.1.7 PBS / 2% FCS

PBS	490mL
FCS	10mL

2.2.1.8 Freezing media 10% DMSO FCS

DMSO	5mL
FCS	45mL

2.2.2 Western blotting

2.2.2.1 RIPA buffer

Per mL	
DTT (1mM)	1µL
Protease Inhibitor (25X)	40µL
Phosphatase Inhibitor (10X)	100µL
RIPA buffer	859µL

*2.2.2.2 10X TBS buffer**

NaCl	876.6g
Tris	121.1g
dH_20	10L

**1X solution was made using 100mL of the 10X solution with the addition of 900mL of dH_20 supplemented with 10mL of Tween-20.*

2.2.2.3 1X Transfer buffer

10X Tris-Glycine-SDS Buffer	100mL
dH_2O	900mL

2.2.2.4 Running buffer

10X Tris-Glycine Buffer	100mL
dH_2O	900mL

2.2.2.5 5% (w/v) Milk/TBST blocking solution

1X TBST	50mL
Powdered Milk	2.5g

2.2.3 Flow cytometry

*2.2.3.1 DAPI (1X)**

DAPI (1000X)	50mg
PBS	1mL

**DAPI stock solution was diluted at a ratio of 1:50 in PBS to give 1000X solution which was stored at -20^{o}C. Prior to use, 1000X DAPI stock was further diluted in PBS to give a 1X solution with a concentration of 1µg/mL.*

2.2.3.2 Annexin V

Annexin V APC/PE	5µL
HBSS supplemented with 1X DAPI	95µL

2.2.3.4 Fixation/Permeabilisation buffer

Paraformaldahyde (4%)	100µL = 0.4%
HEPES (0.05mM)	20µL = 10mM
Saponin (1%)	20µL = 0.02%
ddH_2O	840µL

2.2.3.5 Wash Buffer

FBS	50µL = 5%
Sodium azide	5µL = 0.05%
Saponin (1%)	10µL = 0.02%
EDTA (11mg/L)	0.375µL = 55mg/L

2.2.4 PCR

2.2.4.2 PCR Mix

PHD1 Flox	**µL**	**PHD2 Flox**	**µL**	**PHD3 Flox**	**µL**
PHD1 Fw Primer	0.1	PHD2 WT FL Fw Primer	0.1	AF Fw Primer	0.1
PHD1 Rv Primer	0.1	PHD2 WT FL Rv Primer	0.1	BR Rv Primer	0.1
MangoMix™	12.5	PHD2 FL excised Primer	0.1	MangoMix™	12.5
Nuclease free H_2O	8.3	MangoMix™	12.5	Nuclease free H_2O	8.3
		Nuclease free H_2O	8.2		
PHD1 Muliplex	**µL**	**PHD3 Muliplex**	**µL**	**Vav**	**µL**
PHD13 Fw Primer	0.1	PHD3 WT KO Fw Primer	0.15	Vav Fw Primer	0.1
PHD14 Rv Primer	0.1	PHD3 WT Rv Primer	0.1	Vav Rv Primer	0.1
PHD KO Fw Primer	0.1	PHD3 KO Rv Primer	0.1	MangoMix™	12.5
PHD KO Rv Primer	0.1	MangoMix™	12.5	Nuclease free H_2O	10.3
MangoMix™	12.5	Nuclease free H_2O	10.15		
Nuclease free H_2O	8.1				

Table 2.8. Details of PCR primer mixes.

2.2.4.1 2% (w/v) Agarose TBE

1X TBE	125mL
Agarose	2.5g

**Preparation of 2% agarose gel is described as an example. However, different genes required different concentrations of agarose and gels were also prepared at concentrations appropriate to the gene, which was decided during optimisation of assay.*

Gene	**Agarose concentration**
Vav	2%
PHD1 Multiplex	2.5%
PHD2 Flox	2%
PHD3 Multiplex	2%
PHD1 Flox	3%
PHD3 Flox	2%

Table 2.9. Details of agarose gel concentration used for individual primers.

2.2.5 Transfection

2.2.5.1 2X HEPES-buffered saline (HBSS)

NaCl	8g
KCl	0.37g
$Na_2HP0_42H_20$	106.5mg
Dextrose	1g
HEPES	5g
dH_20	to 500mL

pH 7.05 to 7.1

2.2.5.2 2M $CaCl_2$

$CaCl_2$	147g
dH_20	to 500mL

2.2.5.3 Transfection Solution/10cm^2 plates

dH_20	437.5µl
2 X HBSS	500µl
2M $CaCl_2$	62.5µl
VSV-G	3µg
Plasmid (Meis1 or Hoxa9)	10µg

2.2.5.4 0.1% (w/v) Gelatin solution

Gelatin powder	1g
dH_2O	1L

2.2.6 Plasmid preparation

2.2.6.1 Ampicillin (100mg/mL)

Ampicillin sodium salt	5g
dH_20	50mL

2.2.6.2 LB broth

Miller's LB base®	20g
dH_20	up to 1L
Ampicillin (100mg/mL)*	1mL

**Solution was autoclaved immediately following preparation and prior to supplementation with Ampicillin.*

2.2.6.3 LB agar plates

Miller's LB base®	20g
Microagar	7g
Ampicillin (100mg/mL)*	1mL

**Solution was autoclaved and cooled in a waterbath at 50°C. Cooled solution was supplemented with ampicillin and added to sterile petri dished. Dished were stored at 4°C.*

2.3 Methods

2.3.1 Animal work

2.3.1.1 Ethics

All animal work was in accordance with the Animals Scientific Procedures Act 1986 and UK Home Office regulations. Animals were housed at the Beatson Institute for Cancer Research or at the Veterinary Research Facility at the University of Glasgow.

2.3.1.2 Mouse models

PHD knockout mice were obtained from Peter Ratcliffe (University of Oxford). Gene targeting of PHD1 and PHD3 were used to delete a single exon, resulting in a non-functional mutant allele (Takeda et al, 2006). A mutant band is detectable by PCR using PHD1 and PHD3 primers. The detection of a mutant or WT band is used to determine the

genotype of the mice. PHD1$^{+/-}$ and PHD3$^{+/-}$ were crossed to generate PHD1$^{-/-}$ and PHD3$^{-/-}$ mice.

PHD2 or PHD1 gene is flanked by loxP sites which are recognised as excision sites by Cre recombinase. Activation of Cre will allow the gene to be excised conditionally. VavCre is expressed in haemopoietic cells only, allowing floxed genes to be deleted only in the haematopoietic system. PHD2$^{+/flox}$ and PHD1$^{+/flox}$ mice were crossed to VavCre^{+} mice to create PHD1$^{flox/flox}$VavCre and PHD2$^{flox/flox}$VavCre mice.

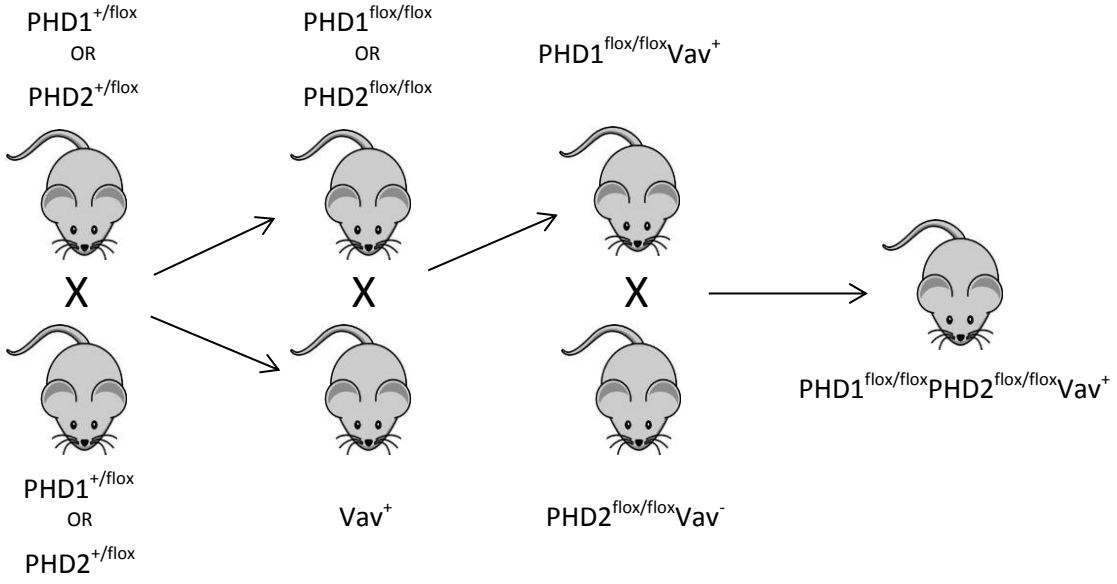


Figure 2.2. Illustration demonstrating mating strategy.

PHD1$^{+/flox}$ mice were crossed to PHD1$^{+/flox}$ mice to generate PHD1$^{flox/flox}$ mice. PHD1$^{flox/flox}$ mice were crossed to Vav^{+} mice to generate PHD1$^{flox/flox}$Vav^{+} mice. Similarly, PHD2$^{+/flox}$ mice were crossed to PHD2$^{+/flox}$ mice to generate PHD2$^{flox/flox}$ mice. PHD2$^{flox/flox}$ mice were crossed to Vav^{+} mice to generate PHD2$^{flox/flox}$Vav^{+} mice. Double knock-out mice were obtained by crossing PHD1$^{flox/flox}$Vav^{+} and PHD2$^{flox/flox}$Vav^{+} mice, generating PHD1$^{flox/flox}$PHD2$^{flox/flox}$Vav^{+} mice.

2.3.2 Cell culture

2.3.2.1 General technique

Tissue culture was conducted in an aseptic manner in which all materials were autoclaved and sterilised with 70% ethanol prior to use. All tissue culture technique was carried out within a laminar air flow hood to ensure aseptic working conditions.

2.3.2.2 Cryopreservation of samples

Primary cells and cell lines were stored at -80^{o}C for future use. Cells were washed several times with ice cold PBS/FBS to remove any residual media. Cells were counted and resuspended at concentrations of no more than 2x10^{6}/mL. Cells were pelleted and resuspended in ice cold FBS with 10% DMSO and dispensed into cryotubes. Cryotubes were placed in a freezing container ("Mr Frosty") containing neat isopropyl to allow gradual freezing of the samples, which were incubated at -80^{o}C overnight. After 24 hours, samples were moved to storage boxes at -80^{o}C.

2.3.1.3 Recovery of frozen samples

In order to ensure maximum recover rates from frozen samples, cells were thawed with care. On removal from -80^{o}C, cells were thawed immediately in a water bath of 37^{o}C. Thawed samples were then moved to 15mL Falcon tubes and 10mL of pre-warmed IMDM supplemented with 10% FBS, was added drop wise to the cell suspension to slowly dilute the freezing medium. Cell solution was centrifuged for 10mins at 400 x g. Cells were then washed with PBS/FBS to remove any residual freezing media. Cells were cultured in IMDM 40/20/20 overnight. Media was changed the following day to remove dead cells or debris after thawing. Cells were passaged as required until re-established.

2.3.3 PCR

Polymerase chain reaction (PCR) is a common technique used in molecular biology to amplify the number of copies of a gene within a sample, allowing that gene to be detected. Primers are designed to anneal to complementary DNA sequences within a target gene. Taq polymerase is also used to allow DNA synthesis of the gene, allowing amplification of the gene of interest. In this study, PCR has been used to determine the genotype of mice and the resulting cell lines that have been derived from them.

2.3.3.1 Primer design

NCBI software was used to design primers using the DNA sequence of the gene of interest. Primers were synthesised commercially by Eurofins MWG Operon. Primers were reconstituted in nuclease-free H_2O to a stock concentration of 100µM and stored aliquoted at -20^{o}C. Working concentration of primers and PCR reagents, along with PCR conditions, were optimised to each primer set. Conditions for primers used in this study are detailed in section 2.2.4.2.

2.3.3.2 Generation of DNA

Mouse ear/tail samples were collected and incubated for several hours or overnight at 55^{o}C in DNA lysis buffer containing Proteinase-K. DNA was extracted from animal samples using a Bioline DNA extraction kit as per manufacturer's instructions.

2.3.3.3 Standard PCR

PCR was used to detect the presence or absence of a gene to determine the genotype of the sample. In this study, the cre-lox system was used as a means to conditionally knock out target genes. PCR was used to distinguish floxed genes from wild type genes, based on the size of DNA fragment that is amplified.

Positive controls were used to determine the success of the reaction. PCR reaction mix without DNA was used to assess contamination of PCR reagents. PCR conditions were optimised for each primer set. DNA fragments produced during the PCR reaction can be analysed using an agarose gel, through which DNA fragments migrate, allowing them to be separated by size. Agarose gels are prepared containing SybrSafe™, which is used to visualise DNA by UV exposure. DNA ladders of known size were run with each gel to help determine the size of DNA fragments and as a control that the DNA had migrated through the gel appropriately.

Agarose gels were prepared to an appropriate concentration, which was optimised for each PCR product. The percentage of agarose used reflects the porosity of the gel. Therefore, larger DNA fragments may require a lower percentage of gel with a higher porosity in order to move efficiently through the gel. Optimisation of this assay found that some genes being analysed achieved clearer results when the percentage of agarose used was altered. Agarose powder was dissolved in 1X TBE which was heated in a microwave. Agarose solution was allowed to cool before SybrSafe™ was added and gel was poured into a gel tray containing combs. Once the gel had solidified, combs were removed, forming wells that were used to load samples into the gel. The gel was placed into an electrophoresis tank containing 1X TBE solution. PCR samples were loaded into the gel and an electric current passed through the tank, causing the DNA to migrate through the gel, separating the fragments by size. Smaller fragments are able to move through the pores in the gel easier that larger one and therefore, are able to migrate further. Following electrophoresis, DNA within the gel can be visualised as bands using UV illumination using the molecular imager Chemidoc™ XRS visualisation system.

2.3.4 Transfection

2.3.4.1 Plasmid production

Plasmid DNA was concentrated and purified using a Qiagen MaxiPrep kit. Agar plates were prepared as detailed in section 2.2.6.3. Plasmid DNA was added to competent

bacteria and streaked onto agar plates. Plates were incubated overnight at 37^{o}C to allow bacterial colony growth. LB broth was prepared as per section 2.2.6.2 and autoclaved to ensure no bacterial growth. A single bacteria colony was picked from the agar plate and added to the LB both. LB broth was placed on a shaker and incubated at 37^{o}C overnight to allow growth of the bacterial culture. Plasmid DNA within the bacterial culture was isolated using a MaxiPrep kit according to manufacturer's instructions. DNA was eluted in nuclease free H_2O and stored at -20^{o}C. DNA was quantified using a NanoDrop™ spectrophotometer Nd-1000.

2.3.4.2 Retroviral production

Retroviral transfection was used to introduce the overexpression of Meis1 and Hoxa9, which are known to play a role in leukaemic development. Genes of interest can be cloned into plasmid DNA structures which can be transfected into packaging cell lines such as commercially available Plat-E cells. Following transfection with plasmid DNA, Plat-E cells package the retroviral DNA into viral particles which are released into the supernatant. Supernatant can then be collected and added to target cells, such as Haemopoietic stem and progenitor cells (HSPCs), in order to transfect these cells with the virus. Retroviral DNA can then integrate into the DNA of the host cell which overexpresses the target gene, in this case, Meis1 and Hoxa9.

10cm^2 culture dishes were pre-coated with 0.1% (w/v) gelatin. Plat-E cells were counted and seeded at a density of 1.8x10^{6}cells/plate and allowed to adhere for 24 hours at 37^{o}C prior to transfection. Transfection solution was prepared as described in section 2.2.5.3 and incubated for 30 mins at room temperature. Fresh, pre-warmed DMEM media was added to cells. Transfection solution was then added drop-wise to the plates which were mixed gently and incubated at 37^{o}C overnight. Following overnight incubation with transfection solution, media was removed and replaced with fresh, pre-warmed IMDM supplemented with 10% FBS, in which viral particles will be collected. Plates were incubated for a further 48 hours to allow cells to produce viral particles into the supernatant. At 48 hours, supernatant was collected and filtered using a 0.44µM sterile

filter. Filtered supernatant containing viral particles was immediately collected on dry ice to ensure that maximum half-life of the virus is preserved in the frozen stock. Virus was stored at -80^{o}C.

2.3.4.3 Testing efficiency of retrovirus

Mouse fibroblast NIH3T3 cells were used to test each batch of virus produced prior to infection of HSPCs. NIH3T3 cells were seeded in 6-well plates at a density of 1 x 10^5 cells/well in 2mL of DMEM media. Cells were cultured for 24 hours at 37^{o}C to allow cells to adhere. Once cells had adhered, media was removed and replaced with 500µL of freshly thawed viral supernatant containing 2µg/mL Polybrene and incubated at 37^{o}C overnight. The following day, media was replaced with fresh DMEM. Forty-eight hours following infection, cells were centrifuged and resuspended in media containing selection antibiotics 1.5mg/mL neomycin and 1.5µg/mL puromycin. Cells that have been successfully transfected with both viruses will express resistance cassettes for the selection antibiotics and will be positively selected. Cells remained in the selection media for 72 hours before selection was complete. At 72 hours, cells were imaged by microscopy to assess if cells had grown in the presence of the antibiotics. Cells that were not infected with any virus were used as a control to ensure that antibiotics successfully prevented the growth of non-transfected cells.

The concentration of neomycin and puromycin were determined through titration experiments in which, NIH3T3 cells were grown in the presence of different concentrations of drug to determine the most efficient dose for selection. The growth of NIH3T3 cells was compared against the growth of NIH3T3 cells that were transfected with virus. Infected cells that grew in the selection media were considered transfected and the virus deemed efficient.

2.3.5 Isolation and culture of HSPCs and pre-LSCs

2.3.5.1 Collection of mouse bone marrow cells

Hips, femur and tibia were extracted from mice following their humane sacrifice by schedule 1 method according to Home Office licence. Bones were crushed using mortar and pestle in PBS containing 2% FBS. Cells were washed several times in PBS/FBS in order to deplete tissue contamination in the bone marrow sample. Bone marrow cells were filtered using a sterile 0.2µM filter to create a single cell suspension. Bone marrow cells were treated with NH_4Cl to lyse any remaining red blood cells to purify the white blood cell population.

2.3.5.2 Isolation of HSPCs by c-Kit enrichment

HSPCs were isolated based on the expression of haemopoietic marker c-Kit, which is expressed on haemopoietic stem cells and early progenitors but not committed progenitors. A c-Kit magnetic bead kit was used to enrich the c-Kit$^+$ population through a column separation method. Bone marrow cell suspension was prepared as in section 2.3.5.1 and counted to ensure an efficient concentration of beads against mouse c-Kit IgG was mixed with the cell suspension. Cells were filtered using a sterile 0.2µM filter to ensure a single cell suspension and added to a magnetic column, to which anti- c-Kit beads coupled to c-Kit$^+$ cells attached. The column was washed several times with PBS/FBS to remove any negative cells. c-Kit$^+$ cells which were selected by the magnetic column were collected and washed. To ensure that the enrichment had worked effectively, selected cells were stained with an antibody for c-Kit and the frequency of c-Kit$^+$ cells determined by flow cytometry.

2.3.5.3 Transduction of primary mouse samples

Primary mouse bone marrow samples were enriched for stem and progenitor marker c-Kit to ensure a primitive population of HSPCs. 1 x 10^6 c-Kit cells were cultured overnight in IMDM 40/20/20 prepared as per section 2.2.1.3. The following day, cells

were collected in 50mL falcon tubes. Frozen viral supernatant was thawed and added to cells along with 2µg/mL Polybrene which increases the efficiency of transfection by neutralising the cell surface and allowing viral particles to bind more efficiently to receptors. Cells underwent spinoculation where tubes were centrifuged at 800 x g for 2 hours at 4^{o}C, maximising the contact of the cells with the viral particles and increasing the chances of infection. Viral media was removed and cells were resuspended in fresh IMDM 40/20/20 and returned to culture at 37^{o}C overnight. The following day, spinoculation was repeated. 48 hours following the initial infection, cells were centrifuged and resuspended in fresh IMDM 40/20/20 containing 1.5mg/mL neomycin and 1.5µg/mL puromycin.

2.3.5.4 CFC assay

The colony forming cell or CFC assay is a common assay in haemopoietic studies and is widely used as a measure of self renewal ability and 'stemness' of bone marrow populations in an *in vitro* setting. Cells are seeded into a semisolid medium where their self renewal capacity allows them to produce colonies. Colonies are replated several times and can be counted at each round to measure the frequency of colony forming cells which possess self renewal capacities. Variations of this assay can be used to monitor proliferative potential of cells in response to drug treatments or genetic manipulation. Furthermore, this technique can be used to evaluate the differentiation of haematopoietic progenitors by characterising colonies of different phenotypes produced in culture.

Cytokines can be added to the culture to support the growth of particular populations and to drive the differentiation of a particular phenotype. In this study, IL-3, IL-6 and GM-CSF mouse recombinant cytokines were added to culture to drive the culture towards a macrophage-granulocyte progenitor population as this is the cell type that is seen to be abnormal within AML.

Primary mouse bone marrow cells were c-Kit enriched and transfected with Meis1/Hoxa9 retroviruses according to section 2.3.5.3. Methylcellulose was prepared as

per section 2.2.1.6. Cells were then added to methylcellulose and vortexed thoroughly to ensure homogenous mixing prior to plating. Cell/methylcellulose mix was distributed into duplicate wells in a 6-well plate using a sterile needle and syringe. Sterile water was dispersed to empty wells within the plate to increase humidity. Furthermore, plates were incubated within a humidifying box to ensure constant humidity and to prevent the media from drying out. Cultures were incubated for a period of 5-8 days (based on growth) at 37^{o}C, 5% CO_2. Colonies were counted and images recorded using standard light microscopy. Colonies were then harvested and resuspended in IMDM. Cells were counted and added to fresh methylcellulose at a density of 1 x 10^4 cells/mL before returning to culture for a second round of replating. This density of cells was chosen as plating with lower densities failed to produce colonies. Procedure was repeated every 5-8 days for up to 5 rounds of replating, with colonies being assessed at each round. A sample was also taken at each round and cells were analysed by flow cytometry to determine their immunophenotype. This assay often generated large numbers of cells and surplus cells were used to generate cell lines or were frozen down for future use as per section 2.3.2.2.

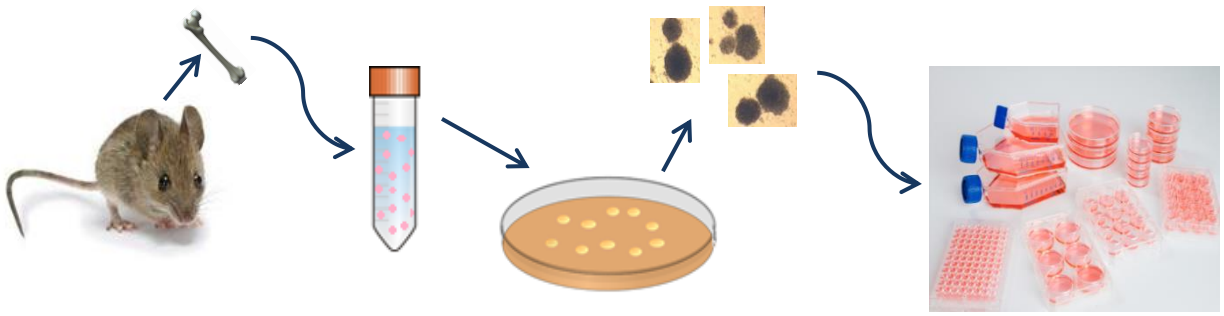


Figure 2.3 Illustration demonstrating preparation of cells by CFC assay.

Bone marrow cells are extracted from mice, which are plated into methylcellulose in a CFC assay. Colonies developed can be transferred to liquid culture to establish new cell lines.

2.3.5.5 Single cell cloning assay

A single cell cloning assay was used to estimate the self renewal capacity and leukaemic potential of cells generated in the CFC assay. Cells were allowed to produce 3 generations of colonies, at which point they were considered fully transformed. Cells from CFC3 were harvested from methylcellulose and washed several times. Cells were stained with fluorescently labelled antibodies against specific LSC cell markers as described in section 2.3.6.2. Cells were analysed by flow cytometry to visualise the pre-LSC population based on the expression of cell markers. Single pre-LSCs were then sorted directly into individual wells of a 96-well plate containing 80µL of methylcellulose (prepared as described in section 2.2.1.6). Single cells for each biological sample were plated in 96 replicates. Plates were spun down to place the cell in the centre of the well. Plates were incubated at 37°C for approximately 10 days to allow colonies to develop. After 10 days, each well was scored for the presence or absence of a colony. Scores were used to determine the frequency of cells within each sample that had self renewal capacity.

2.3.5.6 Culture and expansion of murine cell lines

Cells generated during the CFC assay were used to establish a cell line of transformed pre-LSCs. Cells were replated several times before considered fully transformed so only cells that had formed colonies three times (CFC3) were used to generate cell lines. A minimum of 1 x 10^5 cells were seeded into a liquid culture containing high concentrations of cytokines (IMDM 40/20/20 as detailed in section 2.2.1.3). Media was replaced after 24 hours to remove any dead cells or debris carried over from the colonies, which also contained some dead cells. After a further 24-48 hours (dependent on growth), cells were passaged and resuspended in media containing a lower concentration of cytokines (IMDM 20/10/10 as detailed in section 2.2.1.4). In order to wean cells off higher concentrations of cytokines, cells were passaged several times in IMDM 20/10/10. Once cells appeared to be stabilised, cells were passaged and added to a media containing only IL-3 (IMDM IL-3 as detailed in section 2.2.1.5). Once stabilised, cells

were grown in IMDM IL-3 and passaged every 2-3 days dependent on growth, and resuspended in pre-warmed, fresh media to expand culture. Cell lines were maintained at a density of 2.5 x 10^4cells/mL. Cell lines were frozen at early passages in multiple aliquots, creating a reserve that was used for future experiments.

2.3.5.7 Assessment of viability and cell growth

Counting of the cells and assessment of viability were determined by trypan blue exclusion using a counting chamber or haemocytometer. Trypan blue is incorporated by dead cells that do not have an intact membrane, causing dead cells to appear blue in colour under the microscope. Viable cells with an intact membrane do not incorporate trypan blue and do not take on the blue colour, allowing them to be discriminated against dead cells. Viable cells can be counted in order to determine the cell number and growth of the culture. In order to determine viability, dead cells are counted separately from living cells to calculate the ratio of living/dead cells.

Trypan blue stock solution was diluted 1 in 10 in PBS to give a working solution. Cell suspension was diluted 1 in 2 in trypan blue solution. Cell suspension mixed with trypan blue solution was added to the chamber of a haemocytometer and cells counted under the microscope. To ensure an accurate count, four squares within the chamber were counted and an average of the four squares was used to give the cell count. Cell count was then multiplied by the dilution factor (i.e. 2) and 1 x 10^4 to give the concentration of the cell suspension per mL.

2.3.5.8 Drug treatment of cell lines

Common leukaemia treatment chemotherapeutic agents Doxorubicin and AraC were used to treat cell lines. Doxorubicin (Dox) was available at 2mg/mL and was diluted to give a stock solution of 200ng/mL before use. Dox was diluted 1 in 100 to give a 20,000ng/mL solution. 20,000ng/mL drug solution underwent a further dilution of 1 in

100 to give a 200ng/mL solution, which was distributed into 1ml aliquots and stored at -20^{o}C. Dox was diluted in 0.9% NaCl/H_2O as directed by product datasheet.

AraC was available at 1g/10mL and was diluted to give a stock solution of 24ng/mL before use. AraC stock solutions were determined in a similar manner to solutions of Dox; a 100x solution was made from the original stock and this was then used to make a 1x solution which was used to generate concentrations needed in future experiments. However, as the concentration of AraC is expressed in g/mL, calculations were first done to determine the volume of solution required to generate a 100x solution. 24.32µL of neat drug stock was added to 975.68µL of ddH_2O to give a solution of 2,400ng/mL. 2,400ng/mL solution was then diluted 1 in 100 to give a solution of 24ng/mL (100nM). The concentration of both drugs was determined based on similar studies published in the literature (Pardee et al, 2011). Within this study, AraC concentration is expressed in nM notation and all working solutions have been calculated to be in accordance with the concentrations used in other studies. However, as a comparison between the two drugs used, the concentration of AraC has been expressed as ng/mL, as with Dox. AraC was diluted in ddH_2O as directed by the product datasheet.

1X stock solutions of AraC and Dox underwent serial dilutions to give a range of concentrations which were used to treat cell lines. Cells were counted and viability assessed as per section 2.3.5.6. Only cell lines with good viability for that particular genotype (as determined by previous experiments) were used in the assay. Cells were seeded into 96-well plates at a density of 5 x 10^5/mL with 1 x 10^5 cells being seeded to each well in a total volume of 200µL per well. WT and KO cell lines were tested and each biological replicate was plated in duplicate. The volume required of Dox and AraC solution to make the desired concentrations within a 200µL final volume was calculated and the correct volume of drug stock was added to each well. Plates were incubated at 37^{o}C for 24 or 48 hours without disruption. Cell response was assessed at 24 and 48 hours. Cell growth was assessed by trypan blue exclusion method as detailed in section 2.3.5.7. Apoptosis was also assessed by flow cytometry, as detailed in section 2.3.6.4.

2.3.5.9 Radiation treatment of cell lines

Cell lines were counted and plated at a density of 5 x 10^5/mL in IMDM IL-3. Plates were sealed using parafilm to ensure cells remain in sterile conditions during transportation. Plates were put on ice and transported to The Beatson Institute for Cancer Research in order to use the radiation facilities. Plates were subjected to various doses of gamma radiation. The length of exposure was calculated based on the strength of the machine which decreases over time. Cells were analysed immediately or returned to culture depending on the time points being examined during the experiment.

2.3.5.10 Hypoxia treatment of cell lines

Cell lines were counted and plated at a density of 5 x 10^5/mL in IMDM IL-3. Plates were transported to a specialised hypoxia chamber which allows cells to be cultured at 37^{o}C in an environment with a set concentration of Oxygen. Cells were subjected to 1% Oxygen for 24 hours before being analysed by flow cytometry.

2.3.6 Flow cytometry and cell sorting

2.3.6.1 Flow cytometry

Flow cytometry is a laser-based technology which allows cells to be counted, sorted or detected based on the presence of biomarkers. Flow cytometry has been used throughout this study to detect frequencies of specific cell populations based on the expression of surface markers. In addition, many other biochemical processes can be analysed in this way including apoptosis and cell cycle progression. Flow cytometry can detect the size and granularity of the cells which is used to discriminate different cell types within a heterogeneous population based on these parameters, which is displayed as forward scatter and side scatter, respectively. This can also be used to exclude dead cells from viable populations, as dead cells appear much smaller and denser than larger living cells. Prior to analysis by flow cytometry, cells can be incubated with fluorescently

labelled antibodies which will bind to specific antigens present on the cell. Cells are then passed through lasers which allow fluorescent signals to be emitted by labelled cells, allowing for the quantification of cells that are positive for the particular antigen, based on the fluorescent signal detected by the flow cytometer.

All antibodies and reagents used for this technique have been listed in section 2.1.6. A FACSCanto™ II or LSRFortessa™ cell analyser (both from BD Biosciences) were used for flow cytometry analysis and a FACSAria (BD Biosciences) was used for single cell sorting. Unstained cells, compensation beads and single colour controls were used to compensate for all fluorescent overlap and to optimize gating strategy prior to analysis. All data was acquired using BD FACSDiva (BD Biosciences) and analysis was performed using FlowJo (Tree Star Inc., Ashland, USA) software.

2.3.6.2 Assessment of immunophenotype

The immunophenotype of cells was determined by the simultaneous expression of cell surface markers c-Kit, Mac-1 and Gr-1. An appropriate number of cells were washed in PBS/FBS and incubated in appropriate concentrations of antibody mix on ice and away from light to reduce fading of fluorescence. Cells were washed to remove any unbound antibody and resuspended in PBS/FBS containing DAPI. DAPI is a DNA stain that can be used to distinguish living and dead cells based on the permeabilisation of the dye by dying or dead cells with compromised cellular membranes. Unstained cells and compensation beads were also prepared and kept on ice prior to analysis.

2.3.6.3 Assessment of cell cycle frequencies

Ki67 and DAPI staining was used to determine the frequency of cells in the different stages of the cell cycle. Ki67 is known to be expressed by cycling cells but it is not detectable in non-cycling cells (Endl et al, 1997). Therefore, cells in G0 can be distinguished based on the lack of Ki67 expression. Cells that are positive for Ki67 expression are considered to be actively proliferating. Simultaneous staining with DAPI

allows the other phases of the cell cycle to be identified based on the DNA content of the cells. For example, cells that have completed DNA synthesis during S phase will have a higher concentration of DNA and therefore, a higher incorporation of DAPI and higher fluorescent intensity on flow cyotometry.

Cells were counted and approximately 4 x 10^4 cells were seeded into a round-bottomed 96-well plate. Plates were centrifuged and washed to remove residual media. Cells were resuspended in fixation/permeabilisation buffer as detailed in section 2.2.3.4 and incubated at room temperature for 30 mins. Cells were then washed several times using wash buffer as detailed in section 2.2.3.5. Anti-Ki67 antibody was added to the residual wash buffer within each well and vortexed gently to resuspend cells within the solution. Plates were incubated at room temperature in the dark for 30 mins. Following antibody incubation, plates were centrifuged and cells washed several times before being resuspended in PBS supplemented with DAPI. Samples were kept away from light until analysed by flow cytometry. Unstained cells and an isotype control were used to distinguish a positive result from background fluorescent signal.

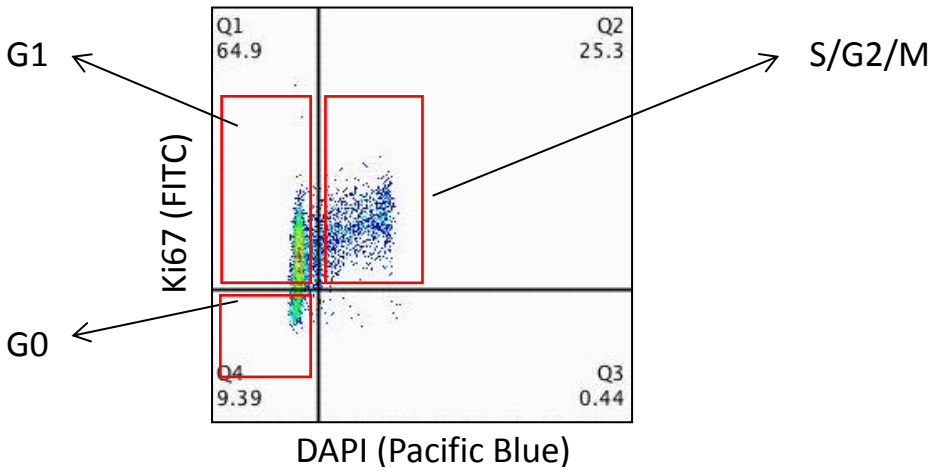


Figure 2.4. Representative plot of cell cycle analysis by Ki67 staining.

DAPI and Ki67 staining is visualised on the X and Y axes respectively. Gates are determines using an FITC isotype control and an unstained control. $Ki67^+$ positive cells are considered as cycling cells and can be further categorised based on the DNA content of the cells as measured by DAPI staining.

2.3.6.4 Assessment of apoptosis

The frequency of apoptotic cells was determined by the detection of Annexin V binding and incorporation of DAPI. During the process of apoptosis, phosphatidylserine (PS) is expressed on the outer membrane of the cell. Annexin V is known to bind strongly with PS and is commonly used to measure apoptosis. Annexin V can be labelled with fluorescent epitopes that can be detected by flow cyotmetry. Annexin V^-DAPI^- cells were considered viable as these cells did not incorporate DAPI or bind to apoptosis marker Annexin V. Annexin V^+ cells were considered to be undergoing apoptosis. However, these can be further discriminated into early and late apoptosis by using DAPI. Cells that were Annexin V^+ but $DAPI^-$ were considered to be in early apoptosis as DAPI was not able to penetrate the cell, suggesting that the cell had an intact membrane. Cells that were Annexin V^+ which were also $DAPI^+$ were considered to be in late phase apoptosis as these cells had lost membrane integrity.

Cells were washed several times and incubated in a solution of HBSS and Annexin V and kept in the dark for 15 mins at room temperature according to manufacturer's protocol. Immediately prior to analysis, cells were further diluted with HBSS containing DAPI. Unstained cells and cells stained with single colours were used to set up gating strategy, allowing for the detection of viable cells and cells undergoing early and late apoptosis.

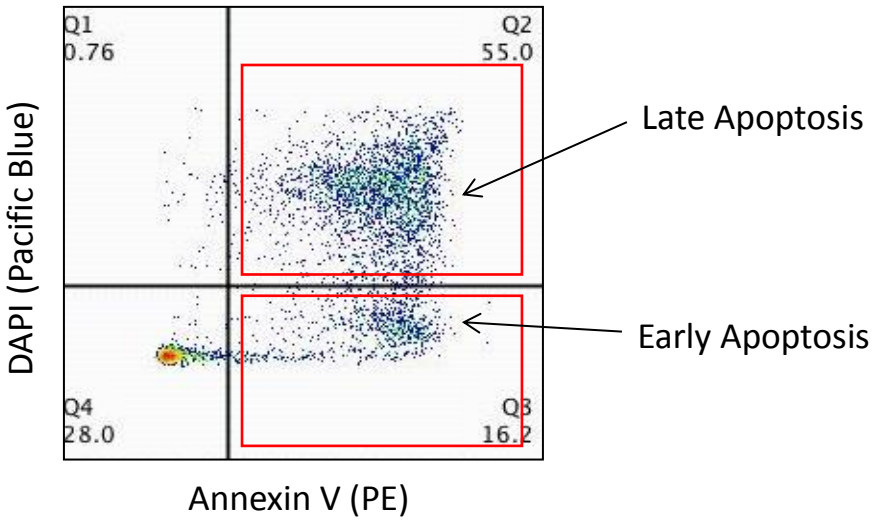


Figure 2.5 Representative plot of Annexin V and DAPI staining to analyse apoptosis.

Annexin V and DAPI can be visualised on the X and Y axes respectively. Viable cells which are negative for both Annexin V and DAPI can be seen in Q4 of the plot. Q3 shows cells that are Annexin V^+ but negative for DAPI, which are considered in the early phases of apoptosis. Q2 contains cells positive for both Annexin V and DAPI, which are considered to be in the late stages of apoptosis. Unstained controls and cells stained with single colours were used to determine the gates.

2.3.6.5 Assessment of functional mitochondria

MitoTracker Green is a fluorescent mitochondrial stain which stains mitochondria within the cell regardless of membrane potential. That is, MitoTracker Green stains mitochondria in both healthy and dying cells. This technique can be used to visualise the number of mitochondria in cells through analysis by flow cytometry. MitoTracker Red works in a similar way but stains intact mitochondria in living cells only and is dependent on membrane potential. Cells can be stained with both MitoTracker Red and MitoTracker Green to distinguish the total number of mitochondria against respiring mitochondria to give the frequency of functional and dysfunctional mitochondria.

1 x 10^5 cells were plated into round-bottomed 96-well plates. Plates were centrifuged and cells washed in PBS. MitoTracker Green and MitoTracker Red stains were diluted in prewarmed IMDM to give a final concentration of 100nM for each stain. MitoTracker Red/Green mix was added to cells and incubated at 37^{o}C for 30 mins according to manufacturer's protocol. Following incubation, cells were washed in PBS to remove excess antibody. Stained cells were resuspended in PBS/2% FBS and analysed by flow cytometry.

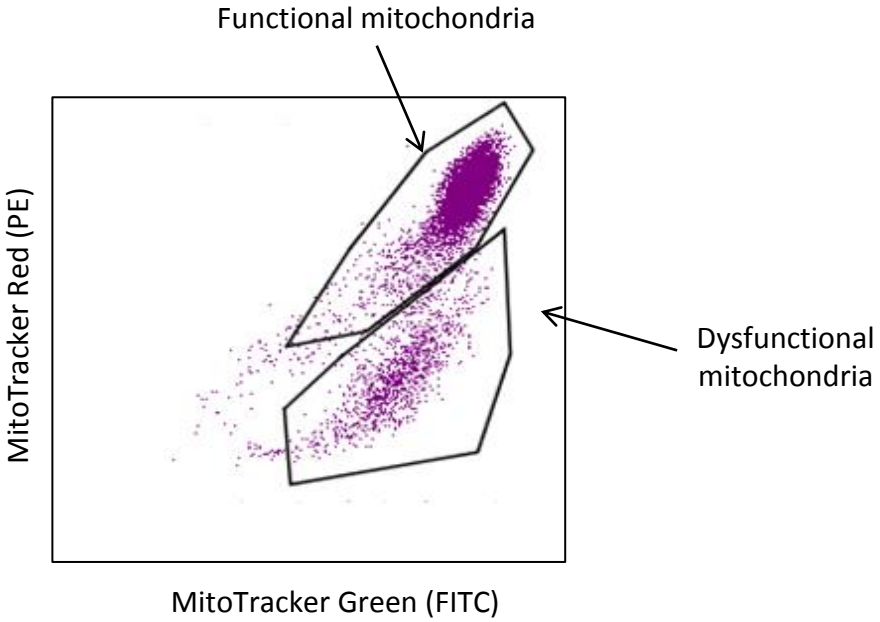


Figure 2.6. Assessment of functional and dysfunctional mitochondria.

MitoTracker Green mitochondrial stain can be used in conjunction with MitoTracker Red stain in order to distinguish functional and dysfunctional mitochondria. MitoTracker Red stains only respiring mitochondria whereas MitoTracker Green stains total mitochondria. A population that stains positive for both colours is considered to be functional mitochondria. However, cells that stain positive for MitoTracker Green only contain dysfunctional mitochondria.

2.3.6.6 Assessment of DNA damage

Cells were fixed and permeabilised following the protocol detailed in section 2.3.6.3. After cells were washed, anti-H2AX antibody was added to the residual wash

buffer within each well and vortexed gently to resuspend cells within the solution. Plates were incubated at room temperature in the dark for 60 mins. Following antibody incubation, plates were centrifuged and cells washed several times before being resuspended in FITC anti-mouse secondary antibody for a further 30 mins. Cells were then washed several times in PBS and resuspended in PBS supplemented with DAPI. Samples were kept away from light until analysed by flow cyotmetry. Unstained cells and an isotype control were used to distinguish a positive result from background fluorescent signal.

2.3.6.7 Flow cytometry cell sorting

Cells to be seeded into the single cell cloning assay (as detailed in section 2.3.5.4) sorted were stained with appropriate antibodies, washed and resuspended in PBS/FBS. Cells were filtered using a sterile nylon filter and samples were kept on ice prior to sorting by FACSAria (BD Biosciences).

2.3.7 Western blotting

2.3.7.1 Preparation of protein lysates

RIPA buffer was used to lyse cells and was prepared on ice as described in section 2.2.2.1 immediately prior to use. Cells were washed twice in ice cold PBS and resuspended in RIPA buffer. Cells in RIPA buffer were incubated on ice and placed on a shaker for 30 mins. Cells were centrifuged at for 30 mins at maximum speed at 4^{o}C to separate the DNA and cellular debris from the supernatant containing denatured proteins. Supernatant was collected and immediately stored at -20^{o}C for short term storage or -80^{o}C for long term storage.

2.3.7.2 Protein quantification

The proteins contained in each supernatant samples were quantified using the BCA™ protein assay kit in accordance with the manufacturer's protocol. Appropriate reagents were added to samples which were incubated at room temperature for 30 mins. A colour change occurred in samples containing protein; the intensity of the colour correlating with the concentration of protein in the sample. The intensity of the resulting colour can then be measured using a spectrometer, which measures the UV absorbance within the sample. Standards, which are included in the BCA assay kit, contain known concentrations of protein. These were used to generate a standard curve which was used to determine the concentration of protein in the unknown samples, based on the absorbance of each sample. The concentrations of protein within the samples were used to determine the volume of sample needed for the western blot and to ensure that an equal amount of protein was loaded for each sample.

2.3.7.3 Gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is a widely used technique used to separate proteins by size. SDS is a negatively charged detergent that linearises proteins and coats them with an uniform negative charge masking the intrinsic charge of the residues such that when an electric current is applied, the proteins to move towards the positive electrode. Proteins migrate through the gel at different speeds, according to their molecular weight. This results in smaller proteins migrating further through the matrix than large ones. A protein marker can be used to mark the position of known sizes of proteins, which appear as bands along the gel. This marker can be used to judge the sizes of proteins that appear in the unknown samples. A protein of interest can be identified based on the presence of a band of the correct size.

Protein samples were prepared in 1X SDS buffer and heated at 95^{o}C for 5 mins. Samples were then loaded into separate wells of the gel alongside a pre-stained protein

ladder. Running buffer (as detailed in section 2.2.2.4) was added to the gel to prevent it from drying out. Samples were run at 120 volts for 1.5 hours.

2.3.7.4 Membrane transfer

Following gel separation, proteins were transferred to PVDF membrane which would allow proteins to be visualised through immunoblotting. The hydrophobicity of PVDF membrane means it must first be soaked in methanol for approximately 30 seconds. The gel containing the separated proteins and the PVDF membrane were pressed together tightly using several layers of sponges and paper which were soaked in transfer buffer (section 2.2.2.3) Layers were compressed in a transfer cassette which was loaded into the electrophoresis tank containing ddH_2O to prevent the system from overheating. The tank was then placed on ice or in a cold room and the transfer was run at 30 volts for 3 hours. Successful transfer was determined by the printing of the protein ladder from the gel onto the membrane. The membrane was kept in TBST solution at all times following the transfer to prevent it from drying out.

2.3.7.5 Immunolabelling

Following transfer of the proteins to the membrane, the membrane was transferred to a blocking solution of 5% (w/v) Milk in TBST to try and minimise non-specific binding. Membrane was incubated overnight at 4^oC with gentle rotation to allow constant motion and to ensure the entire membrane was blocked. The following morning, the membrane was transferred to fresh blocking solution containing the appropriate a primary antibody which will bind specifically to the protein of interest. Membrane was incubated in the primary antibody with gentle shaking for a minimum of 1 hour. Following antibody incubation, membrane was washed several times in TBST to remove unbound antibody. The membrane was then incubated in the appropriate secondary antibody which is used to detect the presence of protein-bound primary antibody for 1 hour at room temperature with gentle shaking. Blot was washed several times before an ECL

detection method was used to visualise the bound antibody and therefore identify the presence of the target protein. Supersignal West Dura ECL kit was used according to manufacturer's instructions. The blot was kept in the dark to avoid loss of luminescent signal. Blots were pressed onto x-ray film, which was developed in a dark room using an x-ray developing machine.

2.3.8 *In vivo* studies

2.3.8.1 Overview

A transplantation assay was used to assess the leukaemic potential of the Meis1/Hoxa9 transformed pre-LSCs which were generated in the CFC assay. Cells used in the CFC assay were harvested from WT and PHD KO mice which were originally bred with B6-Ly5.2 mice which have a $CD45.2^+$ background. The transfer of $CD45.2^+$ cells to recipient mice with a different CD45 background, allows the prevalence of $CD45.2^+$ cells in the bone marrow of recipient mice to be monitored by flow cytometry and distinguished from the bone marrow cells of the recipient. As it is unclear as to whether these cells will have the ability to reconstitute the bone marrow, and due to the difficulty and expense of generating high concentrations of pre-LSCs, support bone marrow can be transplanted simultaneously to ensure the adequate reconstitution of the bone marrow and survival of the recipient mice. Mice can be monitored long term for signs of leukaemic development to determine if genetic differences between the samples injected affect the leukaemic potential of the cells.

2.3.8.2 Cell preparation

The CFC assay as detailed in section 2.3.5.3, was used to generate pre-LSCs which were used in transplantation assays. Samples were plated into the CFC assay as normal but several replicate plates were incubated on the third round of replating to ensure that an adequate number of cells were available for harvest at CFC3. Cells were washed several times in PBS supplemented with 2% FBS to remove any residual media. Cells were

then c-Kit enriched according to section 2.3.5.2 to ensure a population of pre-LSCs. The genotype of each sample was confirmed by PCR prior to transplantation.

During this assay, support bone marrow was harvested from mice with a CD45.1 background. Using mice with a different CD45 background allows both the pre-LSCs and support bone marrow to be tracked simultaneously by flow cytometry following transplantation. Bones were harvested and crushed and red blood cells lysed as detailed in section 2.3.5.1. White blood cells were filtered using a 0.2µM sterile filter and counted using an automated Hemavet 950FS (Drew Scientific) cell counter. Pre-LSCs and support bone marrow was mixed at a ratio of 1 x 10^5 per-LSCs to 2 x 10^5 support bone marrow cells per recipient mouse. Samples were kept in sealed sterile tubes on ice until the time of injection. Samples were analysed by flow cytometry to determine that the frequency of pre-LSCs, as well as the ratio between CD45.2 and CD45.1 cells was similar in each sample.

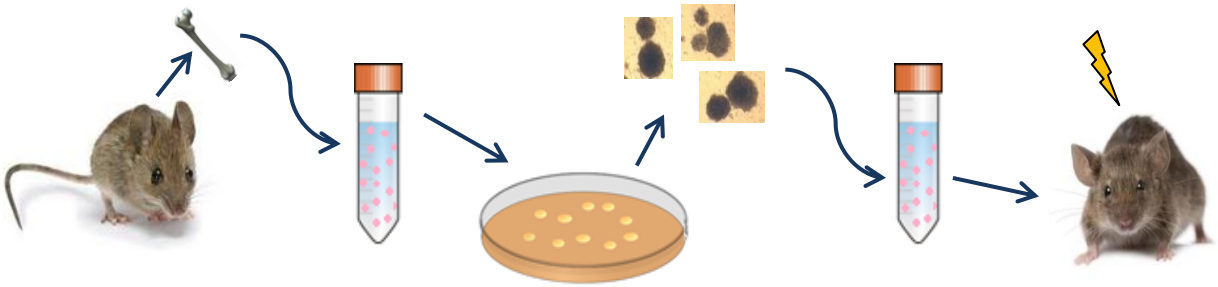


Figure 2.7. Illustration demonstrating cell preparation for transplantation.

Cells are extracted from mice and entered into a CFC assay. Cells produced in the CFC assay are c-Kit enriched and transplanted to lethally irradiated recipient mice.

2.3.8.3 Preparation of recipient mice

Ly5.1/Ly5.2 F1 (first filial generation) hybrid mice, which result from crossing Ly5.1 and Ly5.2 mice, were supplied by University of Edinburgh Biological Services and used as recipient mice. Ly5.1/Ly5.2 F1 hybrid mice are heterogeneous for CD45.1 and CD45.2 and are referred to in this report as $CD45^{het}$. $CD45^{het}$ cells can be positively stained for both CD45.1 and CD45.2 allowing this cell population to be visualised in the double positive quadrant when analysed by flow cytometry. Recipient mice were subjected to 10Gy lethal radiation 24 hours prior to injection to deplete the bone marrow of the mice. Radiation treatment causes the mice to be immunocompromised following bone marrow depletion. Mice were kept in sterile filter-top cages and treated with anti-inflammatory drugs for 4 weeks from the date of radiation, to reduce the chance of infection until the bone marrow was reconstituted by the transplanted bone marrow.

2.3.8.4 Transplantation

Injection to recipients was carried out as soon as possible following cell preparation. Recipient mice received tail vein injections of 200µL of cell suspension under sterile conditions. Mice were closely observed following transplantation and were sacrificed if considered moribund. Following 4 weeks in sterile conditions, mice were transferred to open-top cages and kept in non-sterile conditions.

2.3.8.5 Analysis

Four weeks following injection, peripheral blood samples were taken from the tail vein to assess the background of the cells derived from the reconstituted bone marrow. Blood samples were taken every 2 weeks and analysed by FACS to determine the frequency of $CD45.2^{+}$ cells as an indication of leukaemia. When mice appeared to have a high frequency of $CD45.2^{+}$ cells within the blood, they were considered leukaemic and were sacrificed to prevent suffering of the animal. At this point, mice usually had some

physical symptoms of leukaemia including swelling around the face or torso and lethargic behaviour. Blood, bone marrow, spleen and thymuses were harvested from leukaemic mice for analysis.

2.3.8.5.1 Blood

Blood samples were collected in heparin-coated collection tubes and kept at room temperature to minimise coagulation. Neat blood samples were used to assess cellularity and WBC concentration which was determined by automated cell counter. 10µL of blood was added to 400µL of NH_4Cl to lyse red blood cells and to allow white blood cells to be analysed. Lysed cells were incubated with antibodies against CD45.1 and CD45.2 to determine the frequency of CD45.2 cells. In addition, antibodies against c-Kit, Mac-1 and Gr-1 were used to determine the frequency of cells with a LSC phenotype. Cells were washed and resuspended in PBS before being analysed by FACS.

Ten microlitres of whole blood was also used to create blood smears by spreading the sample onto a microscope slide and fixing the cells in ethanol. Slides were then treated with Giemsa-Wright staining solution which allows cells to be differentiated when analysed under a light microscope. Images were collected from several blood samples to assess the presence of blast-like cells within the blood.

2.3.8.5.2 Bone marrow

Femur and tibia were collected with care from leukaemic mice and bone marrow cells extracted as per section 2.3.5.1. The WBC concentration of the bone marrow cell suspension was counted by automated cell counter. Bone marrow cells were stained with antibodies against CD45.1, CD45.2, c-Kit, Mac-1 and Gr-1 as described in section 2.3.6.2.

Bone marrow cells of leukaemic mice were also further analysed to determine apoptotic frequencies as detailed in section 2.3.6.4.

2.3.8.5.3 Spleen & Thymus

Spleens collected from leukaemic mice were weighed, measured and photographed to evaluate the enlargement of the spleen which is characteristic of leukaemic disease.

Spleens and thymuses were mashed in 12mL & 7mL PBS/2% FBS, respectively, using a sterile plunger and filtered to give a single cell suspension. Cellularity and frequency of $CD45.2^+$ LSCs were determined as described in section 2.3.6.2.

2.3.9 Statistical analysis

The results included in this report are shown as the means of samples including both biological and technical replicates. Error was calculated based on standard deviation. All statistical analyses were performed using the student's unpaired t-test for two sample analysis using Microsoft Excel. A t-test value of $p < 0.05$ was considered significant.

3 Results

3.1 Establishment of Pre-LSCs Lacking PHD Isoforms and the Investigation into Self Renewal Capacity

3.1.1 Optimisation of antibiotic concentration required for selection of transduced cells

In order to investigate the role of PHD in leukaemia, an *in vitro* transformation assay which is well established in the literature was utilised (Kroon et al, 1998; Somervaille & Cleary, 2006; Zeisig & So, 2009; Yeung et al, 2010). This assay facilitates the *in vitro* transformation of haemopoietic cells through retroviral transduction with leukaemia-inducing oncogenes, for example Meis1 and Hoxa9. The successful transduction of cells with oncogene containing retroviruses can be confirmed using appropriate selection antibiotics, based on the presence of antibiotic resistance cassettes carried by the plasmids (as detailed in Chapter 2).

The viruses used for transformation had not previously been assessed in haemopoietic cells, and therefore the assay required optimisation. Puromycin and neomycin antibiotic treatments were used to verify Meis1 and Hoxa9 retroviral transduction, respectively. A concentration-effect assay was conducted to determine the optimal dose of these antibiotics to be used for selection, that is, the concentration which killed non-transduced cells lacking the antibiotic resistance cassette. However, as normal HSPCs are unable to survive in *in vitro* culture for long periods, a commonly used mouse fibroblast cell line (NIH3T3) was chosen for the initial antibiotic sensitivity assay. It should be noted that there are some differences between NIH3T3 cells and HSPCs and that NIH3T3 cells may not be the best representative of HSPC sensitivity. The main differences between these cells are that where HSPCs are primary cells that grow in suspension culture, NIH3T3 cells are adherent cell lines. Despite these differences, NIH3T3 cells were chosen for this assay as they are of mouse origin. Unfortunately, murine suspension cells were unavailable for this assay. NIH3T3 cells were seeded into 6 well plates and allowed to adhere for 24 hours. Puromycin or neomycin was then added to cells in increasing concentrations. NIH3T3 cells were then grown in the presence of antibiotics for 72 hours

to determine which concentration effectively killed non-transduced NIH3T3 cells. The addition of 0.5ng/mL puromycin failed to effectively kill parental, non-transduced NIH3T3 cells (Fig. 3.1.1). However, non-transduced NIH3T3 cells did not survive following addition of 1ng/mL puromycin. Similarly, the addition of 0.5µg/mL neomycin was ineffective; however, 1µg/mL neomycin successfully killed non-transduced NIH3T3 cells.

The drug sensitivity assay was conducted in order to determine the drug concentrations needed to effectively select transduced NIH3T3 cells. NIH3T3 cells were used to test the efficiency of each batch of virus produced and the concentrations used during this selection process were determined by the results of the sensitivity assay. The results of the sensitivity assay were also used to determine an approximate concentration to use for selecting transduced HSPCs. However, as the exact sensitivity of HSPCs to either puromycin or neomycin was unclear, it was decided to use a slightly higher concentration for these cells to reduce the possibility of non-transduced cells evading selection. Based on the results of the NIH3T3 selection assay, it was concluded that 1.5ng/mL puromycin and 1.5µg/mL neomycin were the most effective concentrations to be used to select transformed HSPCs.

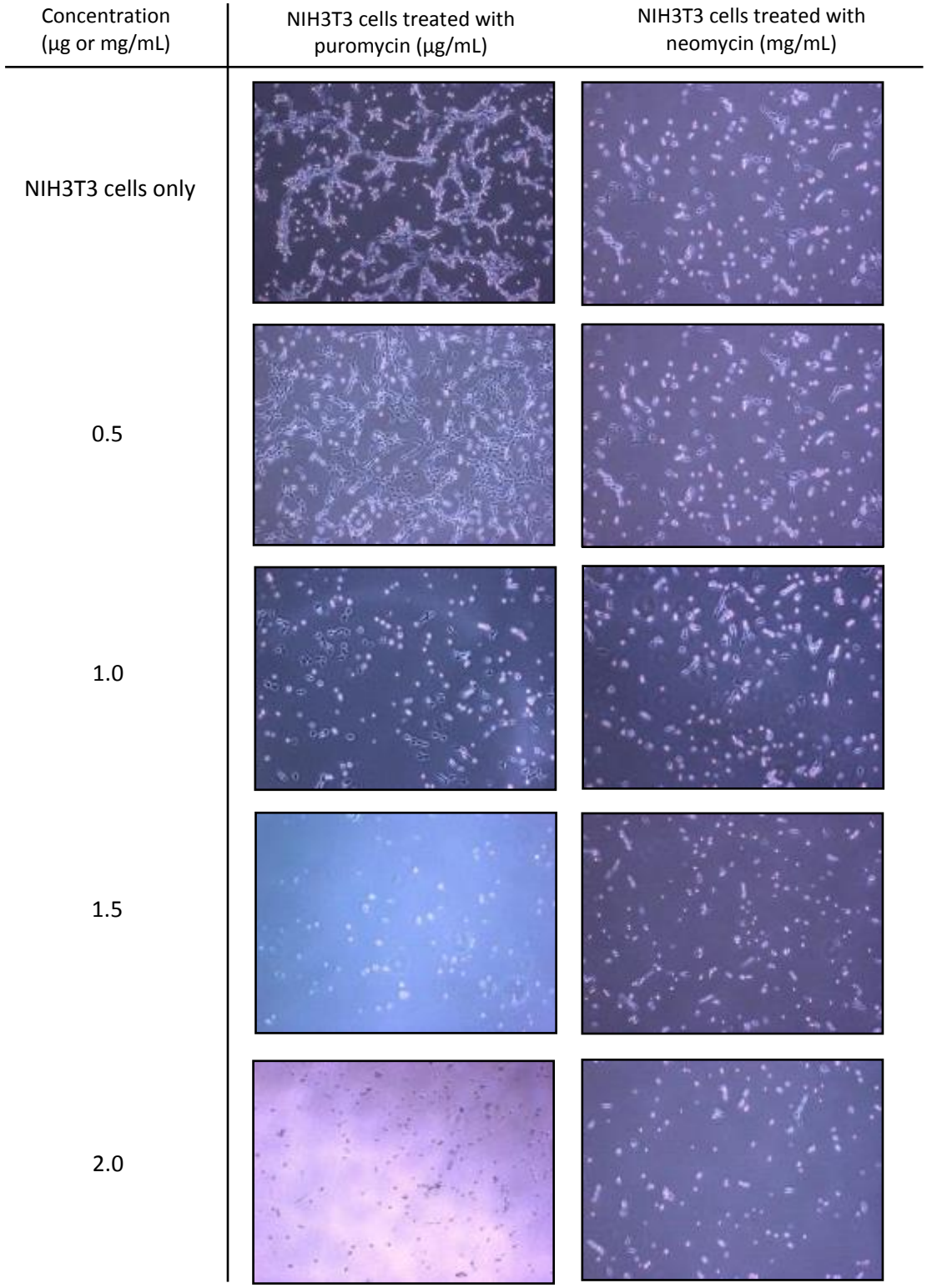


Figure 3.1.1. Antibiotic titration for retroviral selection.

Meis1 and Hoxa9-transduced NIH3T3 cells were cultured in the presence of different concentrations of puromycin and neomycin in order to determine the most effective concentration of antibiotic that would kill the majority of cells, allowing the surviving cells to be positively selected, based on the expression of antibiotic resistance genes. In brief, mouse fibroblast NIH3T3 cells were seeded and cultured overnight in DMEM media to allow adhesion. Adherent cells were cultured in media containing viral particles for 24 hours, allowing cells to be transduced with Meis1 and Hoxa9 oncogenic viruses. Meis1 and Hoxa9-transduced NIH3T3 cells were cultured with antibiotics for 72 hours before survival of the cells was assessed. Cells undergoing selection were imaged and assessed by standard light microscopy.

3.1.2 Verification of successful transduction by Meis1 and Hoxa9 retroviruses

Efficiency of Meis1 and Hoxa9 retroviral transduction was assessed using NIH3T3 cells. NIH3T3 cells were transduced with retroviruses and grown in the presence of selection antibiotics. Their response to the antibiotic was assessed after 72 hours (Fig. 3.1.2). NIH3T3 cells which survived the selection were considered to be fully transduced and so the virus production was verified as successful. Every batch of retrovirus produced was tested in this manner and only virus batches that were confirmed as effective in NIH3T3 transduction were used to transform HSPCs.

3.1.3 Self renewal as a read out for transformation

Self renewal capacity is known to be significantly enhanced in transformed cells and so investigating self renewal in cells lacking PHD would also indicate the requirement of PHD in the transformation of LSCs. In order to assess self renewal, femurs and tibias were first harvested from mice. Bones were crushed to release bone marrow cells, which included the HSPCs, and these were collected in PBS suspension. HSPCs were enriched based on the expression of c-Kit. HSPCs extracted from wild-type (WT) mice were used as controls. HSPCs were also collected from mice lacking individual or multiple PHD isoforms, referred to collectively as PHD knock-out (KO). Following viral transduction, transformation was assayed in the colony-forming cell (CFC) assay that is commonly used in the literature to determine the self renewal capacity of cells *in vitro*. WT and PHD KO cells were each analysed and compared so that the role of PHD in the self renewal capacity of pre-LSCs could be determined.

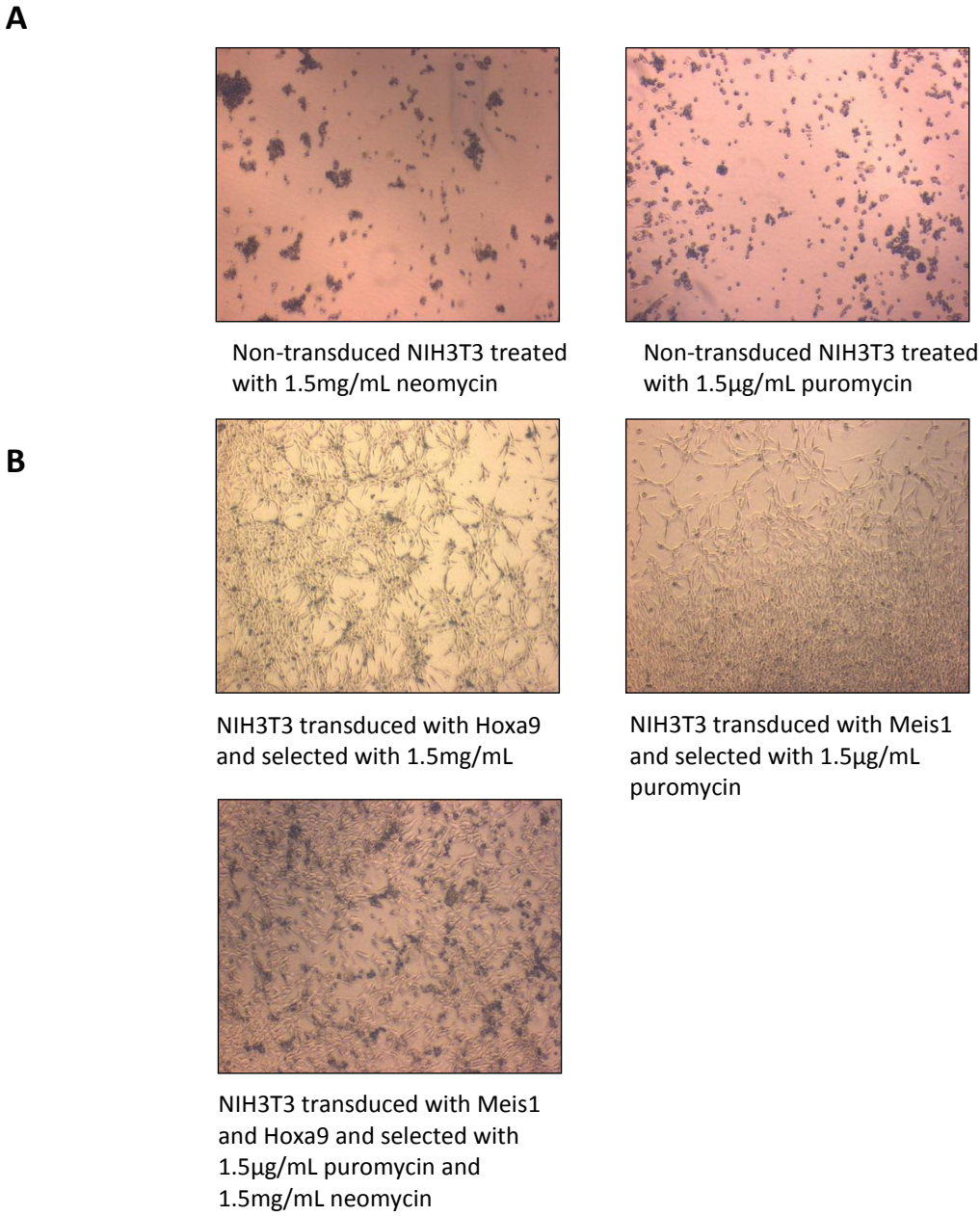


Figure 3.1.2. Retroviral selection for Meis1 and Hoxa9 transduction.

NIH3T3 cells were used to test the efficiency of transduction for each batch of virus produced. NIH3T3 cells were seeded and cultured overnight in IMDM to allow cells to adhere. Cells were cultured for 24 hours in media contain Meis1, Hoxa9 or both Meis1 and Hoxa9 viral particles to allow transduction of NIH3T3 cells. Following transduction, cells were cultured in fresh media containing puromycin or neomycin at concentrations determine by the optimisation experiment, in order to select fully transduced cells based on the expression of resistance cassettes carried by the retroviruses. This method was used to test efficiency of each batch of virus produced to ensure efficient transduction of cells. A. As a control, non-transduced NIH3T3 cells were treated with puromycin or neomycin to ensure selection was efficient and that non-transduced cells do not grow following selection. B. NIH3T3 cells retrovirally transduced with Meis1, Hoxa9 or both Meis1 and Hoxa9 were considered to be successfully transformed based on their resistance to puromycin or neomycin.

A non-transforming control virus was used to determine the survival and self renewal capacity of non-transformed HSPCs. HSPCs were infected with empty vector retrovirus *Mouse Stem Cell Virus* (MSCV). MSCV-transduced cells produced colonies that were well-spread with a distinct morphology as compared to colonies produced by Meis1/Hoxa9-transduced cells. Colonies produced by MSCV-transduced cells appeared to have a similar phenotype regardless of genotype, which is WT or KO background (Fig. 3.1.3). MSCV-transduced cells also produced colonies on the second round of replating. Colony frequency of MSCV-transduced cells was similar regardless of genotype with no significant differences seen. This result suggested that the absence of PHD isoforms did not affect the self renewal capacity of non-transformed HSPCs *in vitro*. All MSCV-transduced cells failed to produce colonies on the third round of replating, suggesting that non-transformed HSPCs have a limited self renewal capacity *in vitro*, as was expected.

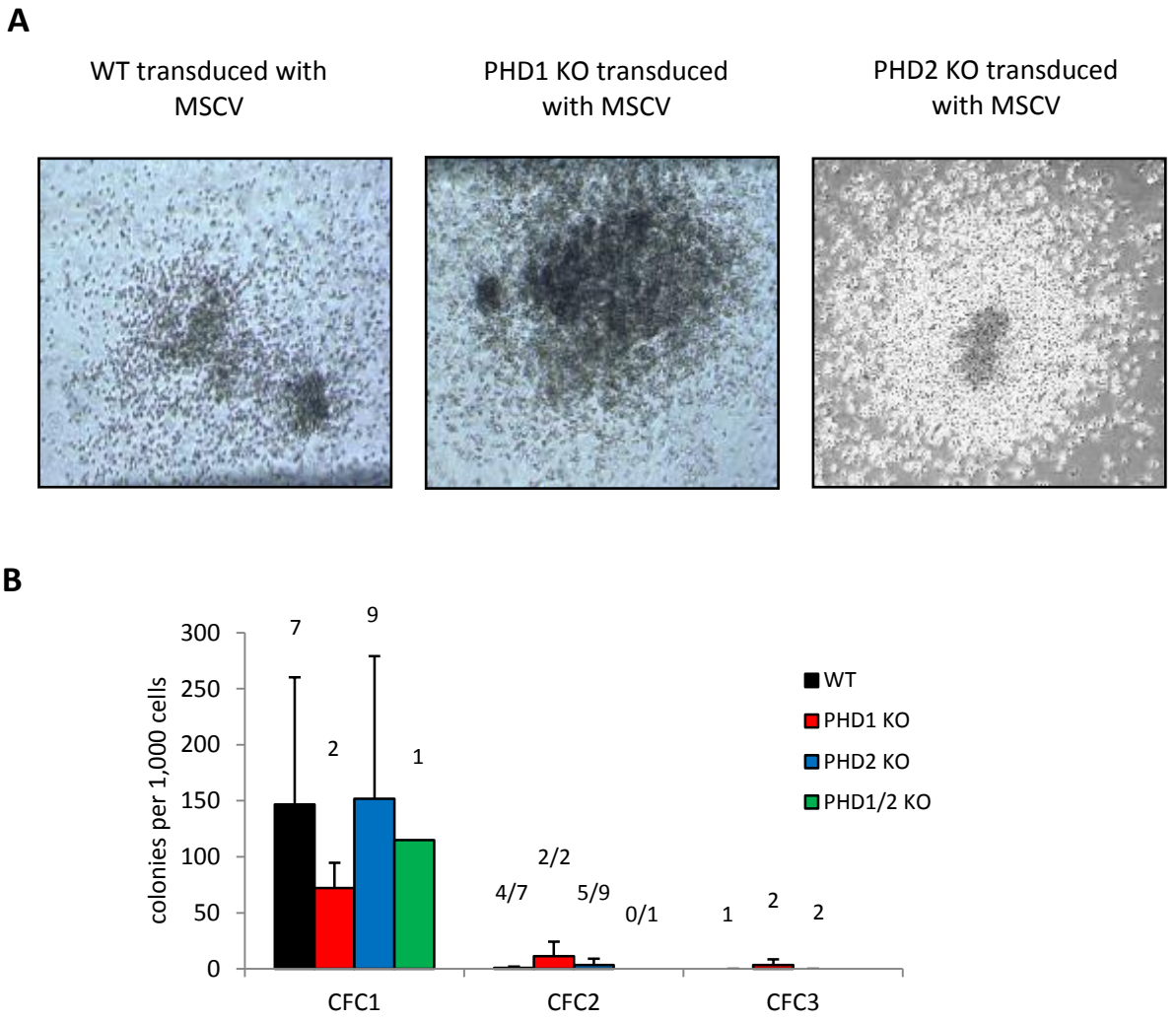


Figure 3.1.3. Transduction of HSPCs with MSCV-neo and MSCV-puro.

Bone marrow cells were extracted from WT and PHD KO mice. White blood cell population was purified by lysing red blood cells. Cells were enriched for c-Kit expression to enrich a HSPC starting population. HSPCs were transduced with MSCV control virus to assess the colony forming abilities of non-transformed HSPCs. Freshly thawed media containing MSCV-neo and MSCV-puro viral particles were added to 1 x 10^6 cells. Cells were centrifuged at 800 x g for 2 hours before being resuspended in fresh media and cultured overnight. Procedure was repeated after 24 hours. 48 hours following infection with virus, cells were cultured in media containing neomycin or puromycin for 72 hours to select fully transduced cells. Following selection, cells were counted and seeded into methylcellulose at 10,000cells/mL. Cells were cultured for 5-7 days and cells with self renewal ability produced colonies. Colonies numbers were recorded before cells were harvested, counted and seeded into fresh methylcellulose for a further 5-7 days. Replating procedure was repeated every 5-7 days until cells expired. A. Images are representative of colonies produced by WT, PHD1 KO and PHD2 KO HSPCs. B. Data presented are the mean total number of colonies produced by MSCV-neo/MSCV-puro-transduced cells during serial replating assay. The number of biological replicates for each genotype seeded into CFC1 is annotated above each data point. Mean colony numbers for CFC2 and CFC3 were based only on plates that produced colonies. The number of plates that produced colonies in CFC2 and CFC3 is annotated in the graph.

Following selection with puromycin or neomycin, WT and PHD KO HSPCs successfully transduced with Meis1 and Hoxa9 all formed dense colonies that had a distinctly different phenotype from cells transduced with MSCV (Fig. 3.1.4). Colonies produced by Meis1/Hoxa9-transduced cells were similar to those detailed in the literature, which are described as hyper-cellular and immature (Takacova et al, 2012). The morphology of the colonies suggested that cells may have had enhanced proliferation and reduced differentiation as a result of Meis1/Hoxa9 transduction.

Colonies produced by Meis1/Hoxa9-transduced cells were counted after one week. Cells were serially replated, with colonies being counted at each replating. In contrast to MSCV-transduced cells, which did not survive after two rounds of replating, Meis1/Hoxa9-transduced cells formed colonies after multiple rounds and were considered fully transformed after three passages (Fig. 3.1.5). Colony frequency can be used to indicate self renewal capacity of the cells. Colony production was comparable across all genotypes and no significant differences were seen. However, this assay proved to be highly variable. In order to overcome this variability, multiple biological replicates were accrued over several experiments. However, it is appreciated that the number of biological replicates completed was insufficient for some of the genotypes analysed and so further work needs to be carried out in order to gain statistical power and significance. Only one biological replicate was available for PHD1/2/3 triple KO, therefore the calculated error was based on intra-experimental technical replicates. Therefore, no solid conclusion could be drawn on these data. Further data collection for this genotype in particular would be very interesting. The functions and interactions of individual PHD isoforms are unclear, but it is possible that there may be some redundancy between them. It would be interesting to address if in the absence of all three PHD isoforms, a situation in which compensation is not possible, self renewal and transformation would be affected. Unfortunately, the lack of availability for this genotype made this impossible.

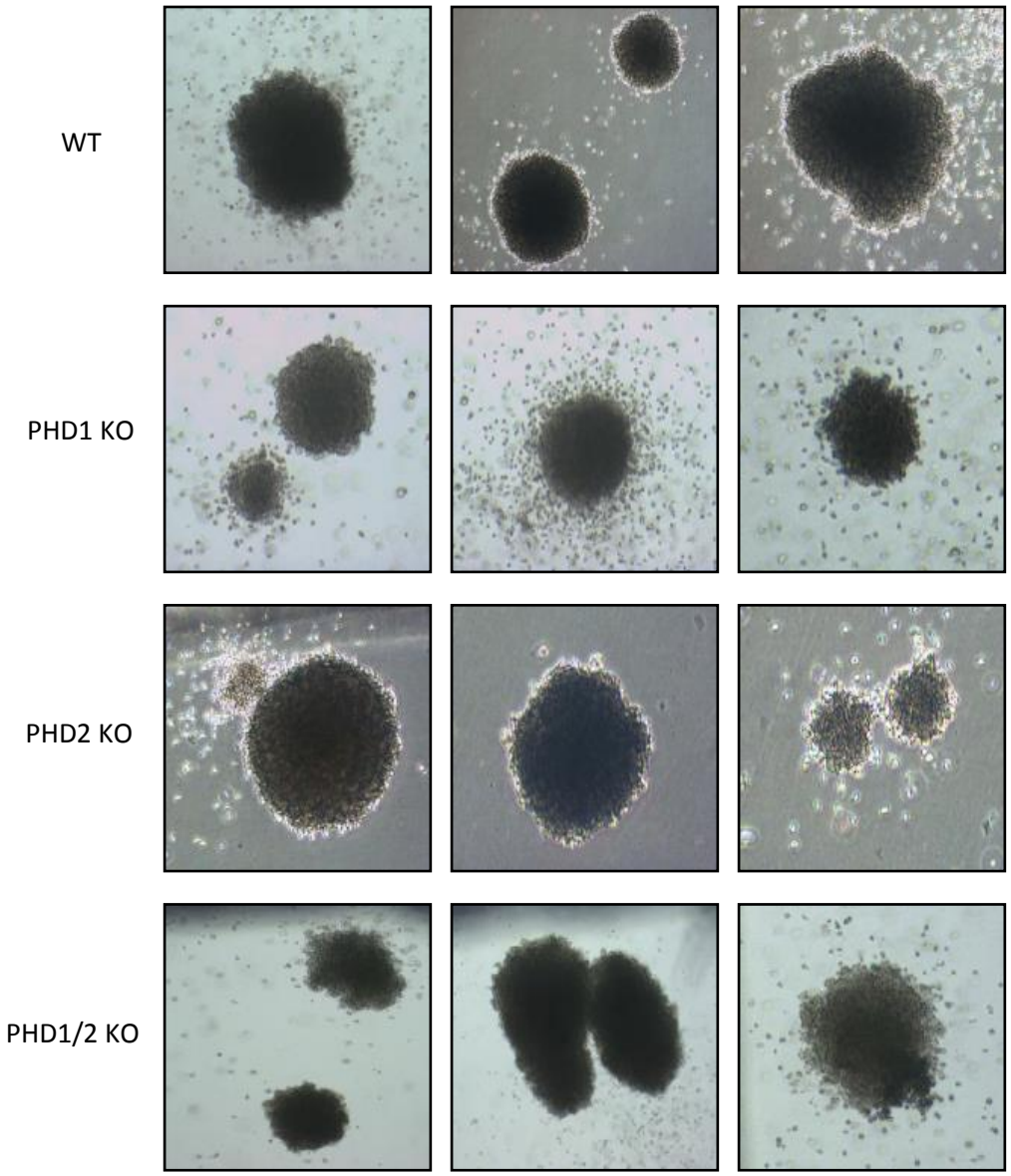


Figure 3.1.4. Colony production of pre-LSCs transduced with Meis1 and Hoxa9.

HSPCs were extracted and purified from mouse bone marrow. Freshly thawed media containing Meis1 and Hoxa9 viral particles were added to 1 x 10^6 cells. Cells were centrifuged at 800 x g for 2 hours before being resuspended in fresh media and cultured overnight. Procedure was repeated after 24 hours. 48 hours following infection with virus, cells were cultured in media containing neomycin or puromycin for 72 hours to select fully transduced cells. Following selection, cells were counted and seeded into methylcellulose at 10,000cells/mL. Cells were cultured for 5-7 days and cells with self renewal ability produced colonies. Colonies numbers were recorded before cells were harvested, counted and seeded into fresh methylcellulose for a further 5-7 days. Replating procedure was repeated every 5-7 days until cells expired. A. Images are representative of colonies produced by WT, PHD1 KO, PHD2 KO and PHD1/2 KO HSPCs transduced with Meis1 and Hoxa9.

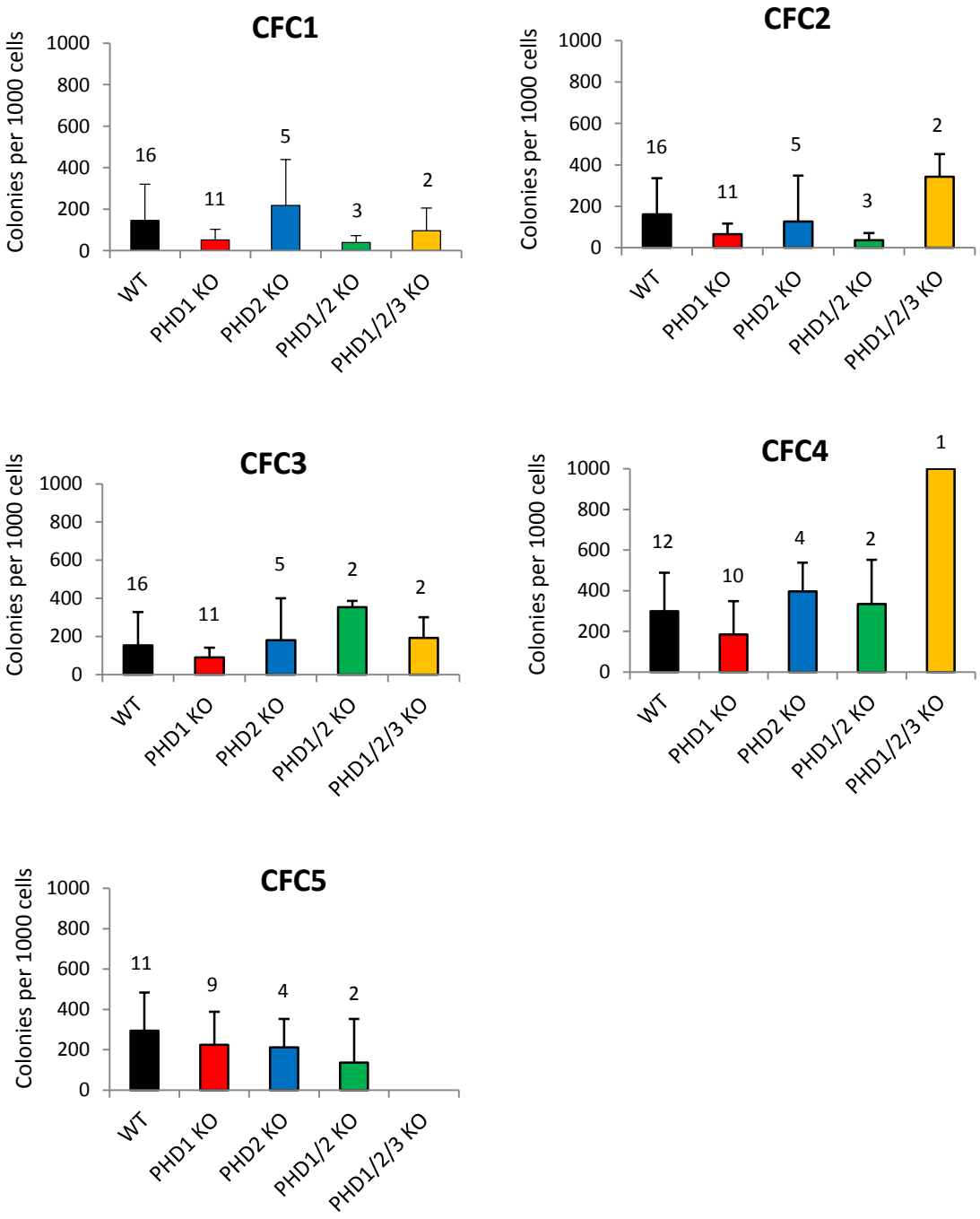


Figure 3.1.5. Colony frequency of Meis1/Hoxa9 transformed pre-LSCs.

HSPCs were extracted from mouse bone marrow, purified, transduced with both Meis1 and Hoxa9 retroviruses and seeded into a methylcellulose serial replating assay as previously described. Colonies were counted at each round of replating. Data presented are the mean total number of colonies produced by Meis1/Hoxa9-transduced cells during serial replating assay. The number of biological replicates for each genotype is annotated within each graph.

Meis1 and Hoxa9 are both known to be associated with the development of AML and are downstream targets of MLL fusion products. Transduction of HSPCs with these oncogenes has been reported in the literature to successfully transform cells *in vitro*, which upon transplantation, have the ability to generate murine leukaemia that is comparable to human AML. These cells are referred to as pre-LSCs. It is believed that although pre-LSCs have leukaemic potential upon transplantation, they require an additional mutational hit *in vivo*, in order to become fully transformed LSCs. Transduction of cells with MLL fusion products is also widely used experimentally. However, the overexpression of Meis1 and Hoxa9 simultaneously is known to induce AML in murine models which is similar to AML induced by MLL transduction, but with a much shorter latency (60 days compared to 200 days), making this a more efficient experimental model (Wang et al, 2006). Since transduction by downstream targets of MLL fusions, such as Meis1 and Hoxa9, provides a more direct and rapid route to disease development, it is possible that this may result in a more aggressive phenotype. Studies by Kroon et al showed that cells transformed with Hoxa9 alone induced AML with a long latency (8 months). Cells transformed with Meis1 alone, were not capable of generating leukaemia. However, cells transformed with both Meis1 and Hoxa9 simultaneously, are known to initiate leukaemic disease within 2-6 months (Kroon et al, 1998; Wang et al, 2006; Gibbs et al, 2012). Furthermore, Meis1 is a known to have a role in HSCs and has been reported to regulate HIF-1α (Simsek et al, 2010; Unnisa et al, 2012). HIF-1α is reported to have a role in leukaemia (Wang et al, 2011; He et al, 2013; Zhang et al, 2012), and it stands to reason that up-regulation of HIF-1α, through overexpression of Meis1, may lead to the enhanced leukaemogenesis seen in this model. The link between Meis1 and HIF-1α makes this an interesting model in which to study the role of hypoxia signalling in leukaemia. As PHD isoforms function to destabilise HIF-1α, it is possible that HIF1-α proteins are stabilised in cells lacking various PHD isoforms, either single or multiple isoforms. Transducing PHD KO cells with Meis1 would cause HIF-1α to be stabilised through both pathways, that is, through PHD ablation and Meis1 overexpression, resulting in a higher expression of HIF-1α in PHD KO cells. A higher expression of HIF-1α may in turn lead to an enhanced Leukaemic phenotype. Therefore, it was interesting to see if PHD KO cells had enhanced leukaemogenesis compared to WT cells.

It has been hypothesised, although not confirmed, that cells transduced with Meis1/Hoxa9 *in vitro* are pre-disposed to leukaemic transformation, but require a second mutational hit *in vivo*, fully transforming them to LSCs which are capable of generating leukaemia (Wang et al, 2005; Chen et al, 2008). *In vitro*, these cells are not considered to be fully capable of leukaemogenesis and are considered to be in a pre-leukaemic state and are therefore referred to as pre-LSCs. Cells generated *in vivo* that are able to induce leukaemia upon transplantation to secondary recipients are referred to as LSCs (So et al, 2010).

Considering the known leukaemic potential of cells transformed with Meis1 and Hoxa9, along with the putative link to hypoxia signalling, these oncogenes were chosen for study in order to transform HSPCs to pre-LSCs.

3.1.4 The expression of LSC markers on Meis1/Hoxa9 transduced pre-LSCs

The simultaneous expression of Mac-1, Gr-1 and c-Kit is unique to LSCs and is commonly used to isolate this population by flow cytometry (Somervaille & Cleary, 2006; Somervaille & Cleary, 2009; Soh et al, 2010). The expression of LSC markers is also seen in pre-LSCs generated *in vitro* in similar studies. These 'LSC-like' cells are suspected to be the population that is capable of generating leukaemia *in vivo*. However, there have been some discrepancies in the requirement for Gr-1 as a pre-LSC marker. Gibbs et al recently performed an in depth study which aimed to identify the cell population responsible for initiating leukaemia *in vivo*. An *in vitro* transformation assay was used to establish a culture of pre-LSCs. Different combinations of LSC markers were then used to isolate populations which were prepared and transplanted to recipient mice. The study concluded that the Gr-1^+ c-Kit^+ cell population had low colony forming ability, (1%) and reduced leukaemic potential. However, they further concluded that populations lacking expression of Gr-1 were still capable of initiation of leukaemia. In contradiction to this study, Somervaille & Cleary found that AML cells that were sorted for Gr-1^+ expression

had full leukaemic capabilities and cells were able to initiate disease upon transplantation (Somervaille & Cleary, 2006).

However, it was observed that cells which were Mac-1$^+$ were also Gr-1$^+$. Therefore, in this study, the definition of 'LSC-like' cell is based on the simultaneous expression of c-Kit, Mac-1 and Gr-1. Flow cytometry was used to identify expression of these LSC markers and the frequency of 'LSC-like' cells was assessed. The frequency of 'LSC-like' cells was used to indicate if this population is increased following transformation with leukaemia-inducing oncogenes and if it is altered by the ablation of PHD genes.

The expression of the LSC markers c-Kit, Mac-1 and Gr-1 by harvested CFC was assessed by flow cytometry. HSPCs transduced with MSCV were used as a control to identify the frequency of cells with an LSC phenotype within the non-transformed cell population. The first analysis was for c-Kit+ cells as a single marker. c-Kit expression alone was assessed as this is a marker of primitive populations. The expression of c-Kit was used to determine if the cells had differentiated or remained primitive. This was assessed in WT cells and PHD KO cells to examine if PHD ablation affects the differentiation of HSPCs. Before cells were plated into methylcellulose (pre-CFC), MSCV-transduced cells showed considerable c-Kit$^+$ expression (range 28.8-62.8%), suggesting that a large fraction of the cells analysed had not differentiated and had remained primitive (Fig. 3.1.6). Colonies formed at each round of the assay were also analysed by flow cytometry for the expression of c-Kit. Colonies analysed following the first round of the assay (CFC1) were seen to have a higher percentage of c-Kit$^+$ cells. It is likely that c-Kit$^-$ cells had already expired at this point. c-Kit$^+$ cells however are more primitive and have increased self renewal capacity and it is possible that this population does not expire as quickly as c-Kit$^-$ cells, allowing the c-Kit$^+$ population to become enriched. As MSCV-transduced cells expired after the second round of replating, very few cells were available for analysis at this point and not all genotypes were available. PHD2 KO and PHD1/2 KO CFC cells were analysed and showed a very low percentage of c-Kit$^+$ cells. This suggested that by this point, c-Kit$^+$ cells had also begun to exhaust, along with c-Kit$^-$ cells.

Next, MSCV-transduced cells were analysed for simultaneous expression of LSC markers c-Kit, Mac-1 and Gr-1 as a measure for the frequency of LSCs. Cells analysed showed a low percentage of cells with an ‘LSC-like’ phenotype prior to the first round of the assay which increased following the first round of replating. This population diminished following the next round, at which point, cells were mostly expired.

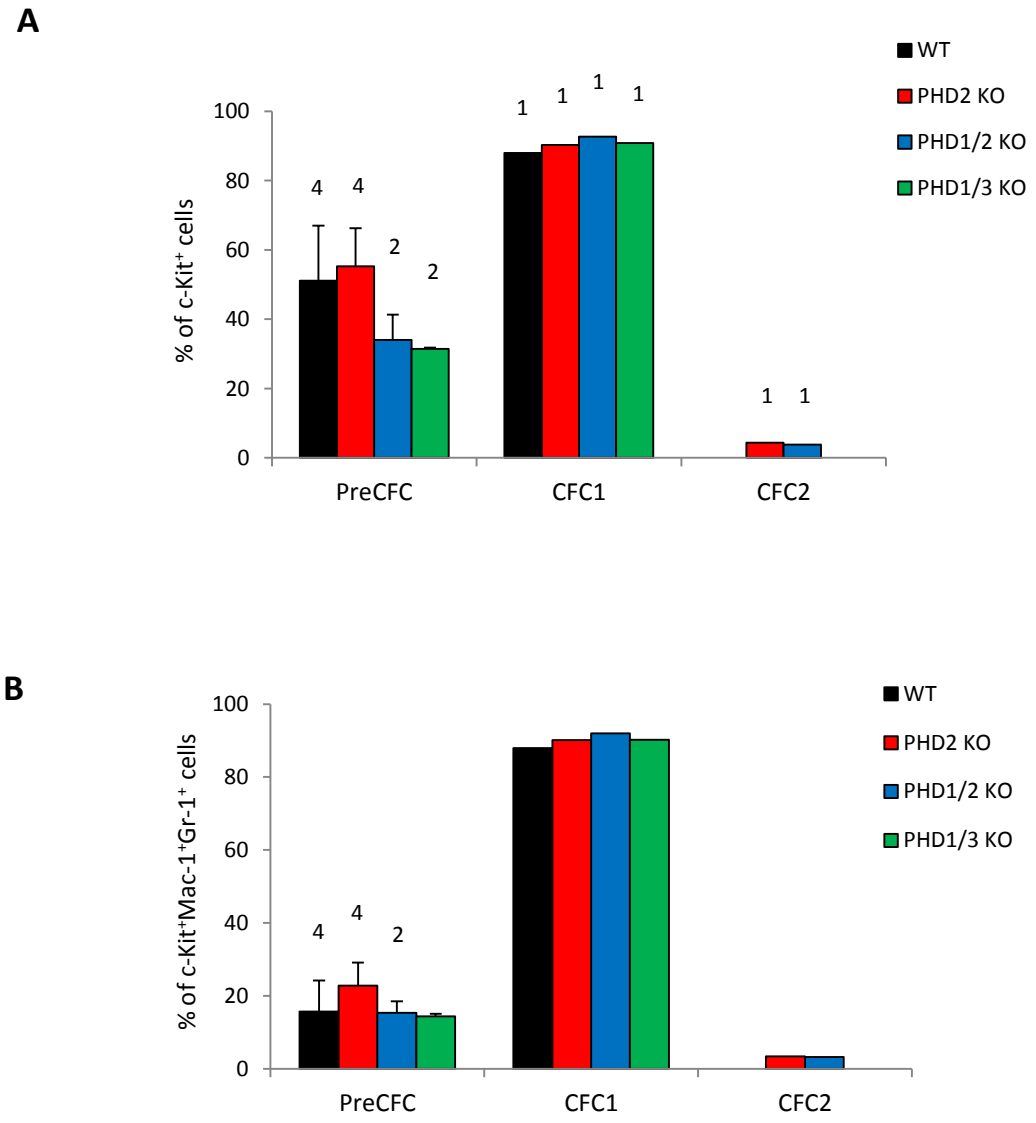


Figure 3.1.6. Immunophenotype of MSCV- transduced HSPCs.

MSCV-transduced cells were harvested from the colonies produced during the serial replating assay. Cells were washed in PBS and incubated in an antibody mix containing antibodies specific for c-Kit, Mac-1 and Gr-1 and analysed by flow cytometry. Cells were analysed for expression of c-Kit (A) and LSC markers c-Kit, Mac-1 and Gr-1 (B) at each round of replating. Data presented represent the total mean of cell frequencies. The number of biological replicates for each genotype analysed at pre-CFC is annotated within the graph. Only one biological replicate was available for genotype presented at CFC1 and CFC2 and therefor data represents a single sample.

Meis1/Hoxa9-transduced pre-LSCs were also analysed at each round of replating and the frequency of cells with an immature phenotype (c-Kit$^+$) as well as the frequency of 'LSC-like' cells were determined. The frequency of c-Kit$^+$ cells remained high throughout multiple rounds of replating, suggesting that these cells had an increased self renewal capacity compared to MSCV-transduced cells, which expired at earlier stages of the assay (Fig. 3.1.7). No significant differences were seen between WT and PHD KO cells. LSC frequency was also investigated in Meis1/Hoxa9-transduced pre-LSCs, which was seen to be low during the first stage of the assay, that is, the first time the cells were harvested before replating. This might suggest that primitive cells are expiring as they have not been fully transformed by the viruses and do not have enhanced self renewal at this stage. However, after the first round of replating, the frequency of c-Kit$^+$ cells increased significantly. This suggested that non-transformed cells have expired, enriching the population with fully transformed pre-LSCs. According to earlier data, cells transduced with MSCV virus (non-transformed) have expired at this point in the assay and so 'LSC-like' cells that survive after this point were considered fully transformed pre-LSCs. After several rounds of replating, all cells had a significant frequency of pre-LSCs which had an 'LSC-like' phenotype (40.6-73.1% between CFC2-CFC5). Statistical analysis preformed showed no significant differences between the genotypes tested. However, the frequencies of pre-LSCs were also highly variable and a larger number of biological replicates would be required before any firm conclusions could be made.

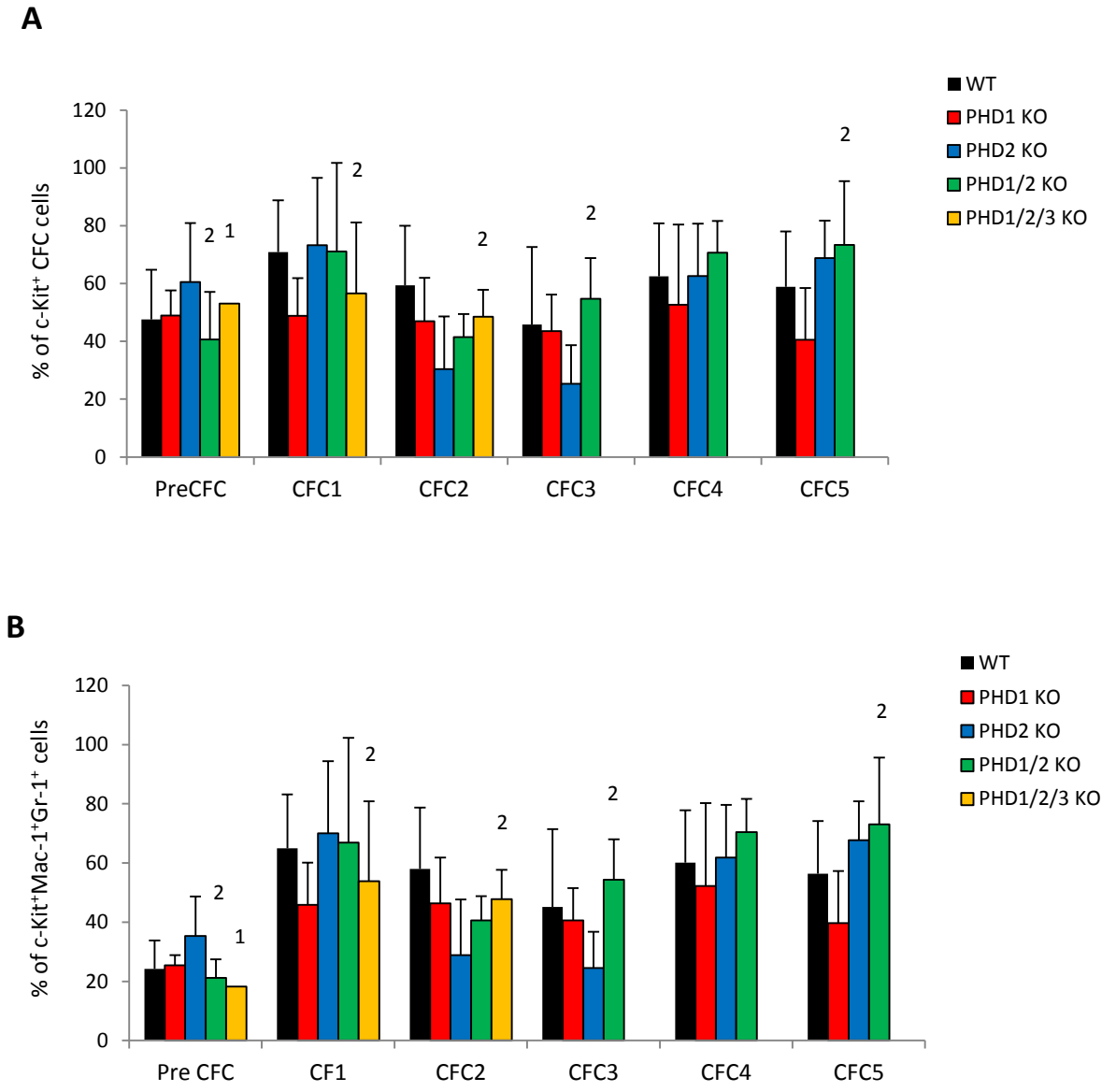


Figure 3.1.7. Immunophenotype of Meis1/Hoxa9 transformed pre-LSCs.

Transduced cells were harvested from the colonies produced during the serial replating assay. Cells were stained with antibodies specific for c-Kit, Mac-1 and Gr-1 and analysed by flow cytometry. Cells were analysed for expression of c-Kit (A) and LSC markers c-Kit, Mac-1 and Gr-1 (B) at each round of replating. Data presented represent the total mean of cell frequencies. All genotypes consisted of at least three biological replicates unless otherwise stated. Genotypes with <3 biological replicates are indicated in the graph.

3.1.5 *In vitro* prediction of leukaemic potential and self renewal capacity

A limiting dilution assay (LDA) is an *in vitro* assay that is used to quantify the frequency of a certain cell property such as self renewal ability. Variations of this assay are often used in conjunction with *in vivo* studies where limiting numbers of pre-LSCs are transplanted into recipients, in order to measure the frequency of leukaemia-initiating cells (LIC) based on the cells' capabilities to generate leukaemia. LSCs are considered to have leukaemia-initiating potential and so this assay was used to calculate the frequency of LSCs.

This assay was utilised to identify the leukaemic potential of cells lacking PHD genes, and as an alternative assay to quantify the frequency of cells with self renewal capacity within the pre-LSCs. Pre-LSCs established during CFC were sorted based on their expression of c-Kit, Mac-1 and Gr-1. WT, PHD1 KO, PHD2 KO, PHD3 KO and PHD1/3 KO cells were available for this experiment. Single pre-LSCs were seeded into methylcellulose and incubated for 10-14 days. Colony development was recorded and used to determine the frequency of cells that were capable of colony formation. Figure 3.1.8 depicts the frequencies of cells that produced colonies and that were therefore considered to be LSCs. Statistical analysis was performed and showed no significant difference between any of the genotypes assessed. However, not all genotypes analysed in CFC were available for this experiment and further work would need to be conducted to complete this study.

Formal LDA and single cell plating have been used to determine the frequency of LSCs *in vitro* by assessing the cloning ability of single AML cells generated *in vivo*, which were sorted based on the expression of LSC markers (Somervaille & Cleary, 2006; Gibbs et al, 2012; Yeung et al, 2010). Following leukaemic development, AML cells were harvested from mice and seeded as single cells. AML cells that form colonies *in vitro* are considered to be LICs. LIC frequencies in Meis1/Hoxa9-induced AML are found to be relatively high at >10% (Gibbs et al, 2012). The cloning efficiencies of Meis1/Hoxa9-induced pre-LSCs have not been calculated. However, similar assays have shown that pre-LSCs transduced with MLL fusion proteins have a cloning efficiency of approximately 18-22% (So et al, 2010).

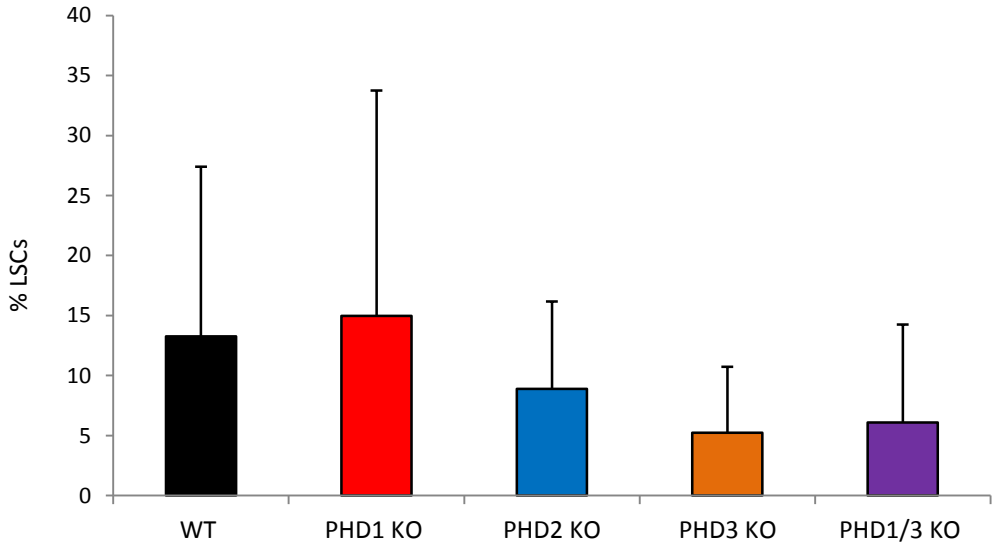


Figure 3.1.8. Cloning frequency of Meis1/Hoxa9 transformed pre-LSCs.

Meis1/Hoxa9-transformed cells were harvested from colonies produced on the third round of plating during the serial replating assay. Cells were stained with antibodies specific for c-Kit, Mac-1 and Gr-1 LSC markers. C-Kit^{+}Mac-1^{+}Gr-1^{+} cells were identified and sorted by flow cytometry as single cells into the wells of pre-prepared 96-well plates containing methylcellulose. Plates were cultured for 10-14 days and colonies were scored to determine the frequency of LSCs, which was based on the cells' ability to form a colony. Data represent the total mean frequency of LSCs calculated from 96 wells per biological sample from 3 independent experiments.

Taken together, these results suggest that the experimental model being used in this study is efficient in the transformation of HSPCs and establishment of pre-LSCs. Furthermore, these results suggest that PHD does not play a role in the transformation of pre-LSCs or their capacity for self renewal *in vitro*.

3.2 Characterisation of Pre-LSCs Lacking PHD Isoforms

3.2.1 Establishment of pre-LSC cell lines

In order to fully characterise pre-LSCs, immortalised cell lines were established. Meis1/Hoxa9-transduced HSPCs were re-plated three times in the CFC assay, at which point, cells were considered fully transformed. Pre-LSCs were harvested and cultured in cytokine rich media. Cells were passaged several times to ensure stability before assays were performed. Cell lines were established for WT, PHD1 KO, PHD2 KO and PHD1/2 KO cells and all genotypes appeared to have a similar morphology (Fig. 3.2.1).

Cell lines make ideal tools for *in vitro* characterisation as these can be grown indefinitely and large numbers of cells are available for analysis. The disadvantage to using cell lines is the emergence of randomly acquired mutations that cannot be controlled. For this reason, all analyses preformed on these cell lines were conducted at early passages to limit the possibility of random mutations. In addition, several biological replicates of each genotype were used to establish separate cell lines which were analysed individually in all experiments. However, it was not possible to analyse all biological replicates of all individual genotypes at one time and it was necessary to repeat cell line experiments several times, in order to accrue multiple biological replicates. Only one biological replicate was available for PHD1/2 KO so statistical analysis could not be performed on any of the cell line based experiments for this specific genotype and therefore no conclusions drawn. Any error bars shown for PHD1/2 KO cell line data have been based on standard deviation between technical replicates only.

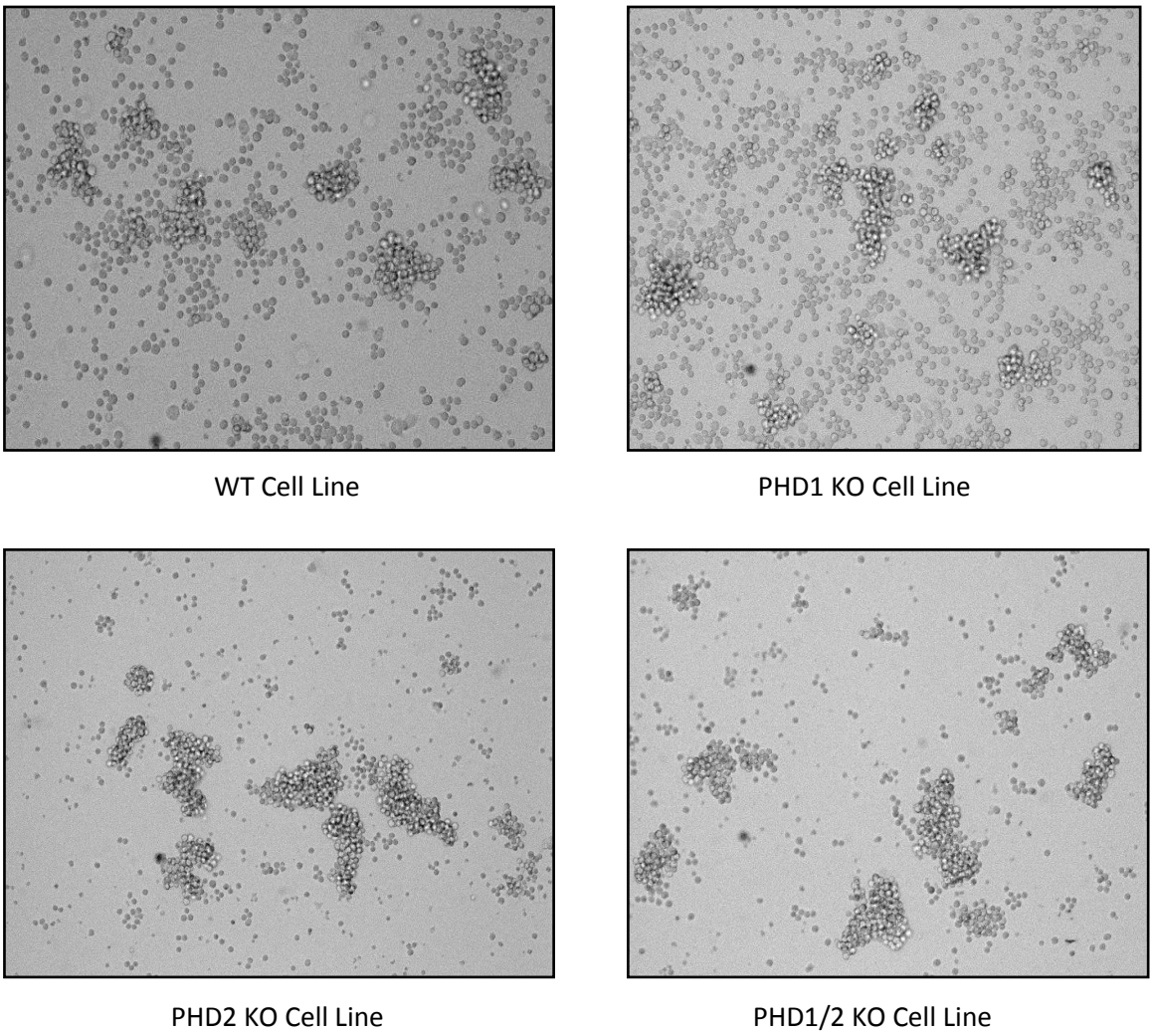


Figure 3.2.1. Establishment of Meis1/Hoxa9-transduced pre-LSC cell lines.

Meis1/Hoxa9-transformed cells were harvested from colonies produced on the third round of plating during the serial replating assay. Cells were seeded into a liquid culture of IMDM supplemented with cytokines and passaged several times until stable cell lines were established. Once cell lines were established, cells were grown in media containing reduced concentrations of cytokines. All cell lines appeared to have a similar morphology, regardless of genetic background, as assessed by standard phase microscopy. Images of cell lines were acquired between passages 8 to 16.

3.2.2 Pre-LSC cell lines show similar expression of LSC markers

Cell lines were analysed for expression of LSC markers to ensure that changing the growth conditions of the cells from semi-solid methylcellulose to liquid culture, did not alter their phenotype. All genotypes of cell lines displayed a comparable expression profile with cells analysed at CFC3 during the serial replating assay, suggesting that the immunophenotype of cells was not dramatically changed following the change in growth conditions (Fig. 3.2.2). In accordance with the immunophenotype seen in CFC3 cells (Fig. 3.1.7), cells had some variability in the expression of c-Kit, although cells had a consistently high frequency of LSCs. Unfortunately PHD1/2 KO cells were not available for analysis.

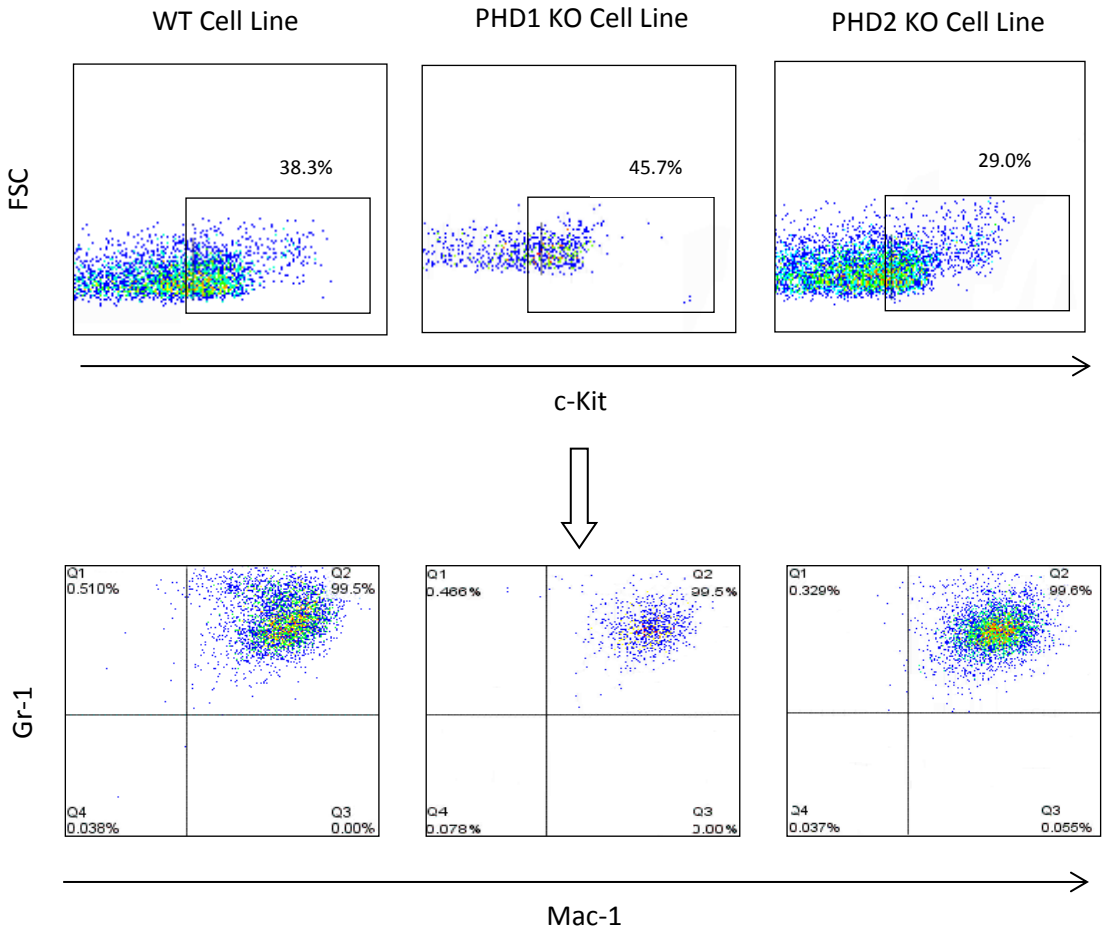


Figure 3.2.2. Immunophenotype of immortalised pre-LSC cell lines.

Pre-LSC cell lines were analysed by flow cytometry to determine the Immunophenotype of the CFC-derived cell lines. Images are representative of flow cytometry plots showing gating strategy for LSC phenotype. Cells were first gated based on c-Kit$^+$ expression. c-Kit$^+$ cells were then analysed for expression of Mac-1 and Gr-1. Frequencies of c-Kit$^+$ cells were variable between samples but are in accordance with data collected from CFC3 cells (cf Fig 3.1.7). Frequency of c-Kit cells that were also Mac-1$^+$Gr-1$^+$, was similar for WT, PHD1 KO and PHD2 KO cells.

3.2.3 The expression of HIF-1α and HIF-2α in pre-LSC cell lines

It was postulated that cells lacking PHD isoforms may overexpress HIF-1α, and as a result, have an enhanced leukaemic potential. Western blotting was used to identify the expression of HIF-1α and HIF-2α proteins in WT and in PHD KO cells. As the exact relationship being HIF-1α and HIF-2α with PHD1 and PHD2 is unclear, both HIF-1α and HIF-2α expression were analysed in cells lacking PHD1 or PHD2 and both PHD1 and PHD2. Cobalt chloride is known to stabilise HIF proteins by inhibiting pVHL binding (Yuan et al, 2003). NIH3T3 cells treated with cobalt chloride were used as a positive control. In the NIH3T3 cells, strong HIF-1α and HIF-2α bands were visible by western blotting. HIF-1α and HIF-2α proteins were undetectable in WT pre-LSC cell lines under normoxic conditions (Fig. 3.2.3). These results were as expected as HIF-1α and HIF-2α are both known to be degraded in the presence of oxygen. In the absence of either PHD1 or PHD2 isoforms, HIF-1α and HIF-2α both remained undetectable in normoxia. Interestingly, HIF-1α and HIF-2α were both strongly detected in cells lacking both PHD1 and PHD2. The stabilisation of HIF-1α and HIF-2α in cells lacking both PHD1 and PHD2, but not in cells lacking single isoforms, suggests that ablation of single PHD isoforms is not sufficient to stabilise HIF. The individual and overlapping functions of PHD isoforms are unclear in the literature. However, these data might indicate that PHD1 and PHD2 may be able to compensate for each other in certain cellular contexts.

Furthermore, as HIF-1α was not stabilised in PHD1 KO or PHD2 KO cell lines, this suggested that the expression of Meis1 and the leukaemic potential of the cells would not be altered through up-regulation of HIf-1a. However, PHD is known to play a role in several other pathways and it is possible that altered regulation of an unidentified non-hypoxia related pathway is involved. However, since HIF proteins were stabilised in PHD1/2 KO cells, it was possible that the leukaemia potential would be enhanced in these cells due to the over expression of HIF-1α. Unfortunately, PHD1/2 KO cells were not analysed for leukaemic potential by LDA as cells were limited, and were utilised for other experiments. However, PHD1/2 KO cells did not show any differences in self renewal capacity or LSC frequency which suggested that the leukaemic potential was not altered.

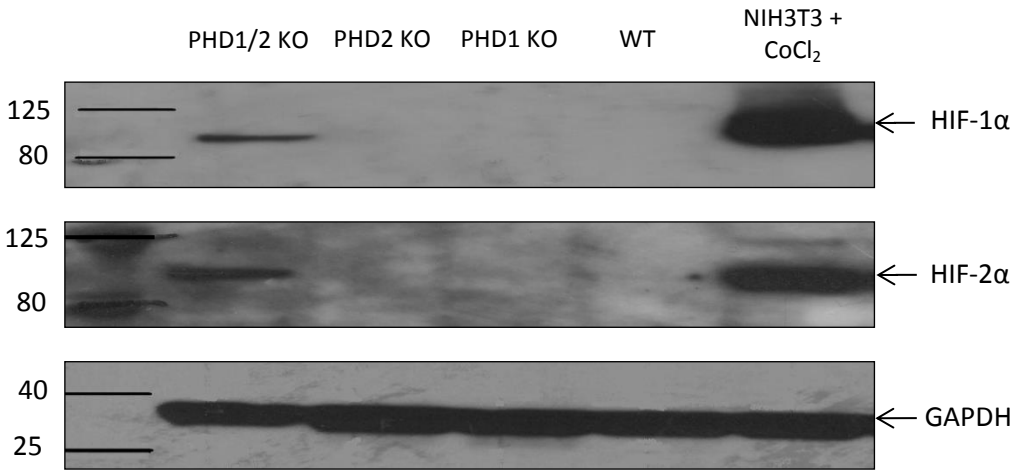


Figure 3.2.3. HIF-1α and HIF-2α protein expression in pre-LSC cell lines.

Meis1/Hoxa9-transduced pre-LSC cell lines were used to investigate the expression of HIF proteins. Protein lysates were prepared from cell lines and the protein was quantified. Equal concentrations of protein were used for each sample which were analysed by western blotting. NIH3T3 cells were treated with $CoCl_2$ for 24 hours and lysates used as a positive control for HIF protein accumulation. Strong bands were visible in both HIF-1α and HIF-2α western blots that were in accordance with band sizes of HIF-1α (approximately 92kDa) and HIF-2α (approximately 100kDa). Bands were also visible in PHD1/2 KO cell lysates but were not seen in WT, PHD1 KO or PHD2 KO lysates. GAPDH was used as a loading control. Results were confirmed by two independent experiments.

3.2.4 Cell cycle distribution and proliferation rate of established pre-LSC cell lines

One of the most fundamental properties of cancer cells is an increased rate of proliferation. Retroviral expression of Meis1 and Hoxa9 in HSPCs is known to stimulate proliferation. One of the aims of this study is to determine if there may be a difference in leukaemic potential between WT and PHD KO cells and this may be evident in the differential distribution of cell cycle phases. Furthermore, PHD1, although not PHD2, has been linked to regulation of the cell cycle. PHD1 has been seen to up-regulate cyclin D1 causing increased proliferation in breast, cervical and renal cancer cell lines (Zhang et al, 2009). However, the role of PHD in the proliferative state of HSPCs or LSCs has not been determined.

Pre-LSC cell lines derived from the CFC assay were used to investigate the frequency of pre-LSCs in different cell cycle phases. At first, the frequency of cycling cells based on the expression of the proliferation marker Ki67 (expressed from G1 through to the end of M phase) was assessed. Additional staining with DAPI allowed determination of the distribution of cells in the separate phases of the cell cycle, based on the DNA content. Figure 3.2.4 shows frequencies of cells in G0, G1 and G2/S/M phases as determined by flow cytometry. Data was collected at 8, 24 and 48 hours after passaging. Data collected at 24 hours was repeated to give three biological replicates (with the exception of PHD1/2 KO). Statistical analysis showed that there were no differences in cell cycle frequencies between WT, PHD1 KO and PHD2 KO cell lines. PHD functions are thought to be cell context specific and although differences were seen in breast, cervical and renal cancer cell lines lacking PHD1, it is possible that PHD1 does not perform this function in the context of the haemopoietic system, or more specifically pre-LSCs. In addition, the similar frequencies of cell cycle phases between WT and PHD KO cells suggested that Meis1 expression was unaffected by the absence of PHD and that Meis1 and Hoxa9 expression were likely to be comparable across the different groups.

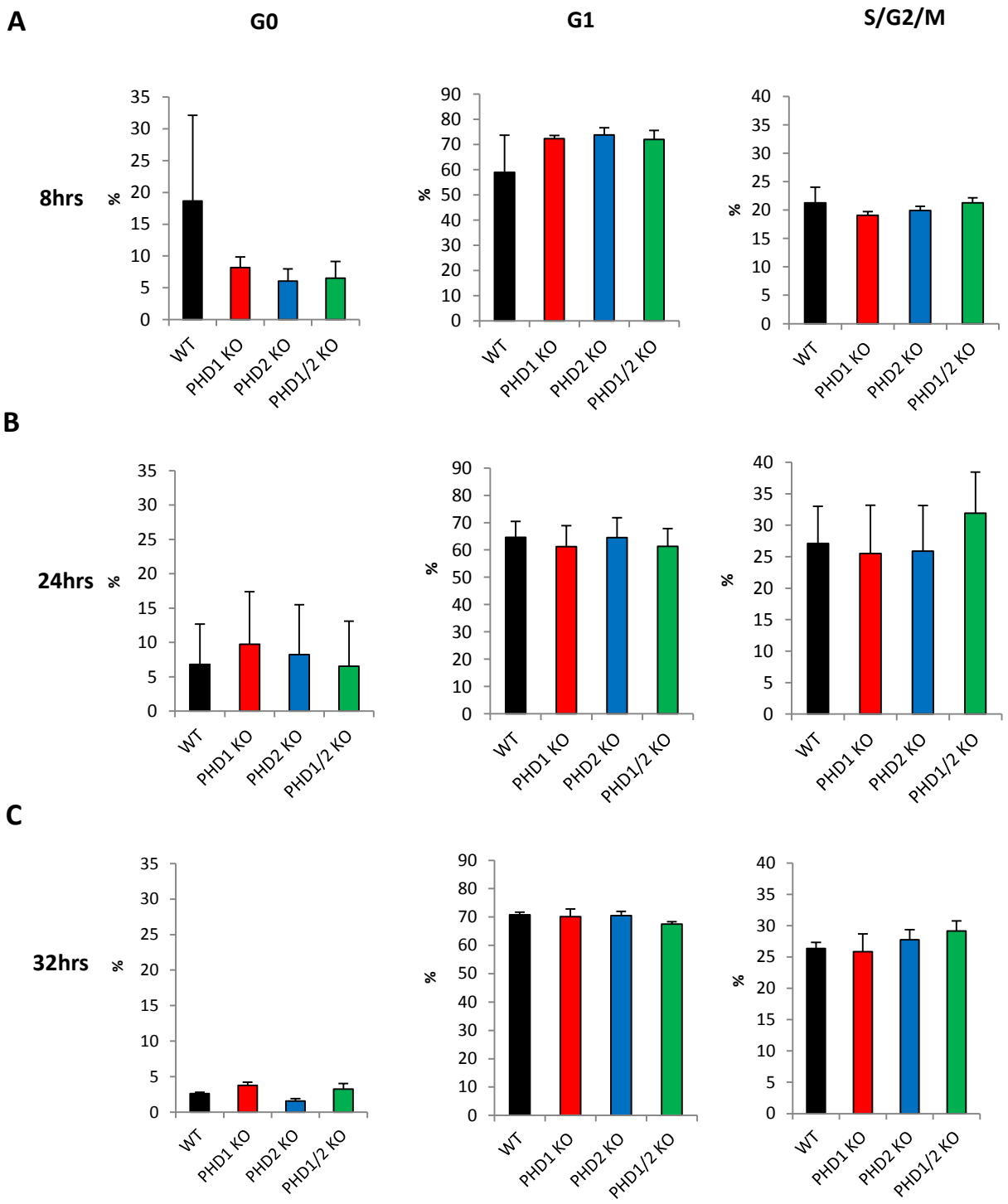


Figure 3.2.4. Cell cycle frequencies of pre-LSC cell lines.

Pre-LSC cell lines were cultured for 8, 24 and 32 hours. 4 x 10^4 cells were fixed and permeabilised in 96-well plates, then stained with an antibody specific for Ki-67, then washed, resuspended in DAPI and analysed by flow cytometry to determine the frequencies in different phases of the cell cycle. Data presented show mean frequencies of cells in G0, G1 and S/G2/M phases at (A) 8, (B) 24 and (C) 32 hours.

Next, the growth rate of pre-LSCs was investigated. Cells were plated and counted after 24 and 48 hours. Dead cells were excluded using trypan blue staining and only live cells were counted. At 24 hours, WT cells had a higher growth rate than all cells lacking PHD isoforms (Fig. 3.2.5). Statistical analysis showed that PHD1 KO cells and PHD2 KO cells both had a significantly lower growth rate than WT cells. PHD1/2 KO cells also appeared to have a lower growth rate compared to WT cells, although no statistical analysis was performed. However, data collected at 48 hours showed that growth rates were highly comparable between WT, PHD1 KO and PHD2 KO. PHD1/2 KO continued to show a reduced growth rate at 48 hours, although the statistical significance is not known and biological meaning of this trend is unclear. Given that no differences were observed in proliferation, it was possible that the differences in cell growth were attributed to altered cell viability which was then examined.

Dead cells that cannot exclude trypan blue were counted and used to calculate the overall viability of the cell population. At 24 hours, WT cells and PHD1 KO cells had comparable percentages of viable cells in the culture, with no significant differences seen between genotypes (Fig. 3.2.6). However, at 48 hours cell viability appeared higher in cells lacking PHD1, compared to that of WT cells (81% compared to 71%) and this difference was statistically significant. Considering that PHD1 KO cells did not show a difference in cell cycle distribution at 24 hours, yet, appeared to have a lower growth rate than WT cells which was statistically significant. It had been hypothesised that the lower growth rate seen in PHD1 KO cells may have been attributed to lower cell viability. Therefore, the observation that PHD1 KO cells had a higher viability than WT cells was unexpected. However, the balance between proliferation and cell death and their effect on overall cell growth is complicated and warrants further investigation.

In contrast, PHD2 KO and PHD1/2 KO cells both had a lower viability (76.1% and 72.5%) compared to WT cells (87.1%) at 24 hours. Statistical analysis preformed between WT and PHD2 KO confirmed that there was a statistically significant difference in cell viability (Fig. 3.2.6). At 48 hours however, this difference was not seen and WT, PHD2 KO and PHD1/2 KO cells had a similar viable cells.

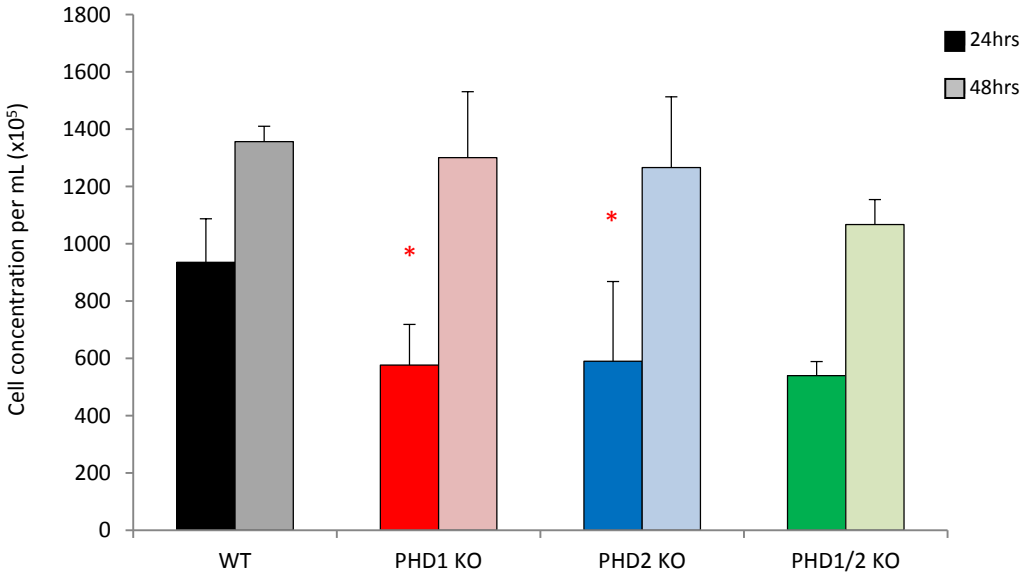


Figure 3.2.5. Cell growth of pre-LSC cell lines.

Pre-LSC cell lines were cultured for 24 and 48 hours at a starting density of 2.5 x 10^5/mL. Cells were harvested and counted by trypan blue dye exclusion method. Data represent the mean cell concentration of biological replicates. (WT n=2, PHD1 KO n=2, PHD2 KO n=4, PHD1/2 KO n=1) Student t-test was performed to determine if the growth rate of PHD KO Pre-LSCs was significantly different to growth rates of WT cells at 24 and 48 hours. *$p<0.05$.

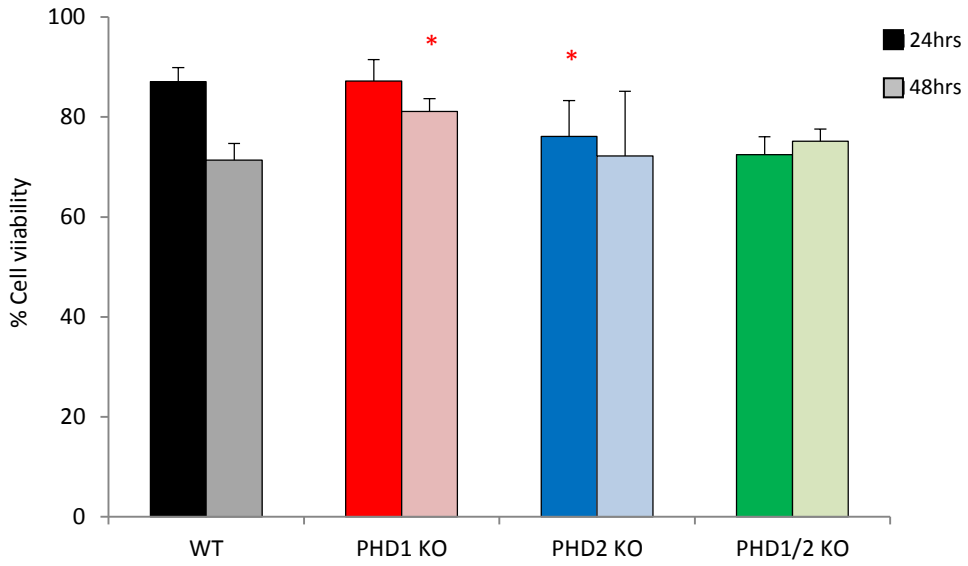


Figure 3.2.6. Cell viability of pre-LSC cell lines.

Pre-LSC cell lines were cultured for 24 and 48 hours at a density of $2.5x10^5$/mL. Cells were harvested and counted by trypan blue dye exclusion to assess the viability of each sample. Data represent the mean percentage of viable cells of biological replicates. (WT n=2, PHD1 KO n=2, PHD2 KO n=4, PHD1/2 KO n=1) *$p<0.05$.

3.2.5 Apoptotic frequencies of pre-LSC cell lines

Changes in cell viability are often attributed to changes in apoptosis. Much like the enhanced proliferative state, an altered apoptotic response is a common property of cancer cells. Cancer cells are often seen to be resistant to apoptosis, forming aggressive cells that are resistant to treatments.

In order to further investigate the differences observed in pre-LSC cell viability, the apoptotic frequency of these cells was assessed. Cells that are Annexin V^+DAPI^- are considered to be in the early stages of apoptosis as they show Annexin V binding to cell surface phosphatidylserine, but have retained membrane potential. Cells that are Annexin V^+DAPI^+ are considered to be in the late phase of apoptosis as they show Annexin V binding and reduced membrane potential.

Flow cytometry analysis preformed 24 hours after passage showed that WT and PHD1 KO cells had comparable frequencies of cells in the early and late phases of apoptosis, as well as overall apoptosis (Fig. 3.2.7). However, PHD2 KO cells showed an increase in early and late phase apoptosis as well as total apoptosis at 24 hours (Fig. 3.2.7). Statistical analysis determined that the difference in early phase apoptosis between PHD2 KO and WT was not significant. However, differences in late phase apoptosis and overall apoptosis were highly significant ($p=0.0002$ & $p=0.0009$). Cells lacking PHD2 also had a significant decrease in viability compared to WT at 24 hours (67.6% compared to 82.5%), which was concordant with the increased apoptosis. This is also corroborative with the reduced viability of PHD2 KO cells as determined by trypan blue exclusion (Fig. 3.2.7).

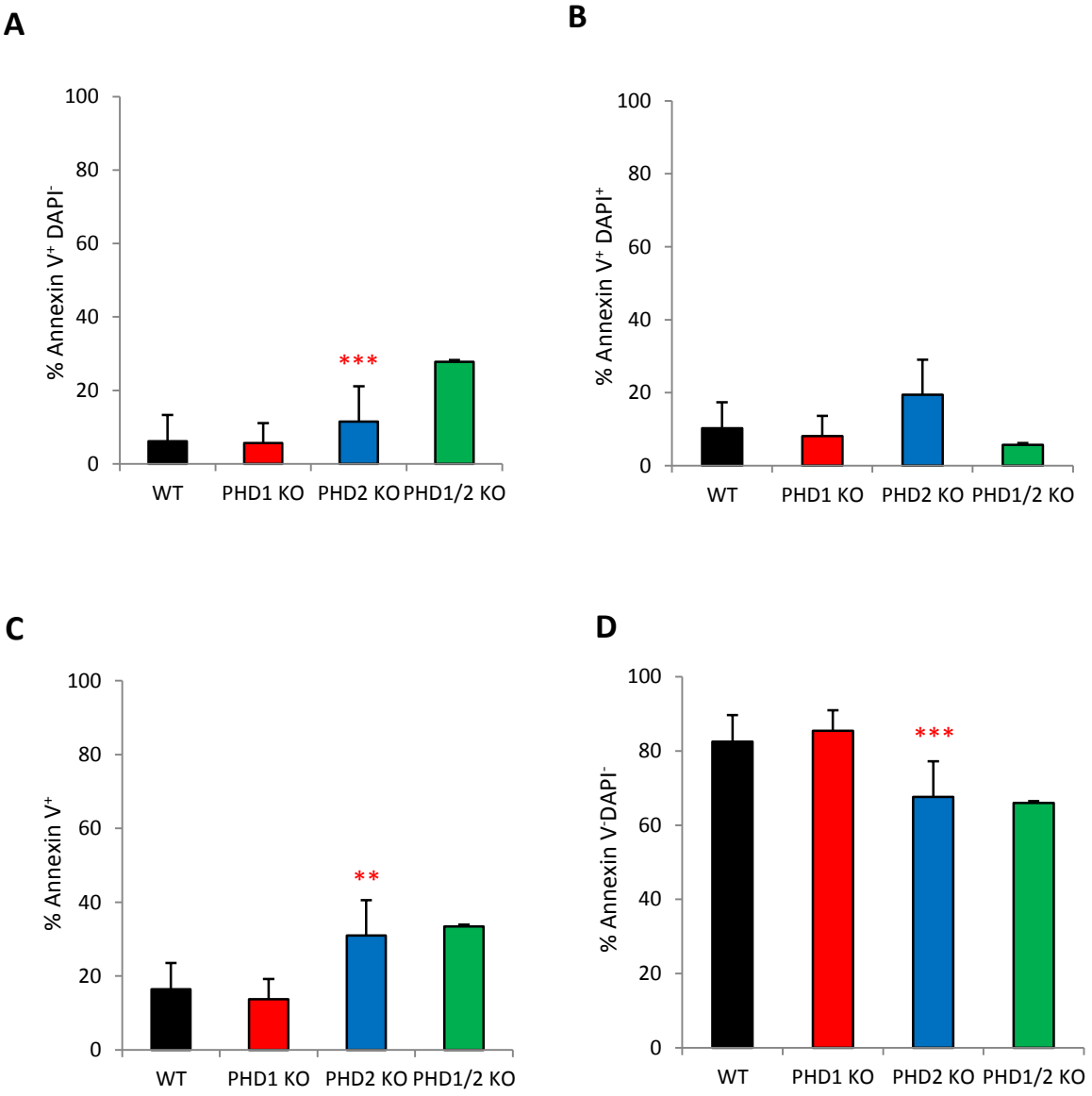


Figure 3.2.7. Apoptotic frequencies of pre-LSC cell lines at 24 hours.

Annexin V binding and DAPI staining was used to determine the frequencies of viable and apoptotic Pre-LSC cells. Annexin V^+DAPI^- cells were considered to be in early apoptosis (A), Annexin V^+DAPI^+ cells were considered to be in late apoptosis (B). Total Annexin V^+ cells (both $DAPI^+$ and $DAPI^-$) were used to measure total apoptosis (C). Cells that were Annexin V^-DAPI^- were considered viable cells (D). Data shown represent the mean frequencies of apoptotic and viable cells. (WT n=6, PHD1 KO n=4, PHD2 KO n=5, PHD1/2 KO n=1.) Student t-test was performed to determine if PHD1 or PHD2 KO genotypes have significantly different frequencies compared to WT Pre-LSCs. *p<0.05, **p<0.005, ***p<0.0005.

However, at 48 hours, PHD2 KO cells showed no differences in apoptosis compared to WT (Fig. 3.2.8), which was in accordance with the previous experiments showing no differences in growth or viability. It was interesting that differences in viability were evident at early but not later time points and it may be that that this difference was related to the density of the population. Interestingly, PHD1 KO cells analysed at 48 hours showed a lower frequency of apoptosis and a higher frequency of viable cells (Fig. 3.2.8). This is in line with previous experiments that also demonstrated a higher viability in PHD1 KO cells at this time point. However, it should be noted that data collected at 48 hours for both trypan blue exclusion and Annexin binding assays consisted of only two biological replicates for both WT and PHD1 KO (n=2) and further replicates would be required in order to perform statistical analysis and verify these results.

In terms of total apoptosis and viability, PHD1/2 KO cells showed frequencies that were comparable to PHD2 KO at both 24 and 48 hours (Fig. 3.2.7 & 3.2.8), yet the frequencies of cells within early and late phase apoptosis did appear to differ. However, this experiment would need to be repeated in order to make any firm conclusions. The similarities in overall apoptosis and viability between PHD1/2 KO and PHD2 KO might suggest that the apoptotic phenotype was due to the absence of PHD2 and was not abrogated further by the loss of PHD1.

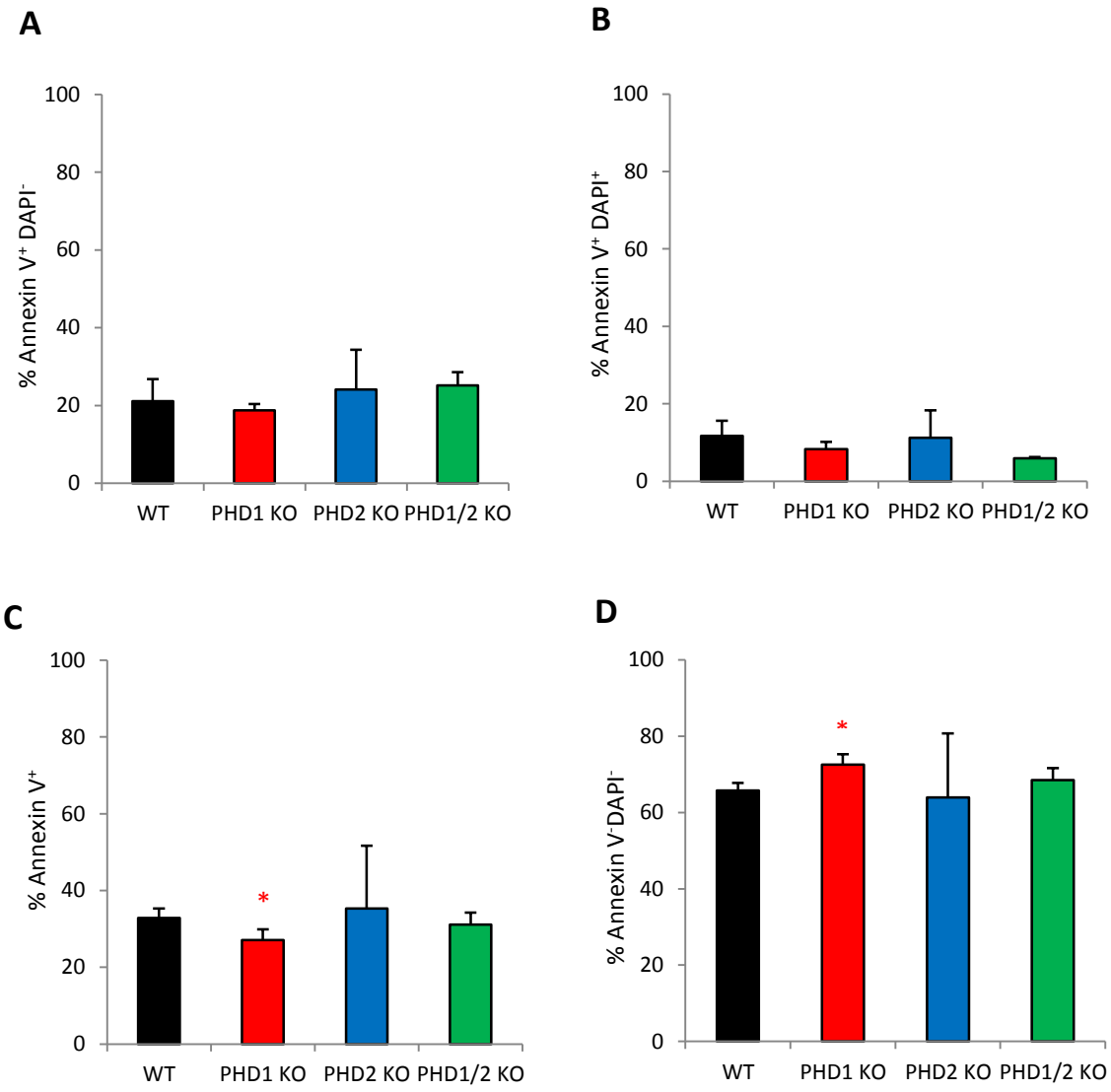


Figure 3.2.8. Apoptotic frequencies of pre-LSC cell lines at 48 hours.

Annexin V binding and DAPI staining was used to determine the frequencies of viable and apoptotic Pre-LSC cells. Annexin V^+DAPI^- cells were considered to be in early apoptosis (A), Annexin V^+DAPI^+ cells were considered to be in late apoptosis (B). Total Annexin V^+ cells (both $DAPI^+$ and $DAPI^-$) were used to measure total apoptosis (C). Cells that were Annexin V^-DAPI^- were considered viable cells (D). Data shown represent the mean frequencies of apoptotic and viable cells. (WT n=2, PHD1 KO n=2, PHD2 KO n=3, PHD1/2 KO n=1.) *$p<0.05$.

3.2.6 Mitochondrial dysfunction and apoptosis in pre-LSC cell lines

Apoptosis is often a response to mitochondrial dysfunction. PHD isoforms are closely linked to the energy production cycles within the cell. It is possible that the absence of PHD may cause the cellular metabolic processes to become dysregulated. Products of the energy production cycle, such as NO and ROS, are known to cause mitochondrial damage (Pieczenik & Neustadt, 2006), and are both known to inhibit PHD, suggesting a clear link between PHD activity and mitochondria dysfunction. Therefore, the frequency of functional and dysfunctional mitochondria within the cells was detected by flow cytometry. WT pre-LSCs had comparable frequencies of both functional and dysfunctional mitochondria compared to PHD KO pre-LSCs (Fig. 3.2.9). These results implied that the increase in apoptotic frequencies seen in PHD2 KO pre-LSCs was not attributed to mitochondrial dysfunction. Unfortunately, frequencies of functional/dysfunctional mitochondrial of PHD1/2 KO cells have not been analysed and further study would be required to detect changes in this cell line.

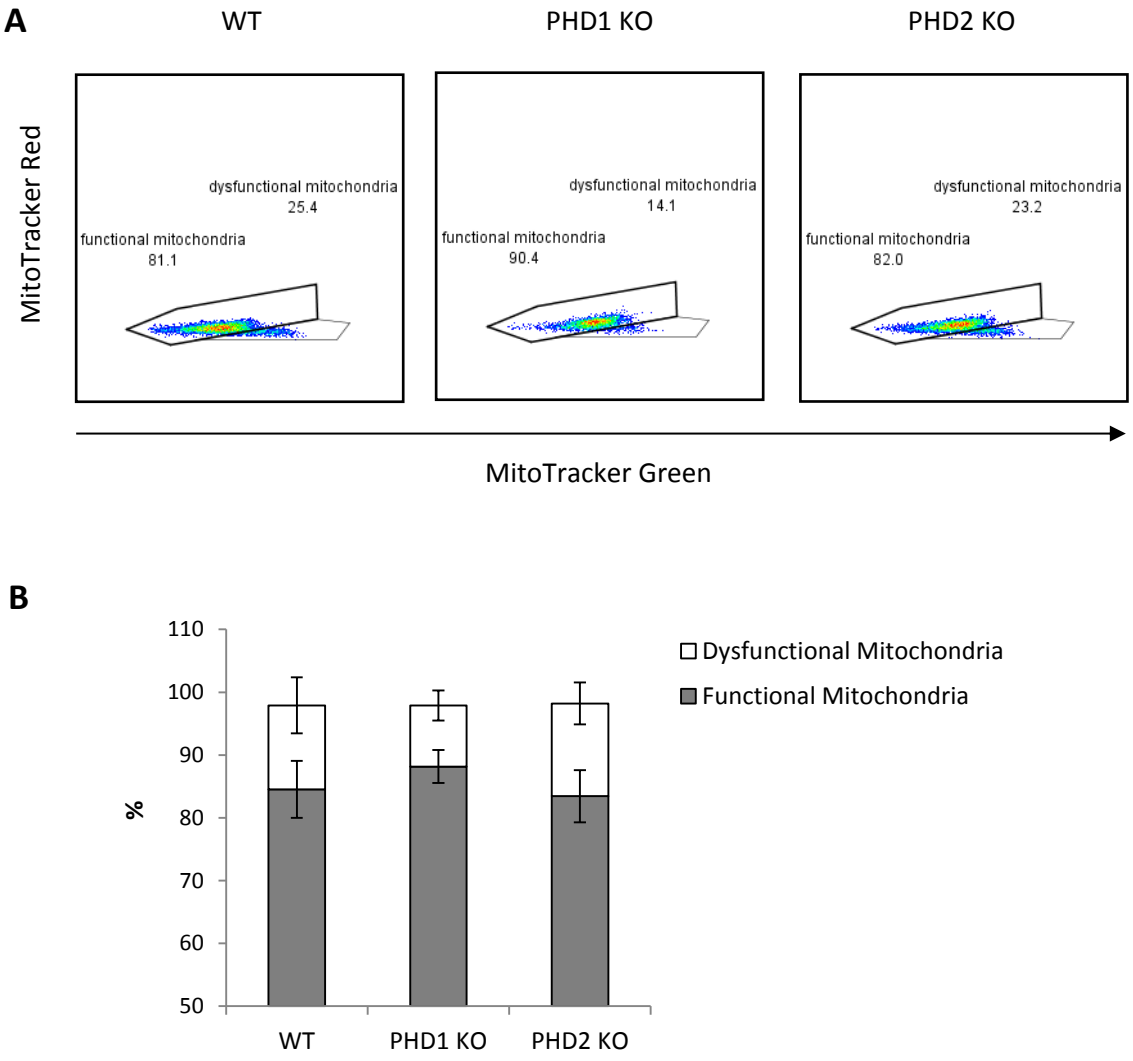


Figure 3.2.9. Mitochondrial function in pre-LSC cell lines.

The frequency of functional and dysfunctional mitochondria was measured in Pre-LSC cell lines. Cells were stained with MitoTracker Green and MitoTracker Red mitochondria dyes which detect total or respiring mitochondria, respectively. Frequencies of functional and dysfunctional mitochondria were analysed by flow cytometry. A. Representative flow cytometry dot plots depicting the gating strategy for functional and dysfunctional mitochondria. Some variations in frequencies were seen between individual samples but no trend was seen between genotypes and no statistical difference was seen. B. Data presented show total mean frequencies of functional and dysfunction mitochondria. (WT n=4, PHD1 KO n=4, PHD2 KO n=3.)

3.2.7 Influence of the bone marrow niche on apoptosis of pre-LSC cell lines

In vitro characterisation of cells lacking PHD isoforms had indicated that the absence of PHD2 may affect apoptosis. However, it was not clear if this was a property of transformed pre-LSCs or if this was a phenotype seen in normal haemopoietic cells within the bone marrow. In order to investigate the apoptotic frequencies of haemopoietic cells *ex vivo*, bone marrow cells were extracted from WT mice and mice lacking PHD isoforms. Cells were enriched for c-Kit expression to ensure that the population isolated was similar to the starting population of transformed cells. WT, PHD1 KO and PHD2 KO cells showed similar percentages of cells undergoing early and late apoptosis (Fig. 3.2.10). Similarly, the overall frequency of apoptotic cells was very low and comparable across all genotypes. The percentage of Annexin V^- (viable) cells in the population was high for all genotypes and no differences were observed.

Mitochondrial function of pre-LSCs *ex vivo* was also analysed by flow cytometry. Frequencies of functional mitochondria were similar across all genotypes (Fig. 3.2.11). Similarly, no differences were seen in the frequency of dysfunctional mitochondria. However, as with the *in vitro* results, data collected from this assay proved variable and would need further verification.

As no differences were seen in apoptotic frequencies in c-Kit^+ cells *ex vivo*, whether WT or PHD KO, this might suggest that the apoptotic phenotype seen *in vitro* in pre-LSCs lacking PHD isoforms was specific to Meis1/Hoxa9 transformed cells. However, it is also possible that this was an artefact of *in vitro* culture and further investigation into this phenotype would be required.

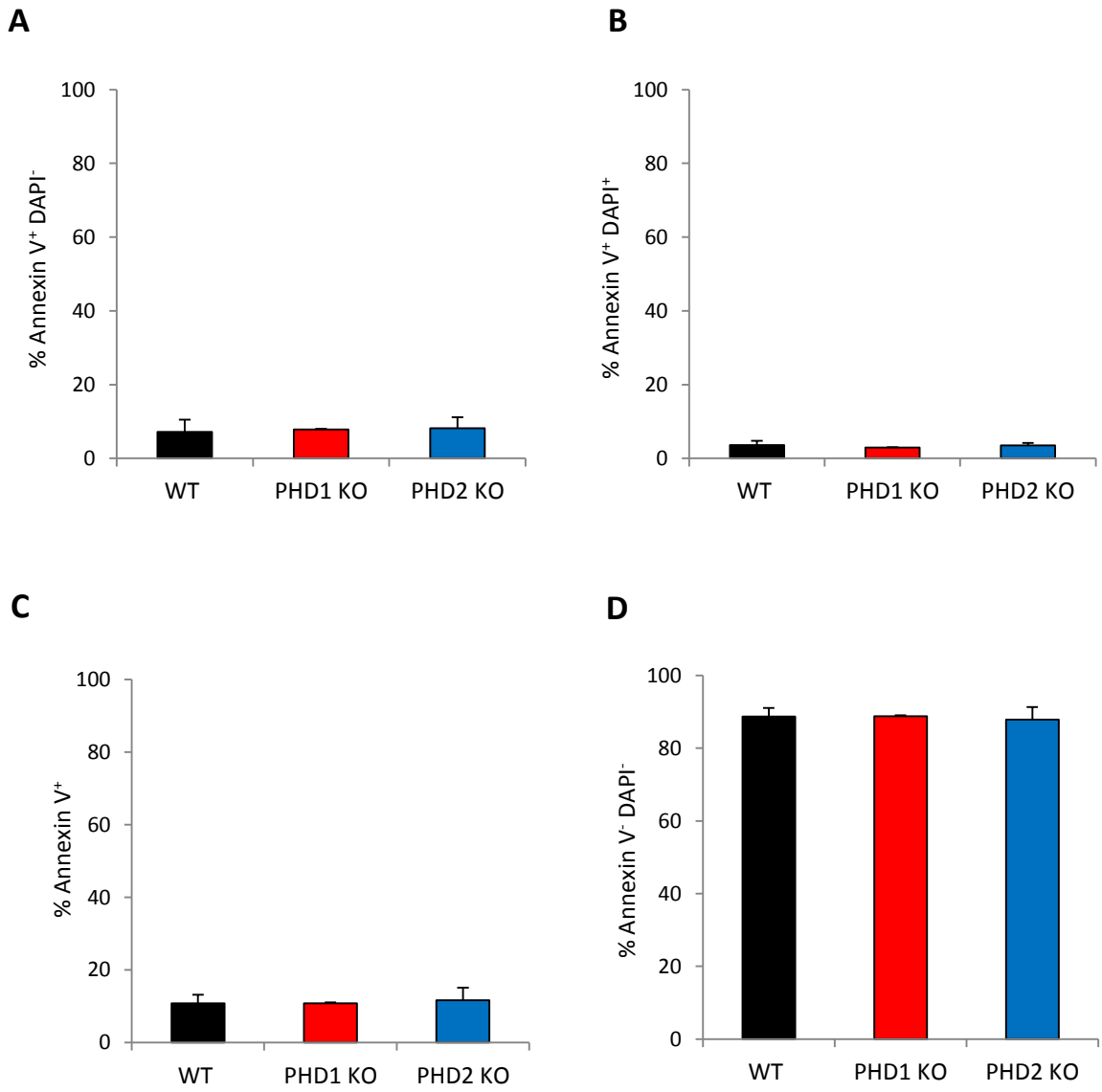


Figure 3.2.10. Frequency of apoptotic HSPCs *in vivo*.

Bone marrow cells were extracted from WT and PHD KO mice. HSPCs were enriched following selection of c-Kit$^+$ cells by column separation method. HSPCs were incubated with Annexin V and DAPI, which was used to determine the frequencies of viable and apoptotic cells within the HSPC population as determined by flow cytometry. Annexin V$^+$DAPI$^-$ cells were considered to be in early apoptosis (A), Annexin V$^+$DAPI$^+$ cells were considered to be in late apoptosis (B). Total Annexin V$^+$ cells (both DAPI$^+$ and DAPI$^-$) were used to measure total apoptosis (C). Cells that were Annexin V$^-$ DAPI$^-$ were considered viable cells (D). Data shown represent the mean frequencies of apoptotic and viable cells. (WT n=3, PHD1 KO n=1, PHD2 KO n=4).

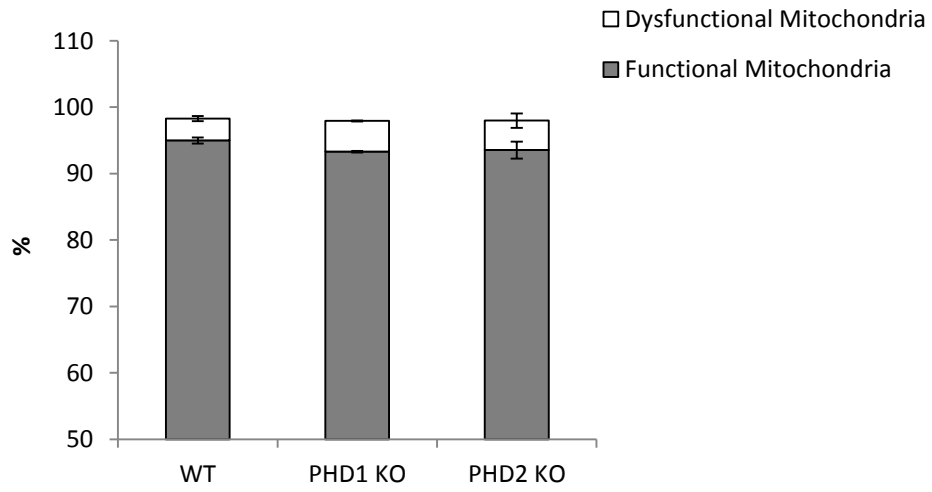


Figure 3.2.11. Mitochondrial function of HSPCs *in vivo*.

Bone marrow cells were extracted from WT and PHD KO mice. HSPCs were enriched following selection of c-Kit$^+$ cells by column separation method. Cells were stained with MitoTracker Green and MitoTracker Red mitochondria dyes which detect total or respiring mitochondria, respectively. Frequencies of functional and dysfunctional mitochondria was analysed by flow cytometry. Data shown represent the mean frequencies of functional and dysfunctional mitochondria. (WT n=3, PHD1 KO n=1, PHD2 KO n=4).

3.3 Investigation into the Leukaemic Potential of Pre-LSCs Lacking PHD Isoforms

3.3.1 Transplantation of pre-LSCs to recipient mice

The leukaemogenic potential of pre-LSCs generated in the CFC assay were assessed, in a single preliminary experiment, using a well-established bone marrow transplantation model (Somervaille & Cleary; Weischenfeldt et al, 2008; So et al, 2010; Gibbs et al, 2012). Due to cost restraints and the desire to repeat this experiment, using larger cohorts of mice and with minor technical modifications, the experiment was terminated early, at 18 weeks, and full analyses on each and every mouse was not performed. Regardless the results were interesting and are therefore presented here. Pre-LSCs were originally derived in BM cells extracted from mice with a $CD45.2^{+}$ background, which were then transformed with Meis1 and Hoxa9 retroviruses to produce pre-LSCs. Pre-LSCs were enriched for c-Kit expression to ensure a population of LIC, in accordance with the literature. WT, PHD1 KO, PHD2 KO or PHD1/2 KO $c\text{-}Kit^{+}$ $CD45.2^{+}$ pre-LSCs were transplanted by tail vein injection to $CD45.1/2^{het}$ recipient mice which were lethally irradiated prior to transplantation. A full schematic diagram of the experimental procedure is depicted in figure 3.3.1.

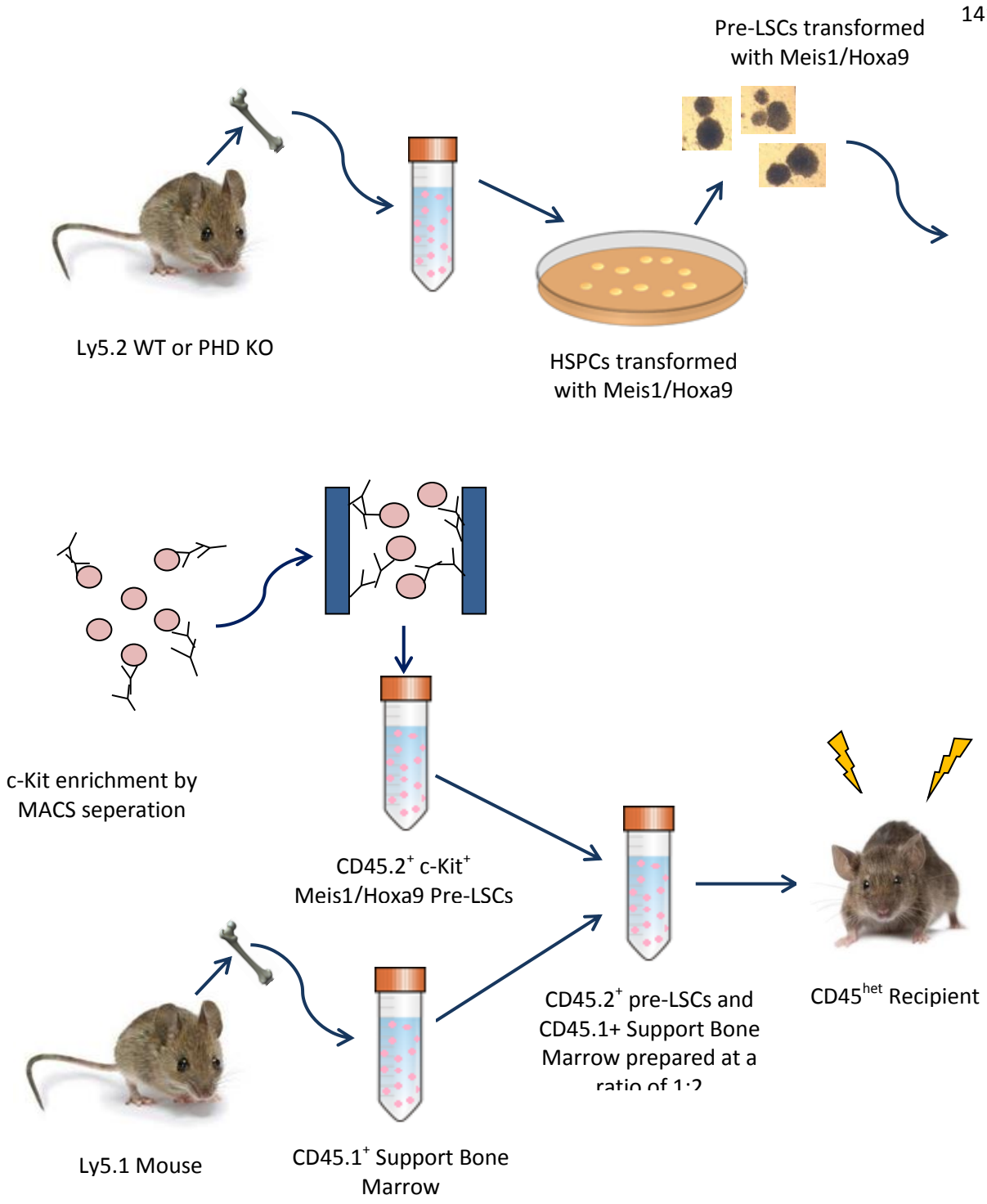


Figure 3.3.1. *In vivo* Experimental Technique.

A CFC assay as described in section 2.3.5.3, was used to produce a population of Meis1/Hoxa9 transformed pre-LSCs. Cells were harvested from B6-Ly5.2 mice with a CD45.2 background. Cells were then c-Kit enriched following incubation with anti-c-Kit magnetic beads and MACS separation technique as described in section 2.3.5.2. The genotype of each sample was confirmed by PCR prior to transplantation. Support bone marrow was harvested from B6-Ly5.1 mice with a CD45.1 background. Pre-LSCs and support bone marrow was mixed at a ratio of 1:2. Ly5.1/Ly5.2 F1 hybrid mice or $CD45^{het}$ mice were used as recipient mice. Recipient mice were subjected to 10Gy lethal radiation to deplete recipient bone marrow. Pre-LCS/support bone marrow cell suspension was then transplanted to recipient mice by tail vein injection.

The genotype of donor pre-LSCs prepared for transplantation was confirmed by PCR using genomic DNA extracted from cell samples. Each sample underwent a PCR reaction along with appropriate primers for PHD1 and PHD2 genes. The resulting DNA fragments were analysed by gel electrophoreses to determine if PHD genes were WT (normal PHD genes) or if genes had been genetically modified, based on the size of the DNA fragments produced. Two different systems were used to generate mice deficient in PHD genes. PHD1 KO cells ($PHD1^{-/-}$) lack both alleles of the functional PHD1 gene. However, following a PCR reaction with PHD1 primers, a smaller DNA fragment can be detected on the gel which represents the mutated PHD1 gene. In this system, one exon of PHD1 has been deleted, resulting in a non-functional shorter gene that can still be detected using primers for PHD1. PHD2 KO cells ($PHD2^{fl/fl}$), lack PHD2 following a conditional deletion using the Cre recombinase system. In this system, PHD2 has been flanked by loxP sites, which are recognised as excision sites by Cre recombinase, allowing the gene to be deleted under specific conditions. The pLox site within PHD2 results in a gene that is longer than WT PHD2 and which can still be detected by PHD2 primers. The band that is excised using this system can also be detected and is represented by a smaller band. This is also the case for PHD1 flox, where PHD1 can be deleted using the Cre system. Due to lethality of complete PHD2 deletion, PHD2 is only deleted in haematopoietic cells. This is achieved by fusing Cre (which is required for the gene excision) to Vav, a gene that is only expressed in the haematopoietic system. Specific VavCre primers are able to detect the presence of VavCre, resulting in a detectable band on a gel. Samples that do not contain VavCre will not produce a band. PHD1 KO, PHD2 KO and PHD1/2 KO cells were originally derived from mice with a $PHD1^{+/-}PHD2^{+/fl}Vav^{+/-}$ background so were genotyped using primers for PHD1, PHD2 flox and Vav. WT cells were derived from mice with a $PHD1^{+/fl}PHD2^{+/fl}Vav^{-}$ background so were genotyped using primers for PHD1 flox, PHD2 flox and Vav (Fig. 3.3.2).

The phenotype of pre-LSCs was analysed by flow cytometry to ensure that the c-Kit enrichment had been successful, and that populations consisted of a high percentage of LSCs, and that the immunophenotypic profile was comparable across all genotypes (Fig. 3.3.3). Within the pre-LSC culture, it was found that an extremely high percentage

(between 90-99%) of c-Kit$^+$ cells were positive for both Mac-1 and Gr-1, therefore enrichment beyond c-Kit expression for LIC phenotype was considered unnecessary. Furthermore, the uncertainty within the literature concerning the requirement for Gr-1 expression then became redundant, considering that cells which were positive for c-Kit and Mac-1 were also positive for Gr-1 expression.

Bone marrow cells were also extracted from CD45.1$^+$ mice and used as support bone marrow, which was mixed with CD45.2$^+$ pre-LSCs before transplantation. Bone marrow transplantation requires a high number of transplantable cells. The number of pre-LSCs generated *in vitro* was limited and support bone marrow was used to ensure efficient reconstitution of the recipient mice following irradiation. The ratio of CD45.1$^+$ support bone marrow to CD45.2$^+$ pre-LSCs was analysed by flow cytometry for each genotype prior to transplantation to ensure equal populations (Fig. 3.3.3).

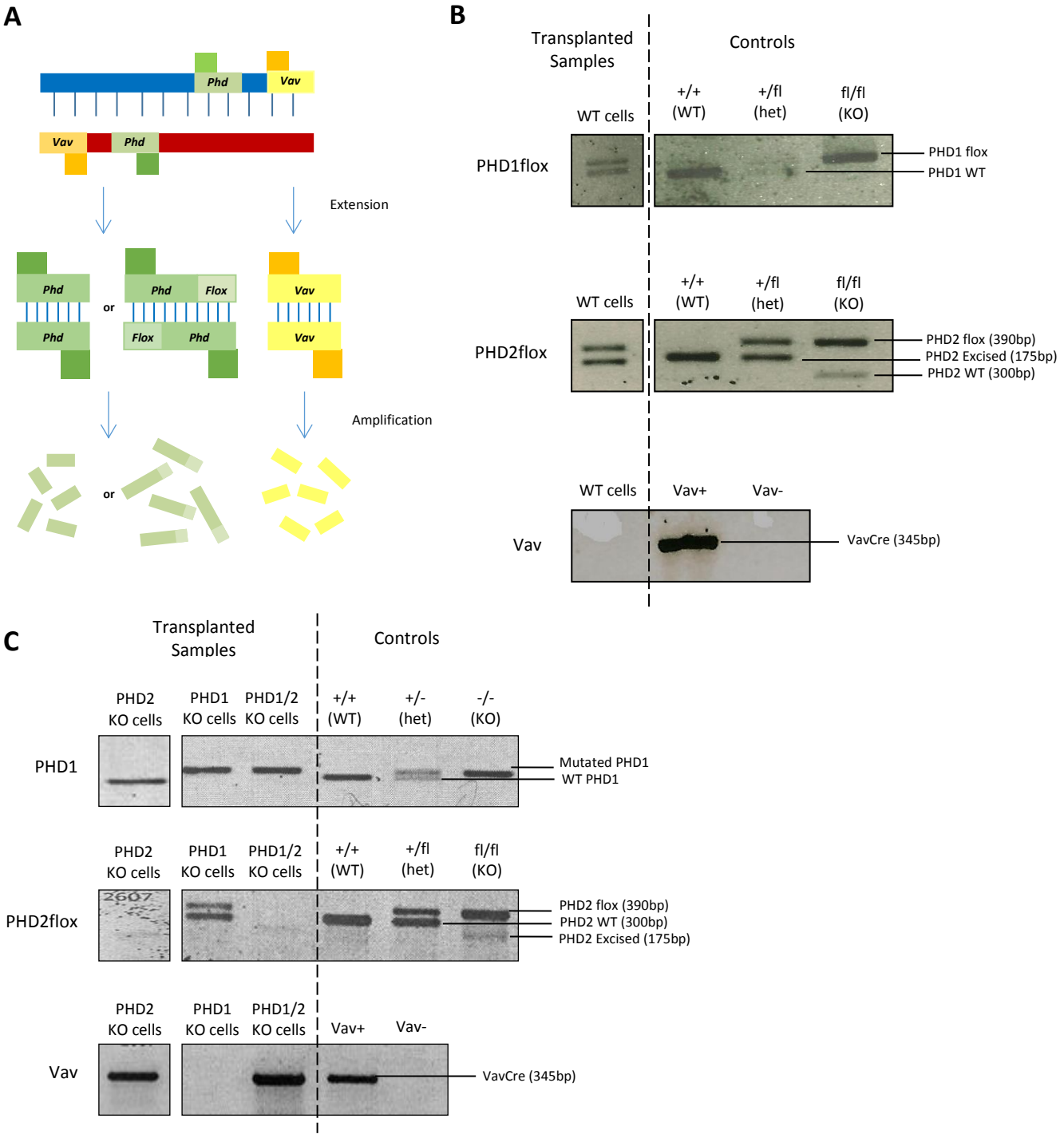


Figure 3.3.2. Confirmation of genotype of transplanted pre-LSCs by PCR using genomic DNA.

A. Schematic diagram of PCR strategy used to confirm the genetic background of the mice used in experiments. DNA was extracted from cells and specific primers were used to amplify PHD and Vav genes in accordance with the initial genetic background of the mice, from which cells were derived. PCR products were separated by gel electrophoresis to confirm the presence of WT or mutated genes in each sample before transplantation. B. WT cells were extracted from mice with a $PHD1^{+/fl}PHD2^{+/fl}Vav^{+/-}$ background and genotype was assessed based on PCR results following reactions containing primers for PHD1flox, PHD2flox and VavCre. C. PHD1 KO, PHD2 KO and PHD1/2 KO cells were extracted from mice with a $PHD1^{+/-}PHD2^{+/fl}Vav^{+/-}$ background and genotype was assessed based on results of PCR reactions containing primers for PHD1, PHD2flox and Vav

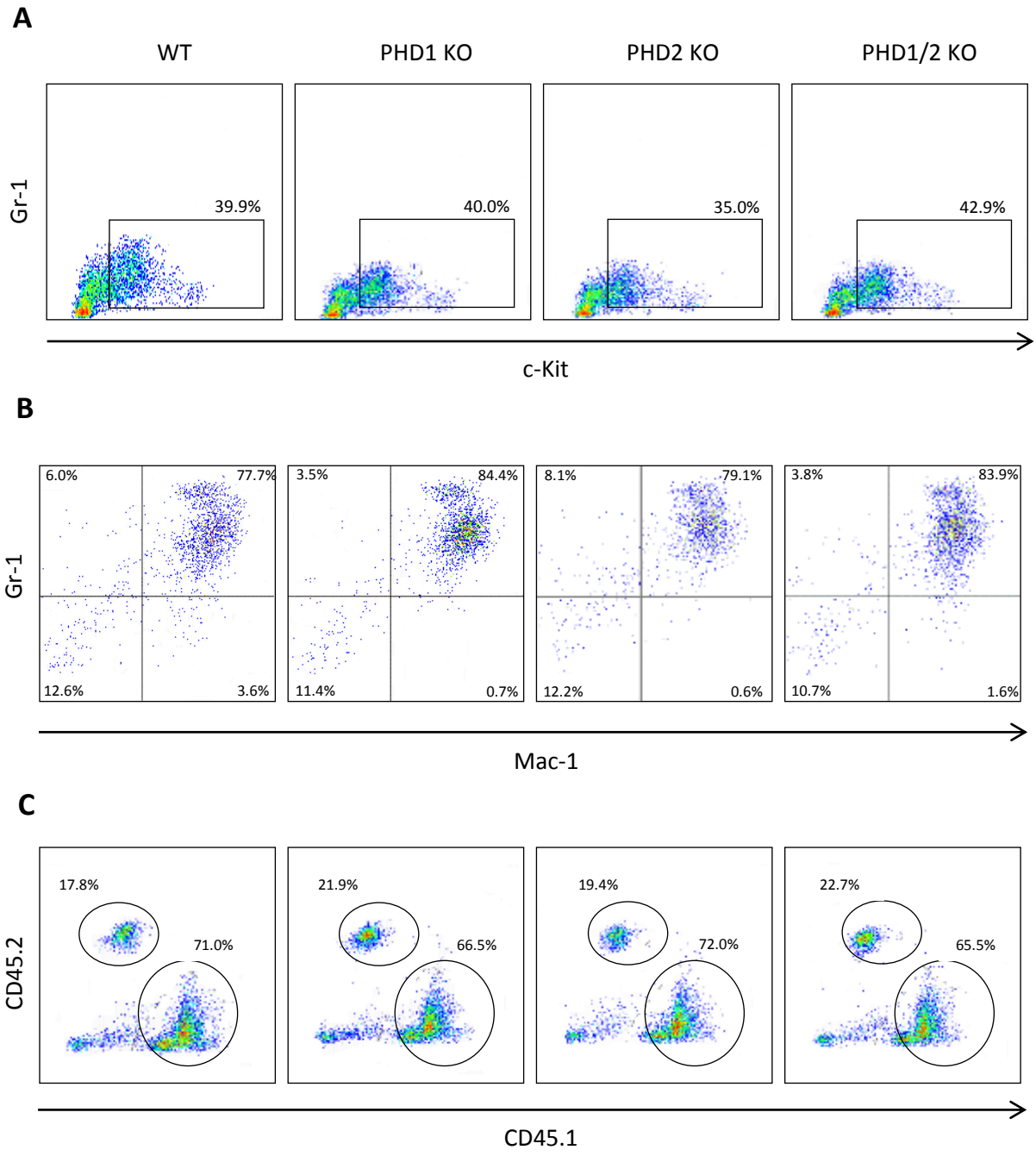


Figure 3.3.3. Immunophenotype of transplanted pre-LSCs.

Pre-LSCs (CD45.2^{+}) and support bone marrow (CD45.1^{+}) cells were prepared for transplantation under aseptic conditions. Pre-LSCs were enriched for c-Kit expression by column separation method. The expression of LSC markers was confirmed by flow cytometry prior to transplantation to ensure that LSC starting populations were similar between groups. A. Cells were analysed for c-Kit expression and c-Kit^{+} cells gated. B. c-Kit^{+} cells were further analysed for the expression of Mac-1 and Gr-1. C. The ratio between CD45.1^{+} support bone marrow cells and CD45.2^{+} pre-LSCs was also confirmed by FACS prior to transplantation.

Following tail vein injection of CD45.1^{+} support bone marrow along with CD.45.2^{+} pre-LSCs, mice were monitored for signs of leukaemic development. The detection of CD45.2^{+} cells within the bone marrow is achievable through bone marrow aspiration. However, this procedure is technically challenging and particularly stressful for leukaemic mice which may be weak and immunodeficient. As an alternative, peripheral blood from recipient mice was analysed fortnightly and the presence of CD45.2^{+} leukaemic cells within the blood was considered a sign of leukaemic disease. Leukaemia develops initially within the bone marrow with cells eventually mobilising to the peripheral blood. As this is a later stage of re-establishment of the haemopoietic system, leukaemic cells were not detected immediately following transplantation. As mice are immunocompromised following radiation, blood samples were not taken until 4 weeks post transplantation, at which point, mice had recovered from radiation treatment and the haemopoietic system had been reconstituted. From the time of transplantation up to 4 weeks post transplantation, mice were monitored closely for signs of illness due to failed reconstitution, in which case, mice were sacrificed humanly in accordance with Home Office regulations.

3.3.2 Detection of leukaemic disease and leukaemic frequency

Flow cytometry analysis of blood samples showed the frequency of CD45.2^{+} pre-LSCs, CD45.1^{+} support bone marrow derived cells and CD45.1/2^{het} cells derived from residual recipient bone marrow (Fig. 3.3.4). This was continuously analysed over the course of the experiment and used to monitor the mice for the development of leukaemic disease. Figure 3.3.3 depicts the time course over which the experiment took place and the development of leukaemia over time. The frequency of CD45.2^{+} cells within the peripheral blood was plotted for individual transplanted mice and showed that overall, 48% of mice developed detectable leukaemia within 18 weeks, which was within the time frame predicted in the literature (Kroon et al, 1998; Wang et al, 2006). This data also shows that the frequency of CD45.2^{+} in the blood increased over time as the disease developed.

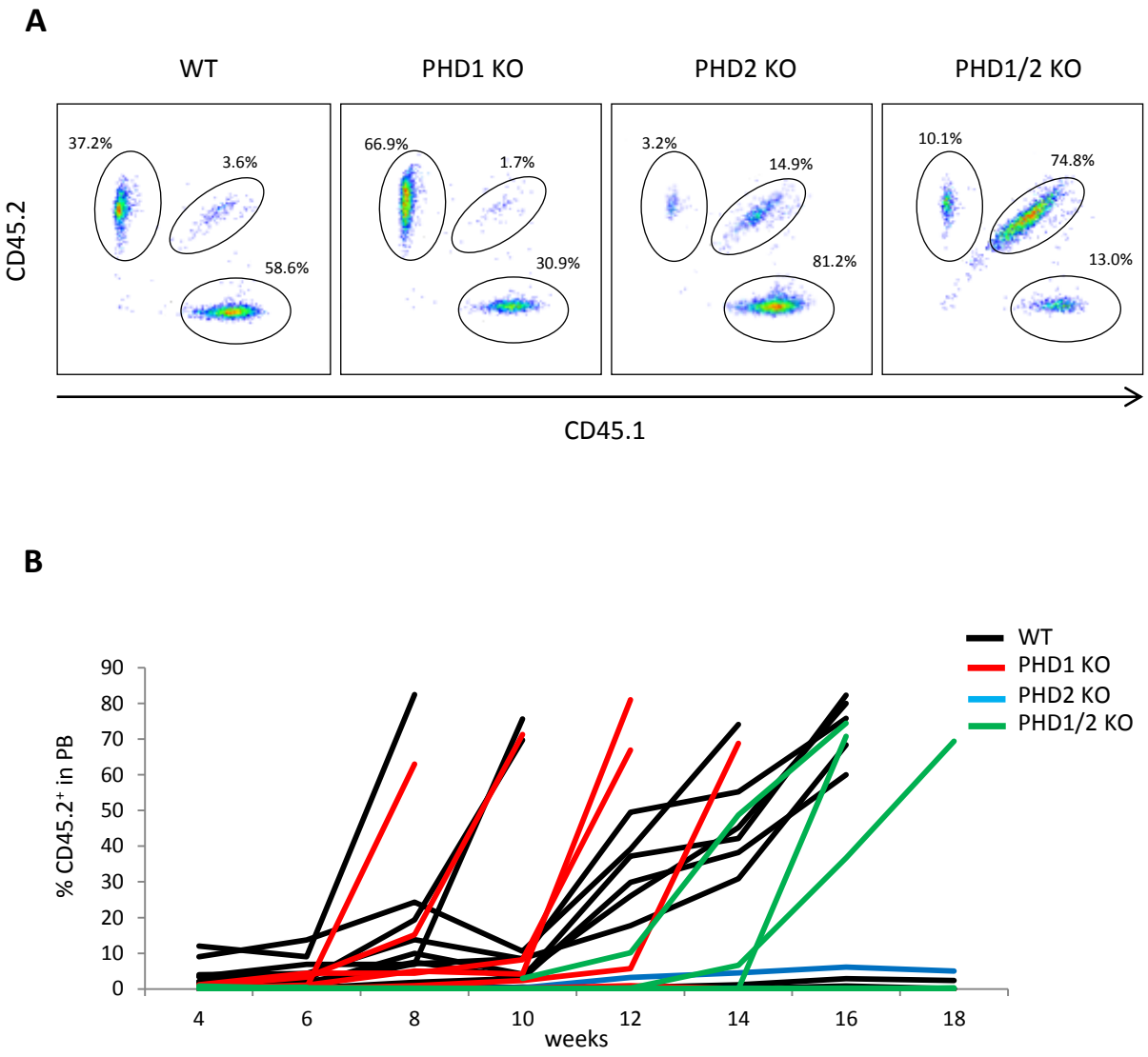


Figure 3.3.4. Detection of CD45.2^{+} donor cells in peripheral blood.

Peripheral blood samples were acquired from recipient mice 4 weeks following transplantation and every 2 weeks thereafter until the experiment was terminated at week 18. Blood was taken via tail vein. Red blood cells were lysed and white blood cells were stained with antibodies specific for CD45.1 and CD45.2. A. Images show representative flow cytometry dot plots showing CD45.1^{+}, CD45.2^{+} and CD.45^{het} cells in peripheral blood of recipient mice. B. Data presented shows the frequency of CD45.2^{+} cells in the peripheral blood as detected by flow cytometry every 2 weeks. Data show that CD45.2^{+} cells detected in recipient mice increase in frequency over the time course. Recipient mice that did not have any detectable CD45.2^{+} cells have not been included. (Recipient mice transplanted with pre-LSCs: WT n=12, PHD1 KO n=9, PHD2 KO n=11, PHD1/2 KO n=7).

The percentage of mice that were positive for CD45.2^{+} cells was calculated for each genotype. Of the 12 mice that received WT pre-LSCs, 10 developed leukaemia within 18 weeks, resulting in 83% penetrance (Fig. 3.3.5). Of the mice that received PHD1 KO pre-LSCs, 56% of mice developed leukaemia. Of mice injected with PHD2 KO pre-LSCs, just 9% of mice developed leukaemia. And of the mice that received PHD1/2 KO pre-LSCS, 43% developed leukaemia. Overall, the percentage of mice that developed leukaemia was significantly higher in mice transplanted with WT pre-LSCs compared to all other genotypes. This was particularly evident when mice were transplanted with PHD2 KO pre-LSCs which had an extremely low penetrance of leukaemia (9%).

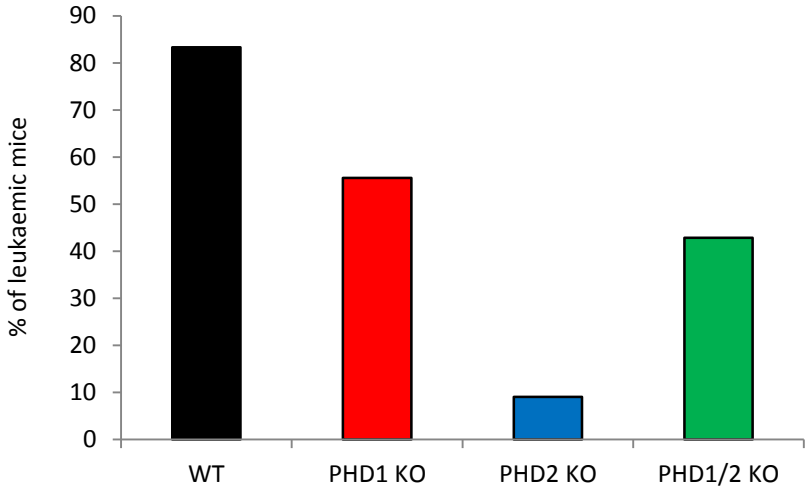


Figure 3.3.5. Leukaemic penetrance in transplanted mice.

Penetrance of leukaemic disease was based on the detection of CD45.2^{+} leukaemic cells in the peripheral blood of the recipient mice. Data presented represent the frequency of recipient mice transplanted with WT, PHD1 KO, PHD2 KO and PHD1/2 KO pre-LSCs that had detectable CD45.2^{+} cells within the peripheral blood up to week 18 when the experiment ended. Recipient mice with detectable CD45.2^{+} cells were considered to be leukaemic. (WT n=12, PHD1 KO n=9, PHD2 KO n=11, PHD1/2 KO n=7).

This was recapitulated when the survival rate of recipient mice was examined. The survival rate of mice transplanted with WT pre-LSCs was 25% at 18 weeks (Fig. 3.3.6). However, one mouse (which appeared to be healthy) was seen to have a low frequency of $CD45.2^+$ cells in the blood (2.34%); it is likely that this mouse would have succumbed to disease at a later time point. Unfortunately, it was not possible to continue the experiment past this point. The survival rate of mice injected with PHD1 KO pre-LSCs at 18 weeks was 44.4%. At the end point of the experiment, several of the mice in this group had a negligible percentage of $CD45.2^+$ cells (<0.1%) and it is therefore impossible to know whether these mice would ever have developed leukaemia. Mice transplanted with PHD1/2 KO pre-LSCs had a significantly higher survival rate than WT (57%). Interestingly, mice that were transplanted with PHD2 KO pre-LSCs had a 100% survival rate at 18 weeks. However, $CD45.2^+$ cells were observed in one mouse in this group at a low percentage (4.99%), so it is possible that this mouse would have developed disease. Regardless of the premature termination of this experiment, the survival rates and penetrance of mice transplanted with pre-LSCs differed between the genotypes. A log-rank test was used to compare the survival rates of the different groups. Mice transplanted with WT pre-LSCs were seen to have a lower survival rate compared to mice transplanted with PHD2 KO cells ($p=0.0004$). WT-transplanted mice also had a lower survival rate than PHD1/2 KO-transplanted mice which was also seen to be statistically significant ($p=0.0354$). WT-transplanted mice survival appeared lower than PHD1 KO-transplanted mice, although this did not prove to be significant. Statistical significance was also seen when comparing PHD1-transplanted and PHD2 KO-transplanted mice ($p=0.0047$). However, these results were based on a single experiment that was terminated early and whilst interesting, it would be imperative that this study be repeated in order to verify these results. In addition, only one biological replicate of each genotype was used for initial transplantation and it would be beneficial to verify these data with additional biological replicates.

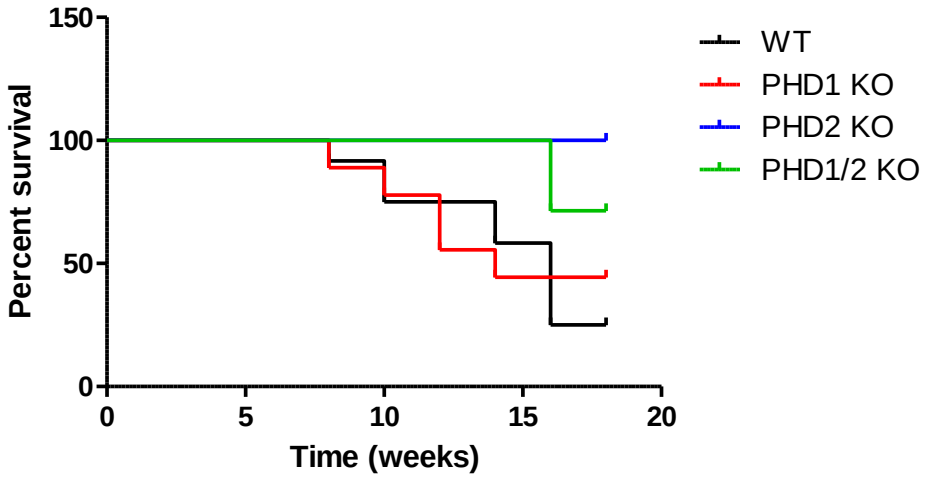


Figure 3.3.6. Survival rate of transplanted mice.

The frequency of recipient mice in each group that became moribund and were sacrificed after succumbing to disease was used to calculate the survival rate of mice in each group which was compared by Kaplan-Meier analysis. Statistical relevance was determined using a log-rank test (WT n=12, PHD1 KO n=11, PHD2 KO n=9, PHD1/2 KO n=7) *p<0.05, **p<0.005, ***p<0.0005.

3.3.3 Characterisation of leukaemic disease

Mice that were positive for CD45.2^{+} cells in the blood were monitored closely and were sacrificed according to Home Office regulations when they were considered moribund. Sacrificed mice were analysed to determine any differences in leukaemic disease between genotypes. However, as PHD2 KO transplanted mice had 100% survival, none of the mice transplanted with PHD2 KO pre-LSCs were available for analysis. Bone marrows and spleens were analysed from mice that were sacrificed because of leukaemia development and were all seen to contain CD45.2^{+} cells (Fig. 3.3.7). However, no differences were observed between groups, suggesting that at the time these mice exhibited signs of leukaemia and had to be culled the leukaemia in each mouse was at a similar stage of development. Leukaemic mice all had enlarged spleens that were characteristic of leukaemic disease (Fig. 3.3.8). Leukaemic spleens sizes ranged from 1.8-2.5cm in length and were larger than spleens taken from non-leukaemic mice which are within the range of 1.2-1.4cm. Next, the spleen weights were used to assess if there were any differences in symptoms. As all of the mice had slightly different body masses at the time of sacrifice, spleen weights were expressed as a percentage of total body mass, which was then used to compare the enlargement of spleens in WT, PHD1 KO and PHD1/2 KO-transplanted mice. The percentage of body mass of the enlarged spleens was comparable across all genotypes analysed (Fig. 3.3.9).

Body weight is also commonly affected by disease. Therefore, the mice were weighed periodically throughout the experiment to measure their general health, and also at the time of sacrifice to assess if any changes in weight had occurred (Fig. 3.3.9). Mice that were weighed more than once appeared to have similar weights suggesting that the disease had not affected their body weight. However, as many of the mice succumbed to disease early on in the experiment, some mice had only been weighed once.

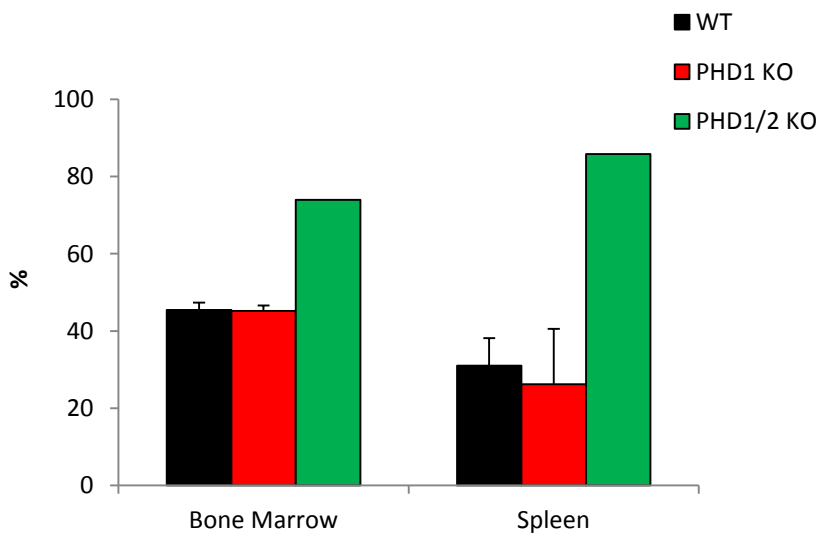


Figure 3.3.7. Detection of CD45.2$^+$ cells in sacrificed mice.

Mice that were thought to be leukaemic (based on the high frequency of CD.45.2$^+$ cells in the blood and the development of physical symptoms), were sacrificed. Bone marrow and spleen were analysed for the presence of CD45.2$^+$ cells as determined by flow cytometry. Femur and tibia of the mice were crushed to extract bone marrow cells. Spleens were extracted and crushed in PBS to give a cell suspension. Bone marrow and spleen cells were stained with antibodies specific for CD45.1 and CD45.2 and analysed by flow cytometry. Data presented represent the total mean frequency of CD45.2$^+$ cells seen in the bone marrow and the spleen of leukaemic mice (WT n=7, PHD1 KO n=4, PHD1/2 KO n=1).

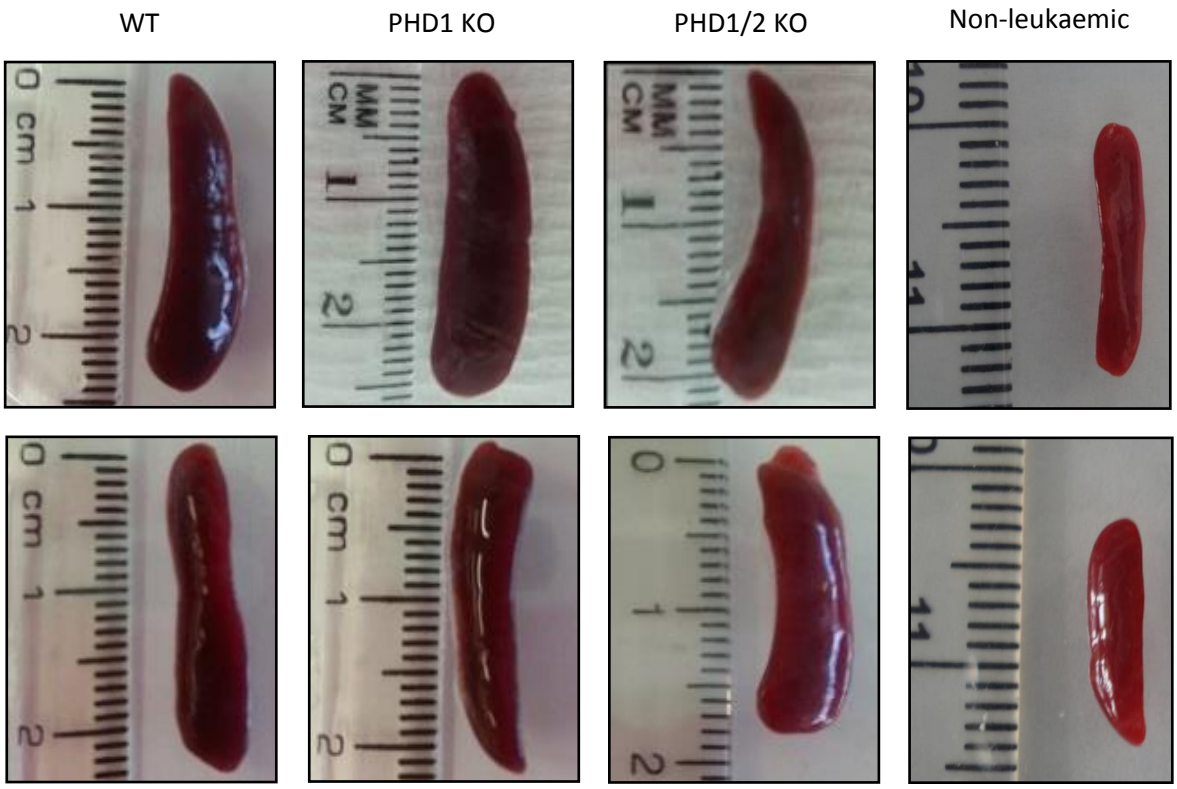


Figure 3.3.8. Leukaemic mice had enlarged spleens.

Spleens were collected, photographed and measured from leukaemic mice at the point of sacrifice to assess if the mice had enlarged spleens, one of the characteristic symptoms of leukaemic disease. Leukaemic spleens were enlarged and characteristic of the disease. Spleens were also collected from several non-leukaemic mice to be used as a control and as a measurement of normal spleen size in healthy adult mice.

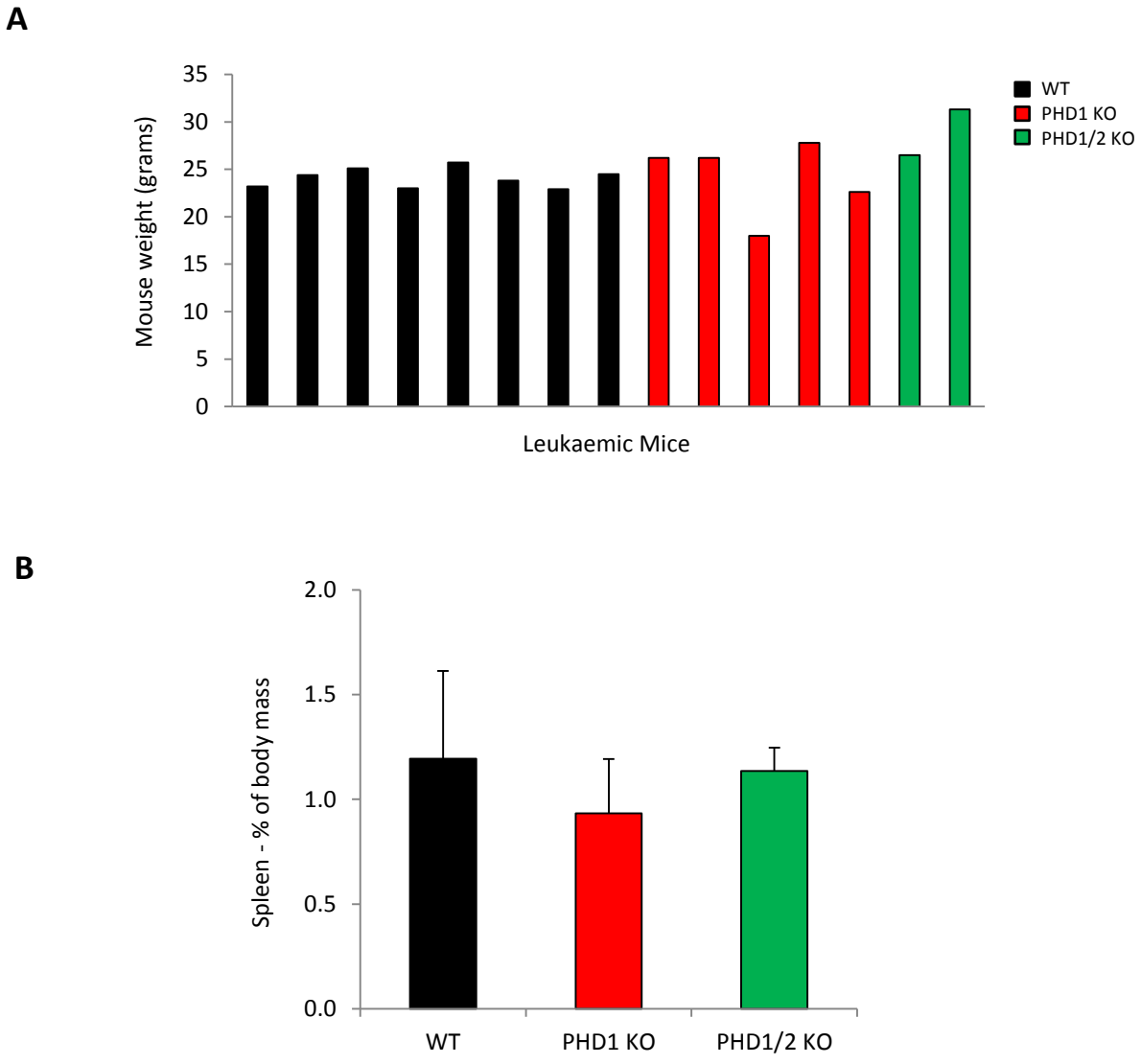


Figure 3.3.9. Body and spleen weights of leukaemic mice at sacrifice.

A. Mice were weighed periodically throughout the experiment to help monitor their health. Graph represents weights of individual mice at the time of death. B. Body weight at death was used to measure the proportion of body mass contributed to by the enlarged spleens. Data shown are mean percentage of spleen contribution. (WT n=8, PHD1 KO n=5, PHD1/2 KO n=2).

Blood obtained from leukaemic mice was stained using a Giemsa-Wright procedure, which is used to differentiate between different types of blood cells and is often used to identify the presence of abnormal, leukaemic cells in the blood. Blood from WT leukaemic mice appeared to have a higher frequency of large blast-like cells compared to blood obtained from WT non-leukaemic mice (Fig. 3.3.10). The presence of leukaemic cells in the blood, as well as visually enlarged spleens in the sacrificed mice, suggests that these mice were leukaemic. No other genotypes were analysed using Giemsa-Wright staining. The purpose of this experiment was to determine if this staining would be advantageous in future experiments and so no quantitative data was collected, nonetheless it would be a good additional test to monitor the leukaemic disease using blood smears.

The concentration of white blood cells (WBC) within the blood was also monitored over the course of the experiment with a high WBC count being indicative of leukaemia. WBC counts of mice that were positive for $CD45.2^{+}$ cells remained within normal range, but increased dramatically at later stages (Fig. 3.3.11). WBC concentration in the blood of leukaemic mice at the point of sacrifice was considerably higher than non-leukaemic counterparts. Cellularity within the bone marrow and spleen were also analysed (Fig. 3.3.11). However, cell counts were variable and no difference was seen between the different groups. Overall, pre-LSCs generated *in vitro* recapitulated disease that was pathologically, morphologically and phenotypically similar to the disease described in other published *in vivo* studies (Somervaille & Cleary, 2006; So et al, 2010).

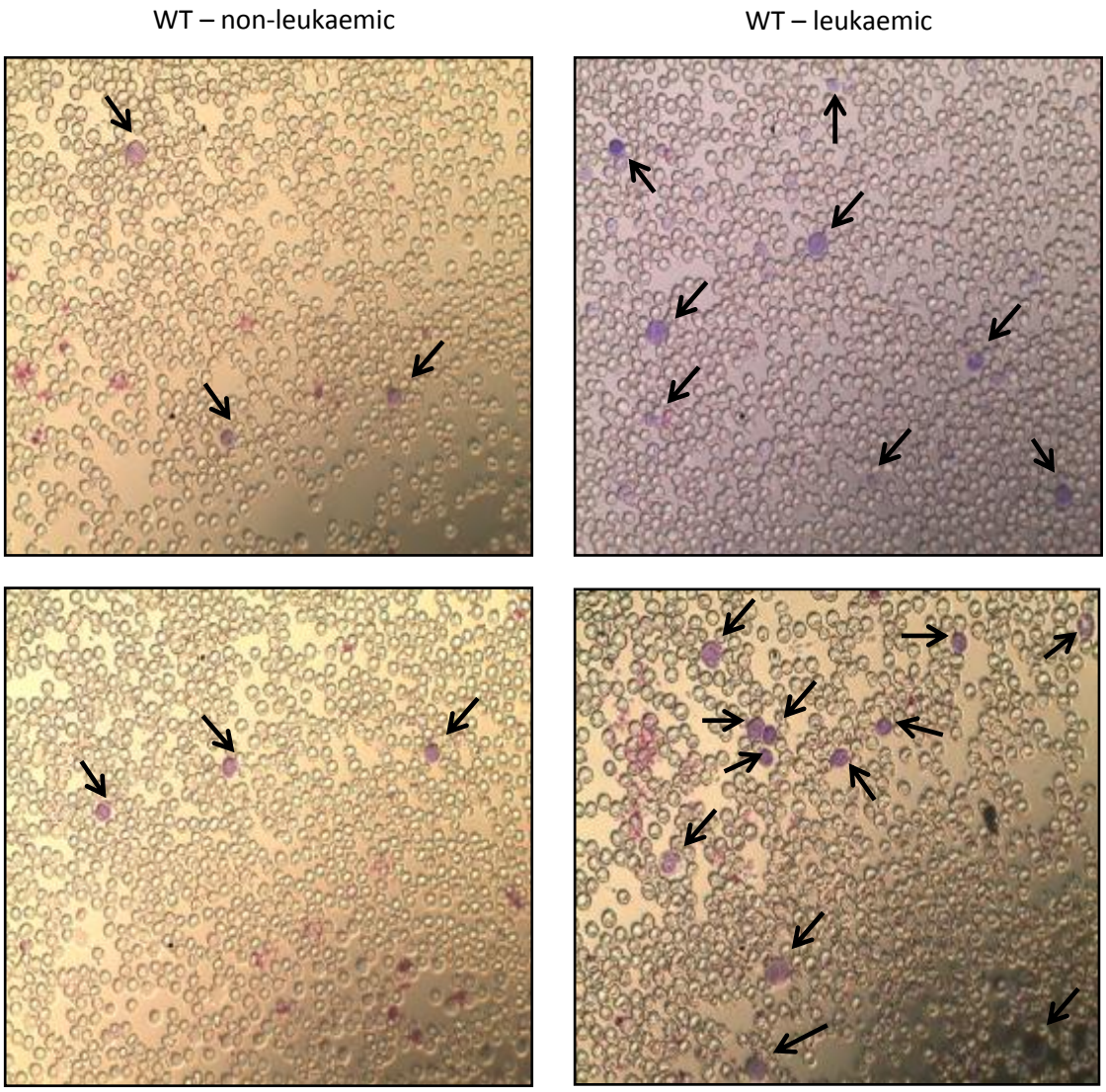


Figure 3.3.10. WBCs in peripheral blood samples.

Peripheral blood samples were acquired from mice via tail vein. Blood was smeared onto glass slides and fixed in ethanol. Fixed blood smears were incubated at room temperature in Giemsa-Wright staining solution. Slides were dried and assessed by standard light microscopy in order to detect the presence of WBCs within the blood sample. The purple colour of the stain is incorporated into WBCs but not RBCs, allowing the two cell types to be distinguished. The number of WBCs and the presence of larger, abnormal WBC-like cells were used to indicate the penetrance of leukaemic disease. Blood samples acquired from leukaemic WT mice appeared to have a higher frequency of WBCs compared to non-leukaemic WT samples. Arrows indicate WBC (WT n=2).

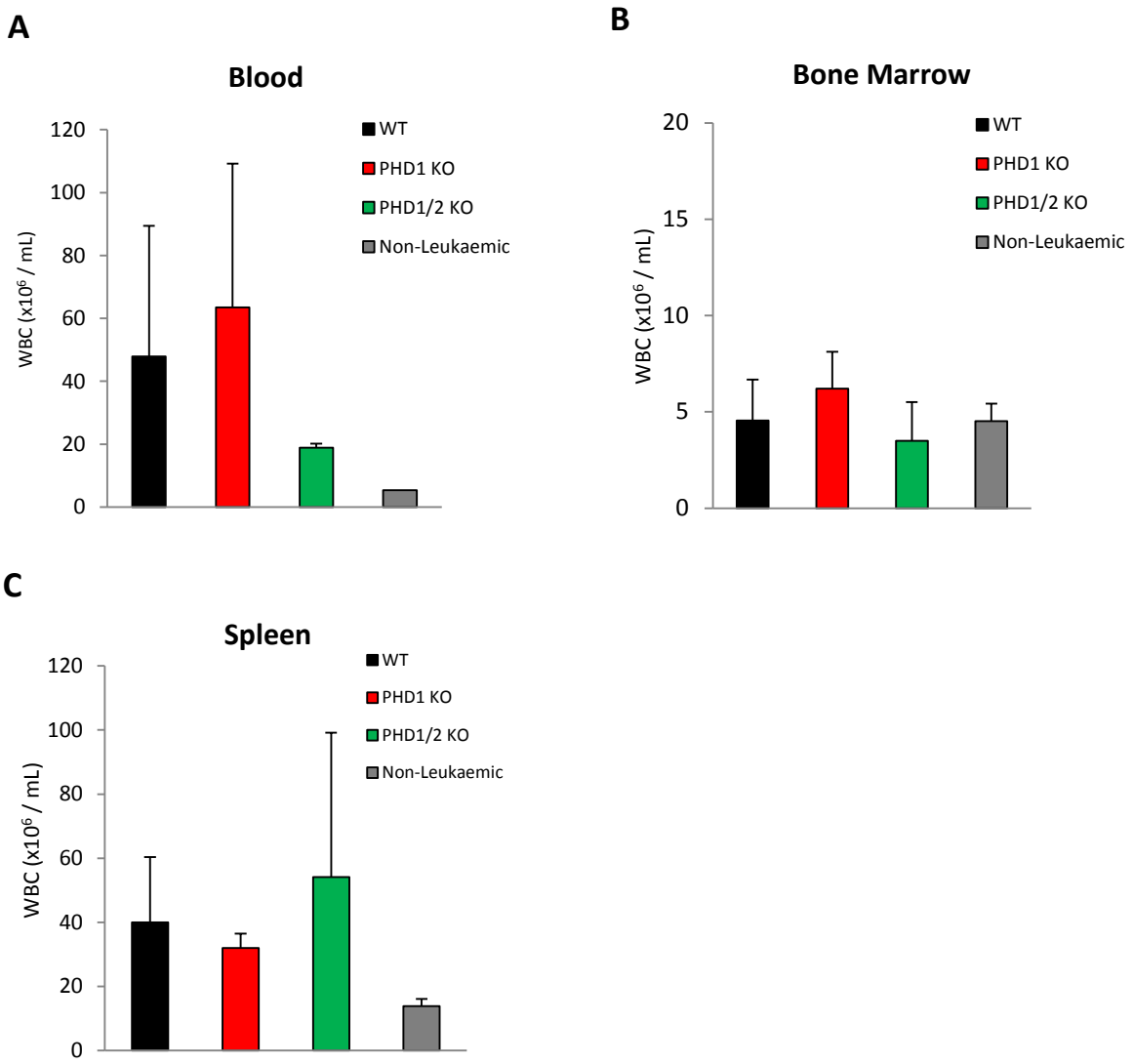


Figure 3.3.11. WBC concentration of blood, bone marrow and spleen.

Blood (A), bone marrow (B) and spleen (C) were analysed using an automated cell counter to determine the concentration of WBCs. Data presented represent WBC concentrations assessed for all leukaemic sacrificed mice. Non-leukaemic WBC concentrations from a previous experiment were used as control. Data shown are total mean WBC concentrations. (WT n=9, PHD1 KO n=5, PHD1/2 KO n=2, non-leukaemic n=6).

3.3.4 Immunophenotype of leukaemic cells

Next the frequency of LSCs was analysed based on the presence of cells that carried the LSC expression markers c-Kit, Mac-1 and Gr-1 simultaneously as analysed by flow cytometry. As a control, CD45.1^{+} non-transduced therefore non-leukaemic support bone marrow was used to determine the frequency of apparent LSC-like cells within the normal bone marrow (background). These individual LSC cell surface markers are seen on normal haemopoietic cells although they are associated with different cell types, making the simultaneous expression of these quite unique to LSC. c-Kit is used as a marker for primitive cells, although Mac-1 and Gr-1 are expressed on more mature cell types. However it is possible that cells exist which express these markers simultaneously and that these may represent a transient population that is going through the differentiation process but, this population is rare.

Cells were first analysed for c-Kit expression. CD45.1^{+} cells appeared to have a low percentage of c-Kit^{+} cells within the bone marrow. This population was undetectable in the blood and spleen (Fig. 3.3.12). c-Kit^{+} cells were then gated and further analysed for simultaneous expression of Mac-1 and Gr-1 and therefore the presence of LSC-like cells. The frequency of LSC-like cells within the CD45.1^{+} population was low (3.74%) within the bone marrow and may represent differentiating cells. LSC-like cells were not detectable within the blood or spleen.

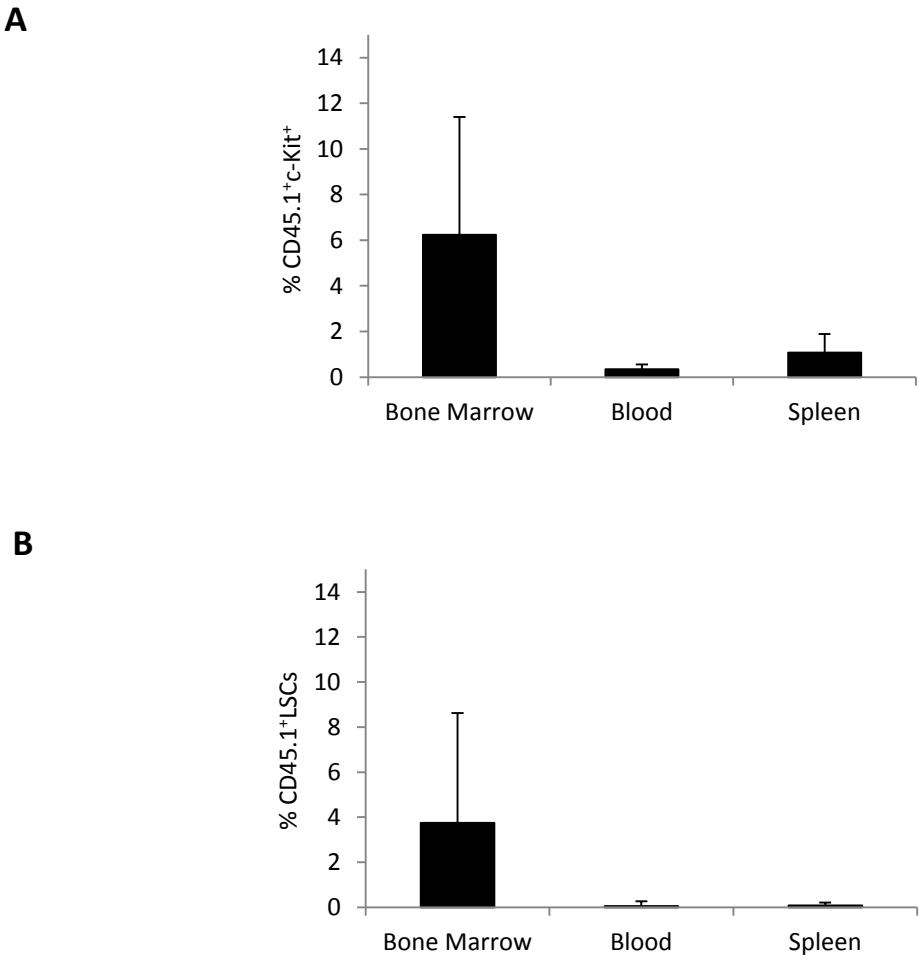


Figure 3.3.12. Immunophenotype of CD45.1$^+$ support bone marrow cells.

Bone marrow, blood and spleen cells were extracted from mice and were analysed as a control to CD45.2 leukaemic cells to determine the immunophenotype of the normal CD45.1 bone marrow cells. Cells were incubated with antibodies specific for LSC markers and analysed by flow cytometry. A. Cells were first assessed to determine the frequency of c-Kit$^+$ cells, representing the normal HSPC population. B. Within the c-Kit$^+$ gate, cells were further analysed for the co-expression of Mac-1 and Gr-1 to determine LSC-like cell frequency within the CD45.1$^+$ control population. Data shown represent the mean frequencies of cell populations. (n=13).

CD45.2$^+$ cells were then analysed for the expression of LSC markers. The CD45.2$^+$ populations within the bone marrow, blood and spleen all contained a higher percentages of c-Kit$^+$ cells compared to CD45.1$^+$ cells, with the highest frequency being seen in the bone marrow (Fig. 3.3.13A). The frequency of c-Kit$^+$ cells was comparable between WT and PHD1 KO cells. Only one sample of PHD1/2 KO cells was available for analysis so no statistical comparison was made between this sample and other genotypes. c-Kit$^+$ cells were then analysed for the expression of Mac-1 and Gr-1. A significant percentage of cells also expressed both Mac-1 and Gr-1, composing a phenotype similar to pre-LSCs produced *in vitro* also in accordance with the literature (Fig. 3.3.13B). However, the immunophenotypic profile of the cells also showed that a population of c-Kit$^+$Mac-1$^+$Gr-1$^-$ cells existed (Fig. 3.3.13C). It is noteworthy that this population did not exist upon transplantation (Fig. 3.3.3), but has developed *in vivo* in correlation with the disease. As the relevance of this population is still unclear, both potential LSC populations were analysed and are referred to herein as LSC-1 (c-Kit$^+$Mac-1$^+$Gr-1$^+$) and LSC-2 (c-Kit$^+$Mac-1$^+$Gr-1$^-$).

The frequency of c-Kit$^+$ cells, as well as the frequencies of LSC-1 and LSC-2 populations, were calculated for CD45.2$^+$ leukaemic cells, only in those mice culled following development of leukaemia. Blood, bone marrow and spleen were all analysed and frequencies were compared across the groups to determine if the absence of PHD isoforms had an effect on the development of LSCs. Frequencies of c-Kit$^+$, LSC-1 and LSC-2 cells were comparable across all groups and no differences were seen.

Next, the frequency of CD45.2$^+$c-Kit$^+$, CD45.2$^+$LSC-1 and CD45.2$^+$LSC-2 cells were determined within the total WBC population of the recipient mouse (Fig. 3.3.14). Each group was compared to determine the effect of PHD ablation. However, no differences were seen in the overall development of CD45.2$^+$ LSCs in the blood, bone marrow or spleen.

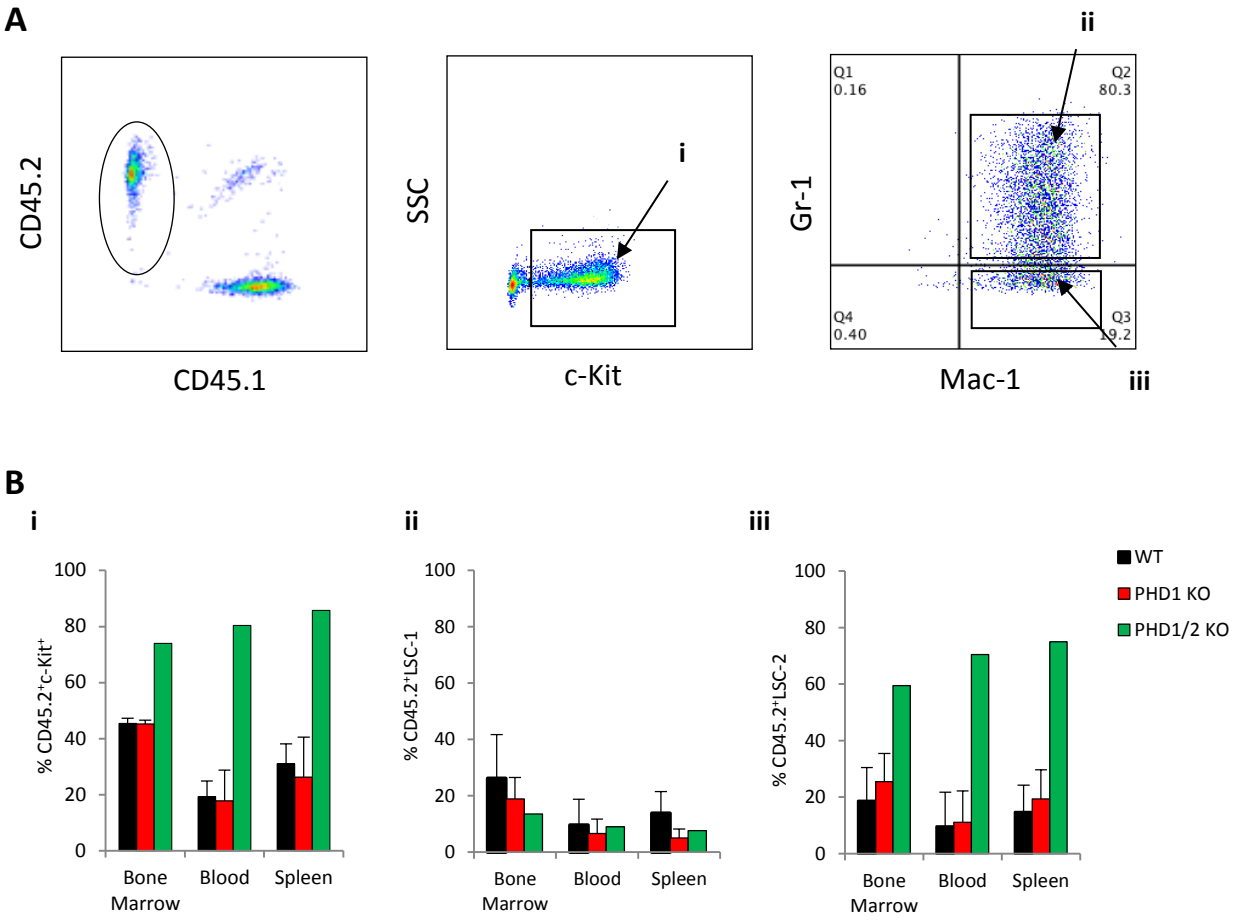


Figure 3.3.13. Immunophenotype of CD45.2$^+$ leukaemic cells.

Bone marrow, blood and spleens were analysed by flow cytometry to determine the immunophenotype of CD45.2$^+$ cells. A. CD45.2$^+$ cells were analysed for c-Kit expression; within the c-Kit$^+$ gate, cells were analysed for co-expression of Mac-1 and Gr-1. Cells that were c-Kit$^+$Mac-1$^+$Gr-1$^+$ were referred to as 'LSC-1'. The frequency of CD45.2$^+$ cells which were c-Kit$^+$Mac-1$^+$Gr-1$^-$ was calculated and referred to as 'LSC-2'. B. Data presented represent the mean frequency of CD45.2$^+$ cells which were c-Kit (i), LSC-1 (ii) and LSC-2 (iii). (WT n=7, PHD1 KO n=4, PHD1/2 KO n=1).

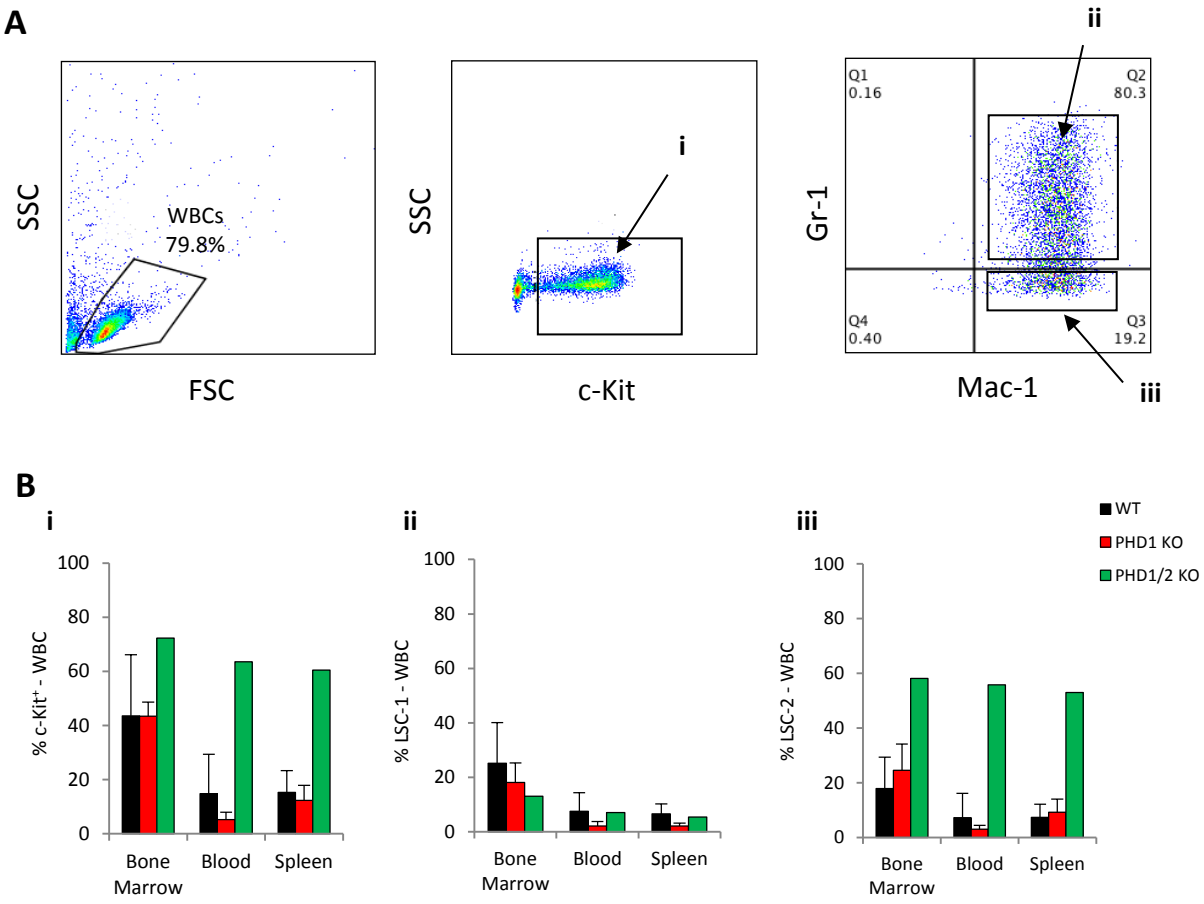


Figure 3.3.14. Frequency of CD45.2$^+$ LSCs within WBC population.

Bone marrow, blood and spleens were analysed by flow cytometry to determine the frequency of c-Kit$^+$, LSC-1 and LSC-2 cells within the WBC population of the recipient mouse. A. WBCs were gated to determine frequency of WBCs that were c-Kit$^+$, LSC-1 and LSC-2 phenotype. WBCs were analysed for c-Kit expression. Within the c-Kit$^+$ gate, cells were analysed for co- expression of Mac-1 and Gr-1. Cells that were c-Kit$^+$Mac-1$^+$Gr-1$^+$ were referred to as LSC-1. The frequency of WBCs which were c-Kit$^+$Mac-1$^+$Gr-1$^-$ was calculated and referred to as LSC-2. B. Data presented represent the mean frequency of WBCs which were c-Kit (i), LSC-1 (ii) and LSC-2 (iii). (WT n=7, PHD1 KO n=4, PHD1/2 KO n=1).

3.3.5 Apoptotic frequency of leukaemic cells

Due to the potentially apoptotic phenotype observed in transformed cells *in vitro*, leukaemic cells within the bone marrow were analysed for early and late stage apoptosis and viability based on Annexin V binding. The bone marrow of analysed mice was almost entirely composed of CD45.2^{+} cells (95.7%) and therefore, it was not necessary to gate this population. C-Kit^{+} cells were gated in order to analyse the frequency of apoptosis in haemopoietic cells (including HSPCs and LSCs). Frequencies of c-Kit^{+} cells undergoing apoptosis were compared across the available groups to determine if any differences in apoptotic frequencies were observed within an *in vivo* leukaemic setting. Apoptotic frequencies appeared to be somewhat variable (between 1.26-5.02% in WT cells) and no differences were seen between groups (Fig. 3.3.15). However, as mice transplanted with PHD2 KO pre-LSCs did not succumb to disease, none were available for analysis (healthy mice were not analysed on the day of termination of the experiment). Considering the pro-apoptotic phenotype seen in PHD2 KO pre-LSCs during the *in vitro* study, it would have been interesting to see if this phenotype prevailed in an *in vivo* setting.

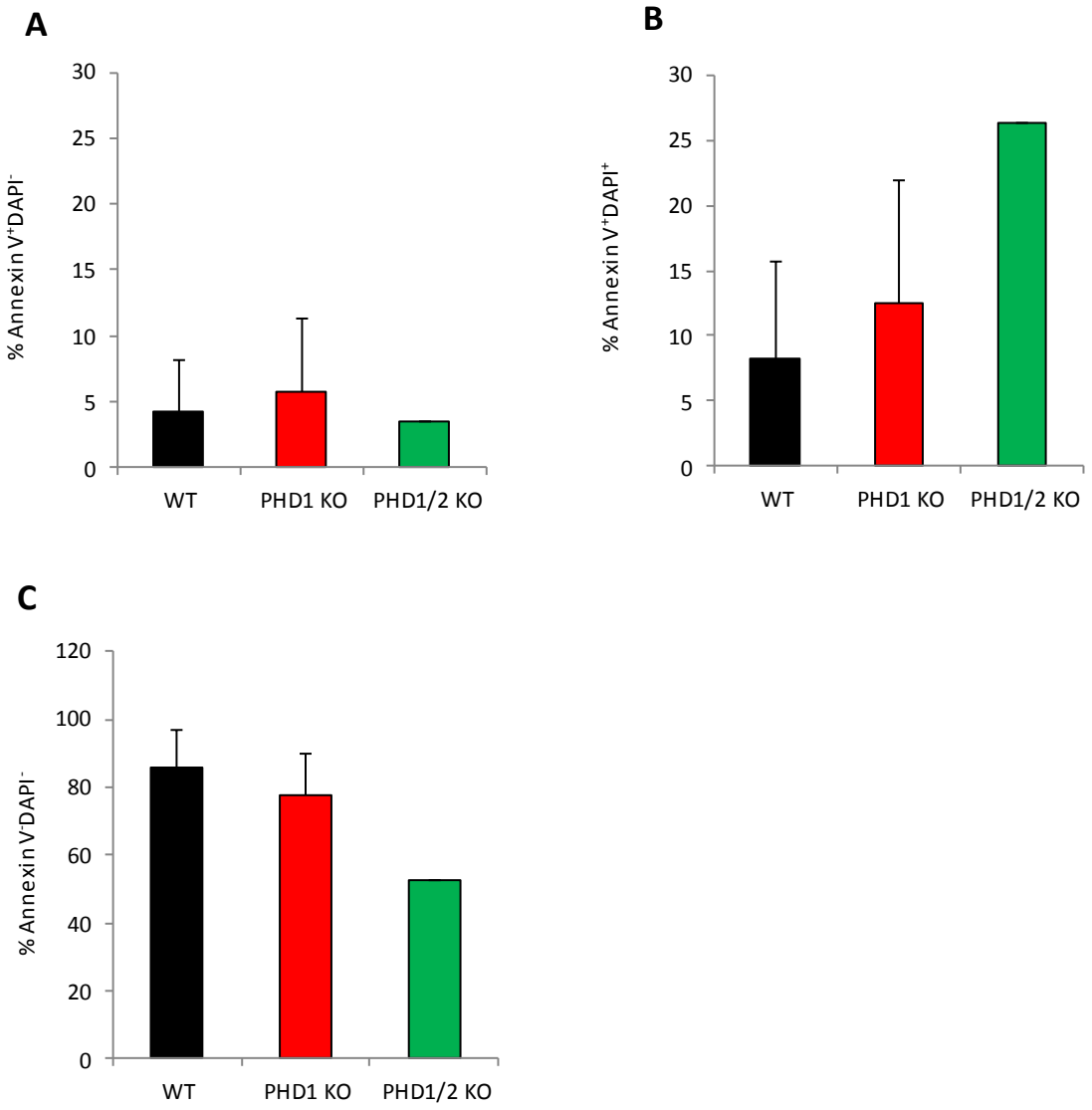


Figure 3.3.15. Frequency of apoptosis within WBC population.

Bone marrow of leukaemic mice was analysed by flow cytometry; Annexin V binding and DAPI staining was used to determine the frequencies of WBC undergoing apoptosis. Annexin V^+DAPI^- cells were considered to be in early apoptosis (A), Annexin V^+DAPI^+ cells were considered to be in late apoptosis (B). Cells that were Annexin V^-DAPI^- were considered to be viable (C). Data presented represent mean frequencies of apoptotic and viable cells. (WT n=7, PHD1 KO n=4, PHD1/2 KO n=1).

3.3.6 Conclusion

The ablation of PHD genes, particularly PHD2, appeared to reduce the leukaemogenic potential of pre-LSCs. Overall, the results suggested that PHD2 was required for leukaemic development and that overall survival rate of the transplanted mice was dramatically increased in the absence of PHD2. However, the data were also suggestive that the disease, when it occurred, following transplantation of pre-LSCs was comparable regardless of PHD absence. Analysis of leukaemic samples suggested that once the disease developed within the host, it was unaffected by the absence of PHD, indicating that PHD may not play a role in the maintenance of the disease. However, as leukaemic frequency and overall survival did appear to be affected by the loss of PHD, it is likely that PHD has a role in the establishment of the disease. However, analysis of PHD2 KO transplanted mice would be required to confirm this. These results are entirely novel and PHD2 has not previously been reported to have a role in leukaemogenesis. It would be vital to repeat these experiments to verify these potentially exciting findings.

3.4 Response of Pre-LSCs to Stressful Stimuli

It was previously shown in the *in vitro* assay assessing leukaemic potential, that WT and PHD KO pre-LSCs had comparable frequencies of LIC (Fig. 3.1.8). Transplanting HSCs to recipient mice is a common assay used to determine the response of the cells to the stressful conditions of transplantation. Considering this, it is possible that the difference seen in leukaemic potential *in vivo* was attributed to a difference in response to stress by the transplanted pre-LSCs. In order to investigate this possibility, exposure to hypoxia was used to mimic the conditions of the bone marrow to determine if the cells showed a difference in response to the stressful nature of the transplantation. It is noteworthy that PHD isoforms are oxygen dependent so are not active in hypoxic conditions; therefore hypoxia signalling may not be altered by lack of PHD. However, there are other regulatory elements governing hypoxia signalling.

3.4.1 Response of pre-LSCs to hypoxic conditions

WT and PHD1 KO, and PHD2 KO pre-LSCs were cultured in a specialist hypoxia chamber in 1% oxygen for 24 hours. Unfortunately, PHD1/2 KO pre-LSCs were not available for analysis in this experiment. Following treatment, the frequency of Annexin V^+ (apoptotic) cells was analysed. Flow cytometric analysis of cells showed that PHD1 KO cells had a lower frequency of late apoptosis compared to WT cells (13.9% compared to 19.1%) which was seen to be statistically significant (p=0.043) (Fig.3.4.1). Data also showed a trend towards lower total apoptosis in PHD1 KO cells, although this was not significant (p=0.115). The data showed that PHD2 KO pre-LSCs had frequencies of apoptosis that were comparable to WT pre-LSCs. No differences were seen in the frequency of viable cells. This suggests that the hypoxia treatment abrogated the apoptotic phenotype of PHD2 KO seen under steady state conditions. This is likely to reflect the oxygen-dependent nature of PHD isoforms. Hypoxic conditions would result in PHD isoforms becoming inactive in WT cells. Therefore, under hypoxic conditions, WT cells are responding effectively under the absence of PHD and so behaving in a similar manner to cells which genetically lack PHD.

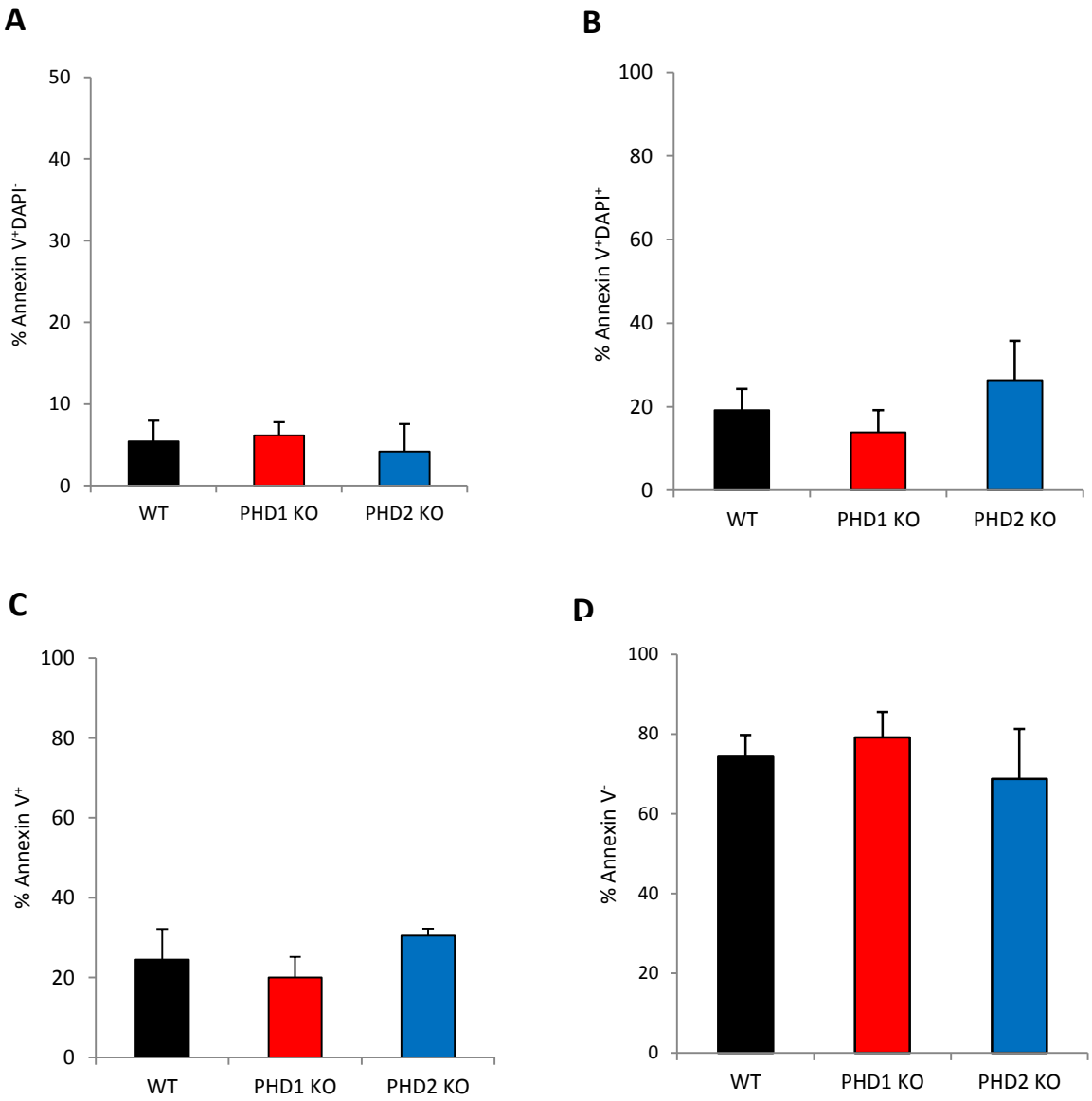


Figure 3.4.1. Apoptotic frequencies of pre-LSC cell lines under hypoxic conditions.

Pre-LSC cell lines were subjected to hypoxic conditions for 24 hours to mimic *in vivo* bone marrow hypoxia. Annexin V binding and DAPI staining was used to determine the frequencies of pre-LSCs undergoing apoptosis in hypoxic conditions. Annexin V^+DAPI^- cells were considered to be in early apoptosis (A), Annexin V^+DAPI^+ cells were considered to be in late apoptosis (B). Cells that were Annexin V^+ (both $DAPI^+$ and $DAPI^-$) were included when calculating total apoptosis (C). Cells that were Annexin V^-DAPI^- were considered to be viable (C). Data presented represent mean frequencies of apoptotic and viable cells. (WT n=3, PHD1 KO n=3, PHD2 KO n=2).

Next, the frequencies of cycling cells were determined by Ki67/DAPI staining (Fig. 3.4.2). Cell cycle frequencies were comparable between WT, PHD1 KO and PHD2 KO pre-LSCs, suggesting that PHD deficiency did not affect the cell cycle under hypoxic stress.

Overall, these experiments suggest that cells do not respond differently to hypoxic stress in the absence of PHD isoforms. This might indicate that the differences seen *in vivo* were not attributable to a cellular response to hypoxic conditions within the bone marrow, but rather reflected a genuine reduction in leukaemic potential in the absence of PHD isoforms.

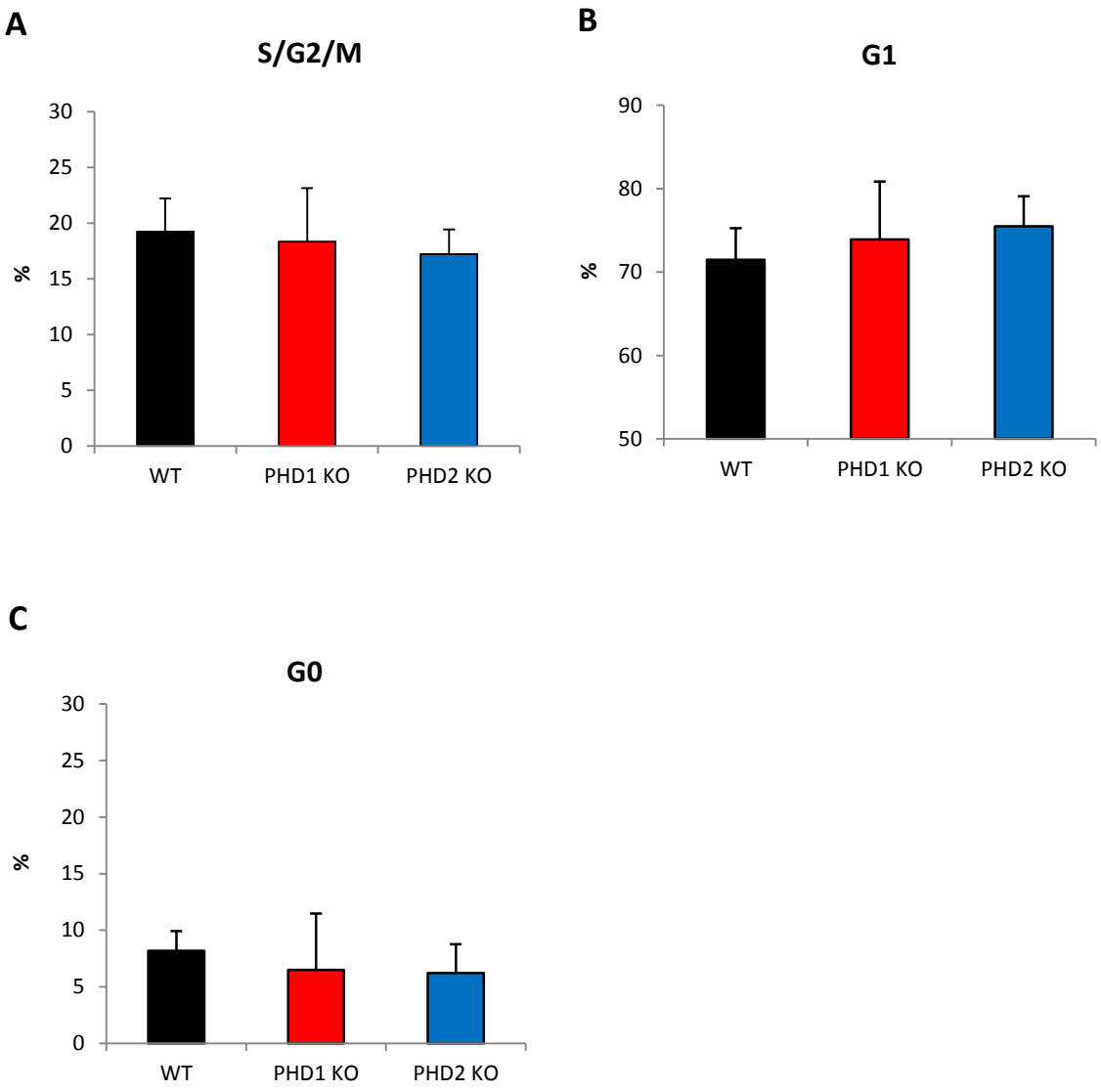


Figure 3.4.2. Cell cycle frequencies of pre-LSC cell lines under hypoxic conditions.

Pre-LSC cell lines were cultured under hypoxic conditions for 24 hours to mimic *in vivo* bone marrow hypoxia and cell frequencies in G0 (A), G1 (B) and S/G2/M phases (C) for WT, PHD1 KO and PHD2 KO pre-LSC cell lines determined by Ki67/DAPI staining. Data shown represent mean cell cycle frequencies. (WT n=2, PHD1 KO n=3, PHD2 KO n=1).

3.5 Response of Pre-LSCs to Chemotherapy and Radiation Treatment

3.5.1 Concentration-effect curves used to select optimal chemotherapy doses

Following the difference seen in leukaemic potential, experiments were conducted to examine if ablation of PHD isoforms could affect cell response to cancer therapy. Absence of PHD2 has already been shown to result in a pro-apoptotic phenotype under normal conditions and it was of interest to see if this apparent phenotype was heightened in response to stress. An increase in apoptosis in response to chemotherapy, coupled with a diminished ability to initiate disease, would reveal PHD2 as a promising therapeutic target that could be utilised in leukaemia treatment.

Doxorubicin (Dox) and cytarabine (or arabinofuranosyl cytidine (AraC)) are chemotherapeutic agents commonly used to treat human AML (Wunderlich et al, 2013). These agents are also used routinely in research to determine chemotherapeutic response in both *in vitro* and *in vivo* studies (Lowe et al, 2011; Pardee, 2012). However, sensitivity to treatment can vary dramatically between different cell types and so assay optimisation depending on the cell lines under study is required. WT pre-LSC cell lines were first used to examine the response to treatment with Dox and AraC, in order to determine the optimal concentration to be used during the assay. The concentration of Dox and AraC used in similar studies in the literature informed the range which should be tested with the pre-LSC cell lines (Lowe et al, 2011).

Cells were treated with increasing concentrations of Dox and counted by trypan blue exclusion after 24 hours (Fig. 3.5.1A). Trypan blue exclusion method can be used to determine both the viability of the cells, and concentration of the cells to give a measure of cell growth. Cell viability is determined by counting both white and blue cells separately to give the frequency of healthy cells. Cell growth is determined by counting only white cells to give the concentration of the cell suspension. Trypan blue exclusion showed that WT cells treated with Dox decreased in viability in a dose-dependent manner. However, it was only at higher doses (10ng/mL & 20ng/mL) that the effect was prominent. Therefore, a second concentration-effect curve was completed this time with cells treated with 0-50ng/mL Dox and counted at 24 and 48 hours (Fig. 3.5.1B). In WT

cells, viability was unaffected at lower concentrations; 50ng/mL Dox was required to initiate a substantial reduction in viability.

Similar results were seen at both 24 and 48 hours suggesting that an appropriate response could be obtained after 24 hours. Cell concentration of WT cells was reduced at both time points following treatment with 10ng/mL Dox. However, a more prominent reduction in cell growth was seen following treatment with higher concentrations of Dox (25 and 50ng/mL). Similar results were seen at the two time points with 25 and 50ng/mL Dox, again suggesting that 24 hours was adequate to observe a response in WT cells. However, the exact response of PHD KO cells was unknown so it was difficult to determine which concentration of Dox was optimal to observe a difference in response. However, based on these results, 25 and 50ng/mL Dox were chosen to investigate chemotherapy response in PHD KO cells.

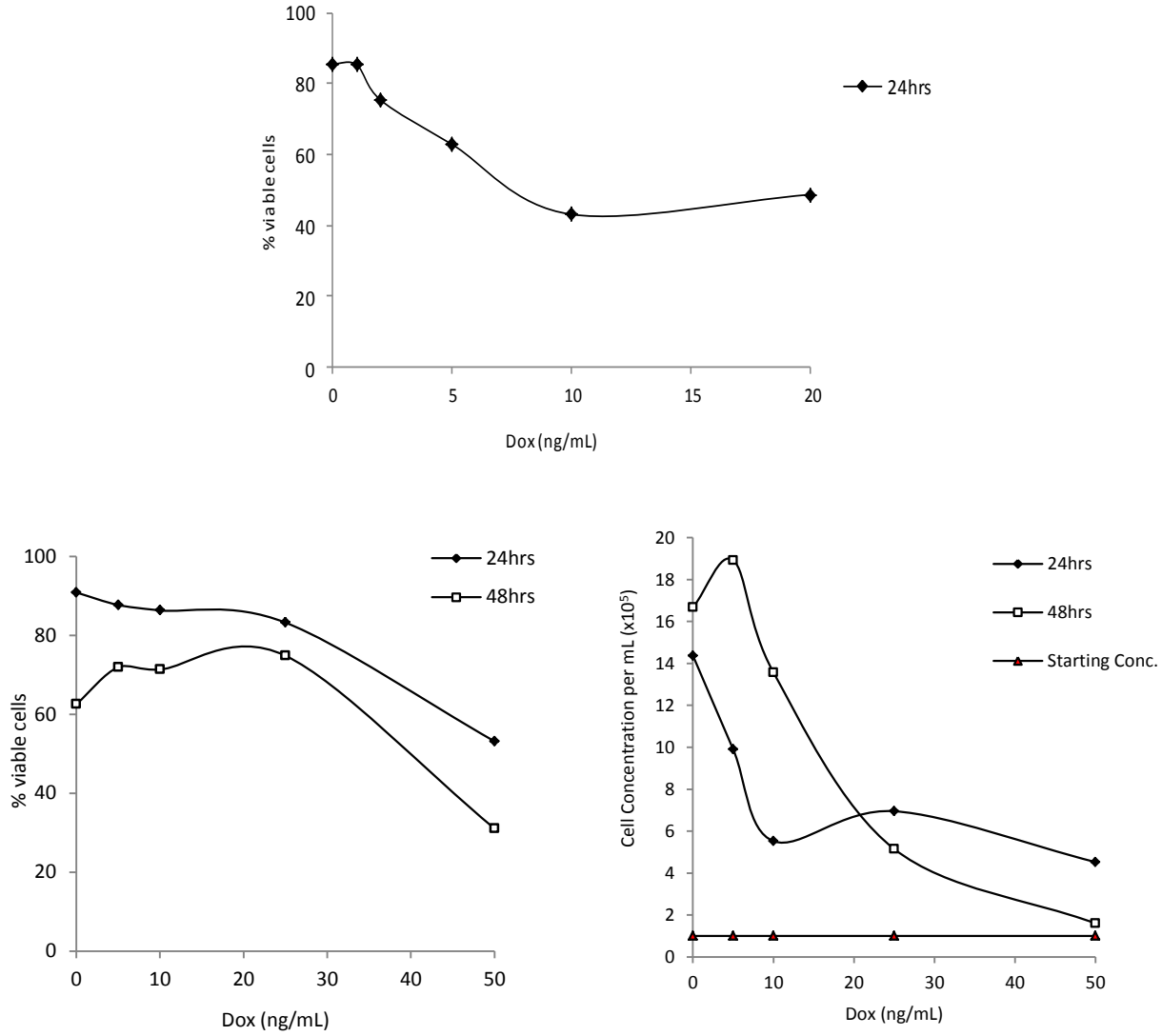


Figure 3.5.1. Concentration-effect curves of WT cells following Dox treatment.

WT cells were treated with increasing concentrations of Dox to determine the most effective concentration for use in further experiments. A. Cell viability was assessed using trypan blue staining following treatment with 0-20ng/mL Dox. B & C. Cells were subjected to a second concentration-effect curve whereby the maximum Dox concentration was increased to 50ng/mL. Cell viability and cell growth were both measured using trypan blue staining.

Next the viability of cells treated with AraC was determined by trypan blue exclusion. WT cells treated with all concentrations of AraC were found to have extremely low viability after 24 hours suggesting that the dose curve of 0-48ng/mL was too high for these cells (Fig. 3.5.2A). Lower (0-2.4ng/mL) concentrations of AraC were then used. However, after treatment with even the lowest concentration of AraC, no cell growth was observed and cell viability was drastically reduced (Fig. 3.5.2B). As the response to chemotherapy *per se* in cells lacking PHD was not being tested in this assay, using a concentration that already caused a dramatic death response in WT cells may not have allowed observation of any subtle differences in sensitivity between WT and PHD KO cells as a consequence of their PHD genotype. Therefore, a concentration that exerted an effective, but not absolute response in WT cells was optimal to allow both increases and decreases in response to be observed. Considering this, a third concentration-effect curve was completed using lower concentrations of AraC (0-0.24ng/mL) being much lower than those reported in the literature. However, drug sensitivity can vary extensively between cell types and the exact sensitivity of the pre-LSC cells had never been examined. Cell growth of WT cells treated with AraC was significantly diminished in all treatment groups (Fig. 3.5.2C). However, 0.12ng/mL AraC treatment was required before an affect was seen in cell viability. Based on these results, 0.12ng/mL AraC was chosen as the optimal concentration to assess response to chemotherapy.

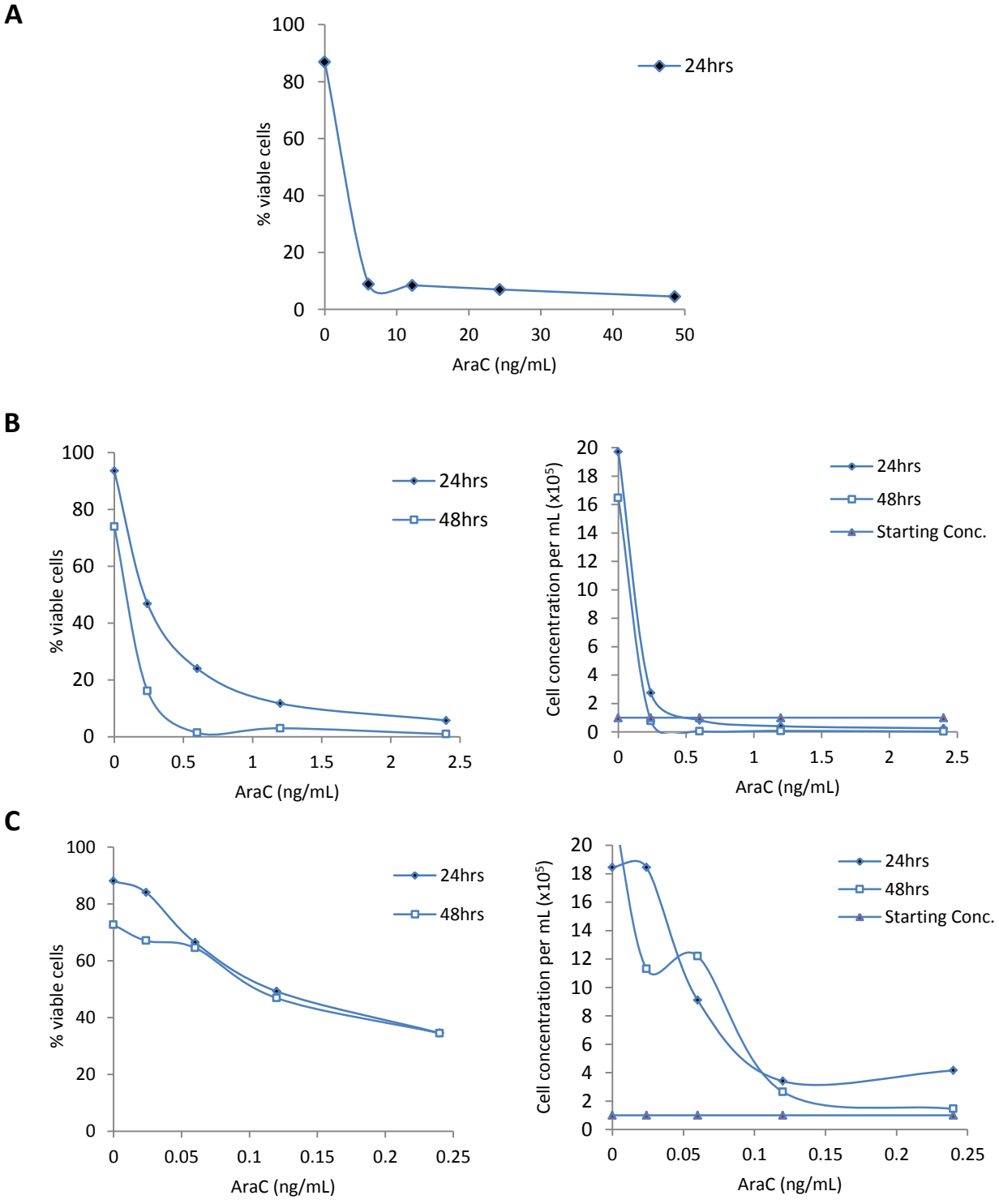


Figure 3.5.2. Concentration-effect curves of WT cells following AraC treatment.

WT cells were treated with increasing concentrations of AraC to determine the most effective concentration for use in further experiments. A. Cell viability was assesses using Trypan blue staining following treatment with 0-48ng/mL AraC. B. Cells were subjected to a second concentration-effect curve whereby the maximum AraC concentration was decreased to 2.4ng/mL AraC. C. A third concentration-effect curve was completed whereby cells were treated with 0.24ng/mL AraC. Cell viability and cell growth were both measured using Trypan blue staining.

3.5.2 Response of pre-LSCs to chemotherapy treatment

In order to determine if cells respond differently to chemotherapy in the absence of PHD genes, WT, PHD1 KO, PHD2 KO and PHD1/2 KO cells were treated with Dox, AraC and a combination of Dox and Ara-C for 24 and 48 hours. For this experiment, only one biological replicate was available for PHD1/2 KO cells and therefore, statistical analysis was not performed on data from these samples. Two biological replicates were available for WT and PHD1 KO. Statistical analyses were performed using two biological replicates along with technical replicates in two individual experiments although statistical analysis would be more conclusive using three or more replicates. Three biological replicates were available of PHD2 KO cells and so these were used to calculate statistical relevance. However, it would be essential to repeat these experiments to increase the number of replicates and data available for analysis, in order to confirm these results.

Trypan blue exclusion method was used to determine the viability of the cells. Untreated cells were used as a control (No Drug Control, NDC). At 24 hours, a difference in viability was observed between NDC groups from different PHD genotypes, that was concordant with previous results (Fig. 3.2.7). As each group had a difference in cell death prior to drug treatments, results are displayed as the percentage of cell death incurred following drug treatment, normalised to the percentage of cell death in each NDC group. Therefore, data presented shows the cell death in response to treatment and not total cell death. Following 24 hour treatment with 25ng/mL Dox, no difference was seen between groups (Fig. 3.5.3). However, in cells treated with 50ng/mL Dox, 0.12ng/mL AraC and a combination treatment of AraC and Dox, PHD1 KO, PHD2 KO and PHD1/2 KO pre-LSCs all had higher frequencies of cell death compared to WT pre-LSCs. Statistical analysis showed that differences between PHD1 KO and WT were significant in cells treated with 50ng/mL Dox, and in cells treated with a combination of Dox and AraC. Differences seen between PHD2 KO and WT cells were significant in all treatment groups with the exception of 25ng/mL Dox. At 48 hours, a similar trend was observed (Fig. 3.5.3). Following 25ng/mL Dox treatment, PHD KO pre-LSCs all showed increased cell death compared to WT. As this was not seen after 24 hours treatment, it is likely that at lower concentrations, this observation will only manifest following longer treatments. At 48

hours, data showed that in all treatment groups, PHD1 KO, PHD2 KO and PHD1/2 KO had a higher frequency of cell death compared to WT. Statistics preformed between WT and PHD1 KO showed that frequencies of cell death were statistically significant in all treatment groups. However, although PHD2 KO cells appeared to have a higher rate of cell death than WT in all groups, only cells treated with 50ng/mL Dox showed a difference that was statistically significant.

Next, trypan blue exclusion was again used to determine the concentration of the cells (growth), which was determined at 24 and 48 hours (Fig. 3.5.4). In accordance with the effect seen in cell death frequencies, cells lacking PHD generally had diminished cell growth in comparison to WT in all treatment groups at both 24 and 48 hours. Statistics between PHD1 KO and WT showed that the difference was statistically significant in all treatment groups except cells treated with 0.12ng/mL AraC. The difference in cell growth seen between PHD2 KO and WT cells also proved to be significant in all treatment groups.

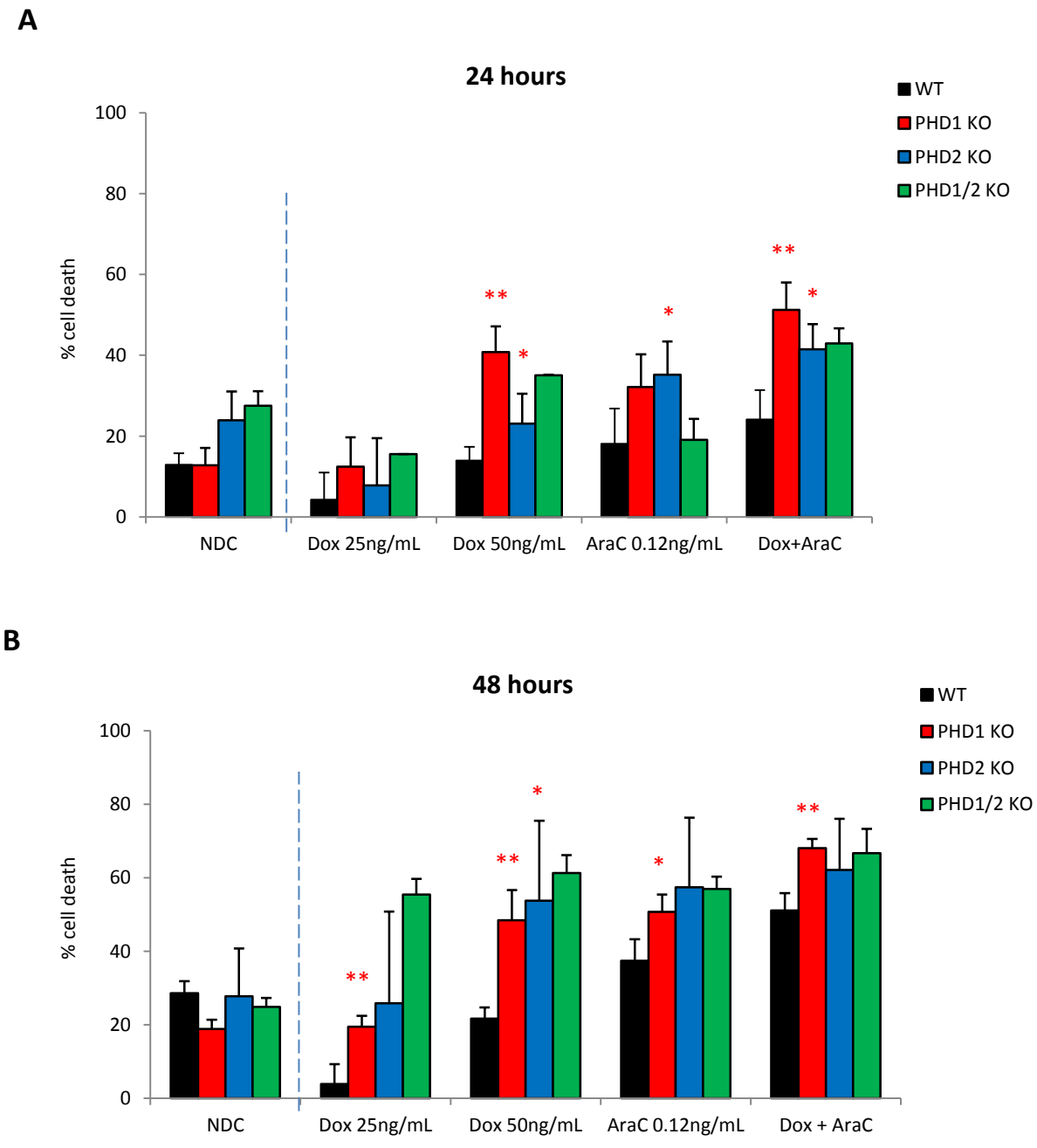


Figure 3.5.3. Cell death of pre-LSC cell lines following treatment with chemotherapy.
Pre-LSC cell lines were treated with chemotherapeutic agents Dox and AraC at concentrations determined in the concentration-effect curves. Cell death in response to drug treatment was normalised against a control group. Cell death was assessed by trypan blue staining which was performed at 24 (A) and 48 hours (B) following treatment. Data shown represent the change in cell death frequency following treatment, relative to the cell death frequency of control cells that did not undergo treatment (NDC). Data represents the mean percentage of cell death induced by treatment which was normalised to percentage of cell death seen in NDC cells. (WT n=2, PHD1 KO n=2, PHD2 KO n=3, PHD1/2 KO n=1). *<p.0.05, **<p.0.005.

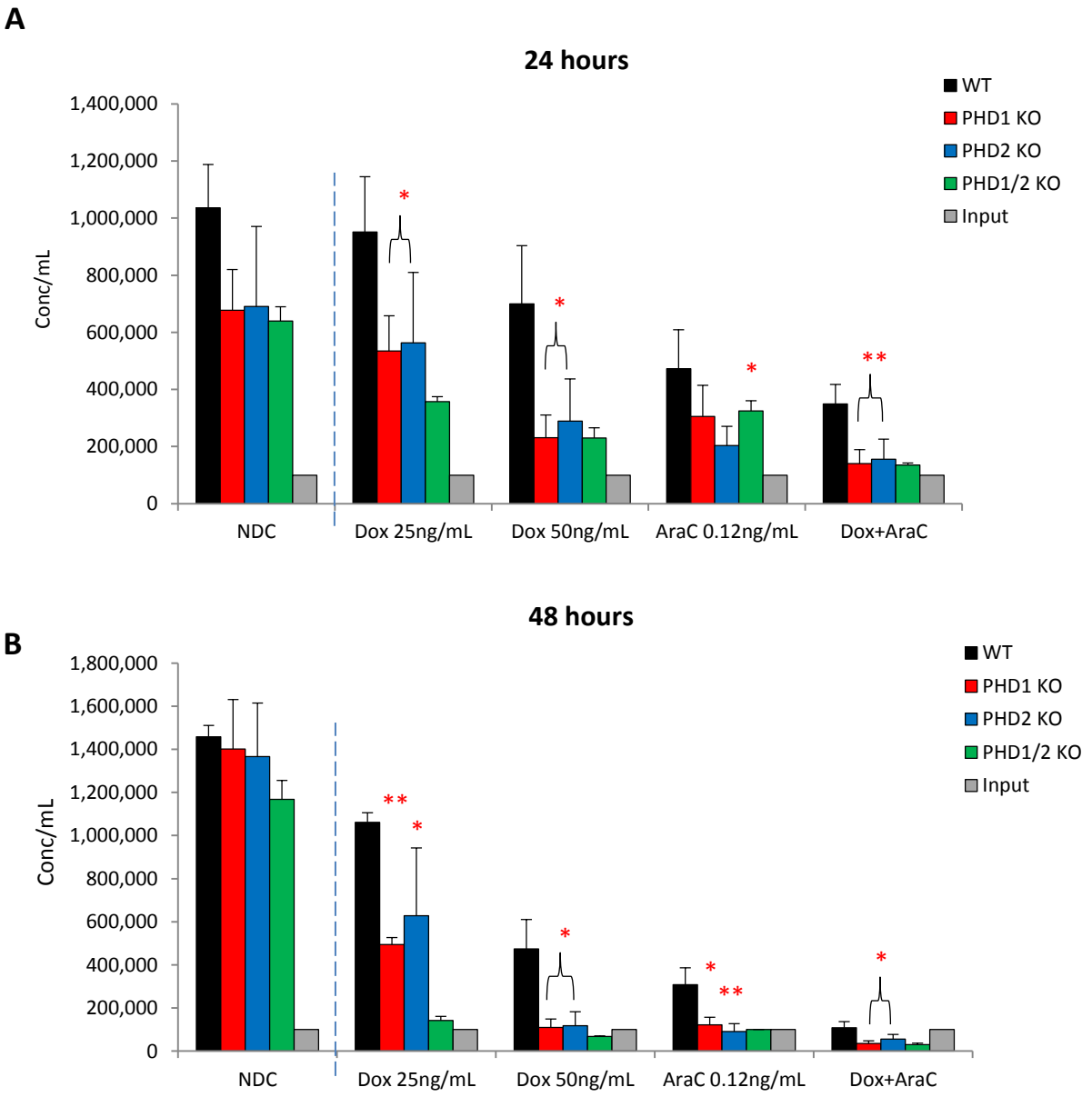


Figure 3.5.4. Cell growth of pre-LSC cell lines following treatment with chemotherapy.

Pre-LSC cell lines were treated with chemotherapeutic agents Dox and AraC at concentrations determined by the concentration-effect curves. Cell concentration was assessed by Trypan blue staining which was performed at 24 (A) and 48 hours (B) following treatment. Data presented represent mean cell concentrations following treatment. Student t-test was performed to determine if PHD KO genotypes have significantly different cell growth compared to WT Pre-LSCs. (WT n=2, PHD1 KO n=2, PHD2 KO n=3, PHD1/2 KO n=1). *<p.0.05, **<p.0.005.

The frequency of cells undergoing apoptosis was also determined by flow cytometry analysis of Annexin V binding. As with the previous experiment, apoptotic frequencies were different in NDC groups so data is presented as the increase in apoptosis following drug treatment normalised to NDC groups. At 24 hours, PHD1 KO, PHD2 KO and PHD1/2 KO pre-LSCs all showed a higher frequency of drug-induced apoptosis compared to WT (Fig. 3.5.5). However, the difference between WT and PHD2 KO cells was not statistically significant. An increase in drug-induced apoptosis was also seen in all PHD KO cells following treatment with 50ng/mL Dox, with statistical significance seen between WT and PHD2 KO. Following AraC treatment, PHD1 KO and PHD2 KO cells showed increased apoptosis, but this was not seen in PHD1/2 KO cells. Following treatment with AraC and Dox combined, PHD1 KO cells showed an increase in apoptosis, but this was not seen in PHD2 KO or PHD1/2 KO cells. Overall, PHD1 KO appeared to have the highest apoptotic response in all drug groups.

At 48 hours, a similar trend was seen. PHD1 KO cells, as well as PHD1/2 KO cells, showed a higher frequency of apoptosis in all treatment groups compared to WT. PHD2 KO cells also showed a slight increase in apoptosis which was statistically significant following treatment with 50ng/mL Dox. However, some variance was seen between PHD2 KO samples causing standard deviation to be considerable and therefore no statistical differences were seen with other groups.

Lastly, viability as determined by the frequency of Annexin V^- cells was also calculated. The frequency of Annexin V^- WT pre-LSCs was seen to be higher across all drug groups at both 24 and 48 hours when compared to cells lacking PHD (Fig. 3.5.6).

Overall these results suggested that pre-LSCs lacking PHDs responded better to chemotherapy than WT pre-LSCs. It would be important to repeat these experiments to ensure that an adequate sample pool is used. However, clarification of these results could hold great therapeutic potential and point to PHD being a promising therapeutic target in treatment of leukaemia.

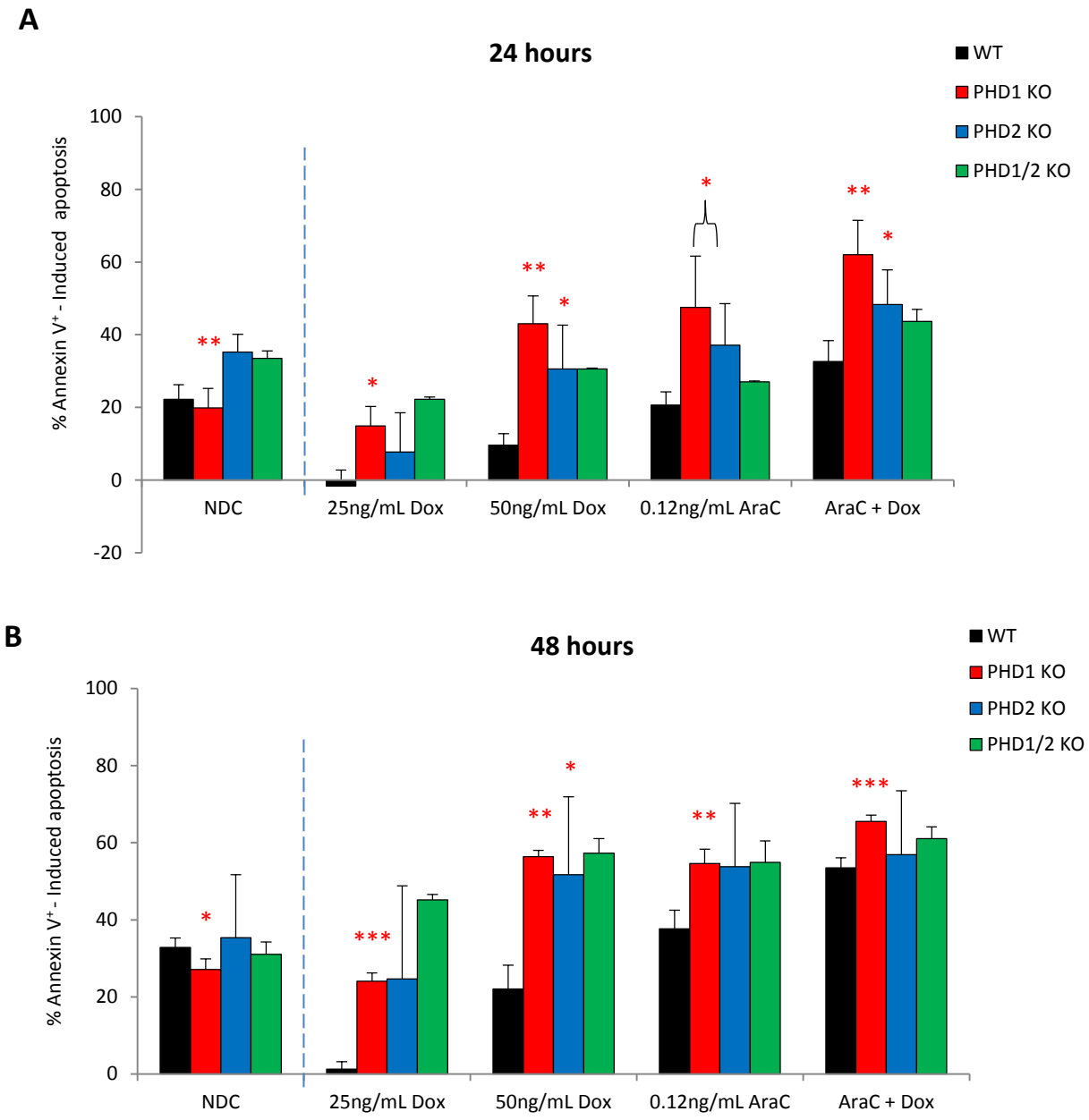


Figure 3.5.5. Apoptotic frequencies of pre-LSC cell lines following treatment with chemotherapy. Pre-LSC cell lines were treated with chemotherapeutic agents Dox and AraC at concentrations determined by the concentration-effect curves. Apoptotic frequencies of pre-LSCs were determined by Annexin V binding and DAPI staining. Annexin V binding was measured at 24 (A) and 48 hours (B) following drug treatment. Data shown represent the change in apoptotic frequencies following treatment, relative to apoptotic frequencies of control cells that did not undergo treatment. Student t-test was performed to determine if PHD KO genotypes have significantly different frequencies compared to WT Pre-LSCs. (WT n=2, PHD1 KO n=2, PHD2 KO n=3, PHD1/2 KO n=1). *p <0.05, **p<0.005, ***p <0.0005.

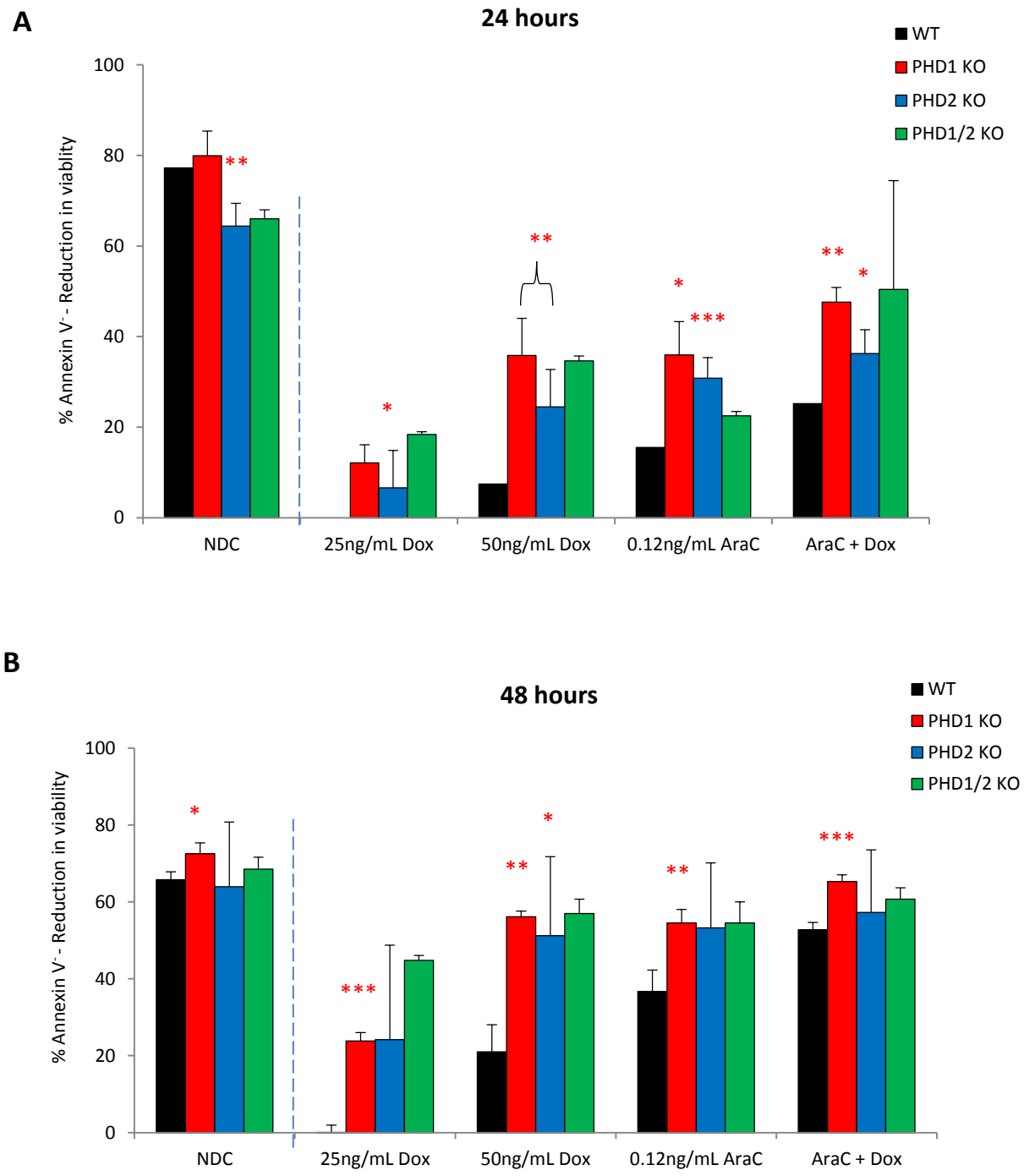


Figure 3.5.6. Cell viability of pre-LSC cell lines following treatment with chemotherapy.
Pre-LSC cell lines were treated with chemotherapeutic agents Dox and AraC at concentrations determined by the concentration-effect curves. Frequencies of viable cells were determined by Annexin V binding and DAPI staining. Annexin V binding was measured at 24 (A) and 48 hours (B) following drug treatment. Data shown represent the change in viability following treatment, relative to the viability of control cells that did not undergo treatment (NDC). Data shown represents mean apoptotic frequencies of all samples. Student t-test was performed to determine if PHD KO genotypes have significantly different frequencies compared to WT Pre-LSCs. (WT n=2, PHD1 KO n=2, PHD2 KO n=3, PHD1/2 KO n=1). *<p.0.05, **<p.0.005.

3.5.3 Response of pre-LSCs to radiation

Radiation therapy is also a commonly used cancer therapy modality. In the haematological setting, radiation is used prior to bone marrow transplantation to ablate host marrow. Therefore, examining the response of pre-LSCs to radiation could give an indication of how cells respond during radiation therapy to treat leukaemia. As with the response to chemotherapy, the sensitivity of these cells to radiation is unknown. WT pre-LSCs were subjected to increasing doses of radiation in order to select the optimal dose at which differences in responses could be observed. Annexin V binding was analysed by flow cytometry to determine the frequency of viable and apoptotic cells. WT pre-LSCs showed increasing frequencies of apoptotic cells that correlated with the dose of radiation (Fig. 3.5.7). Viability of the cells also reduced as the dose of radiation increased. Doses of 4 and 6Gy radiation were selected for additional apoptosis experiments as these doses should allow any positive or negative differences to be seen.

WT, PHD1 KO and PHD2 KO pre-LSCs were then analysed for apoptotic frequencies following dosing with 4 and 6Gy radiation. As with the chemotherapy response experiment, control groups showed varied apoptotic frequencies. Therefore, data is presented as radiation-induced apoptosis and not total apoptosis (Fig. 3.5.8). PHD1 KO and PHD2 KO pre-LSCs both had frequencies of apoptotic cells that were slightly higher than those seen in WT pre-LSCs. In addition, WT pre-LSCs had a slightly higher frequency of viable cells compared to PHD1 KO and PHD2 KO cells. However, these differences were small and did not prove to be significant.

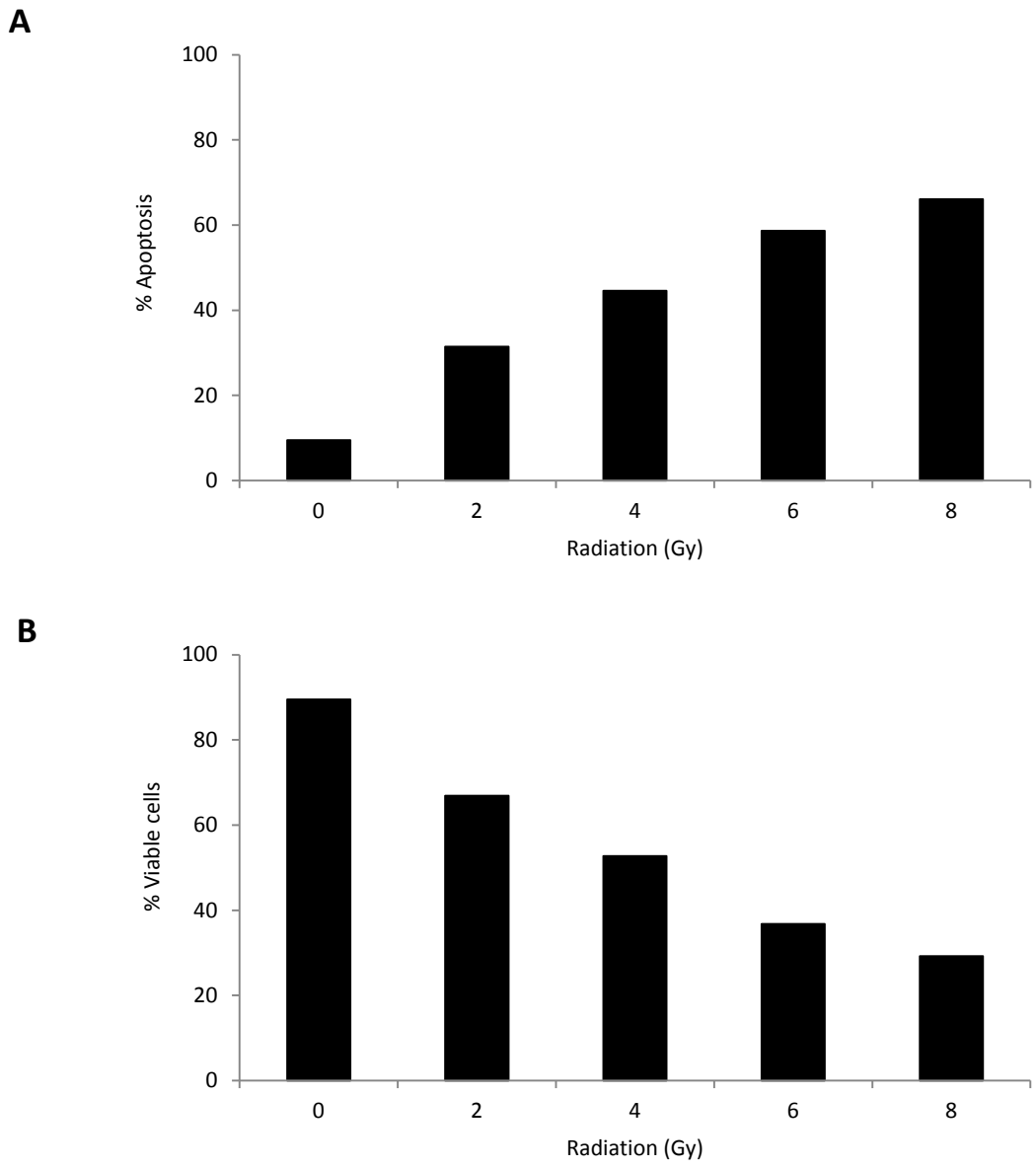


Figure 3.5.7. Dose response curve of WT cells following radiation treatment.

WT pre-LSC cell lines were treated with increasing doses of radiation to determine the most effective dose for use in further experiments. Annexin V binding and DAPI staining were used to assess the frequency of apoptotic cells to determine sensitivity of the cells. A. Total Annexin V^+ cells (both $DAPI^+$ and $DAPI^-$) were used to calculate total apoptosis. B. Total Annexin V^- cells were used to calculate the frequency of viable cells.

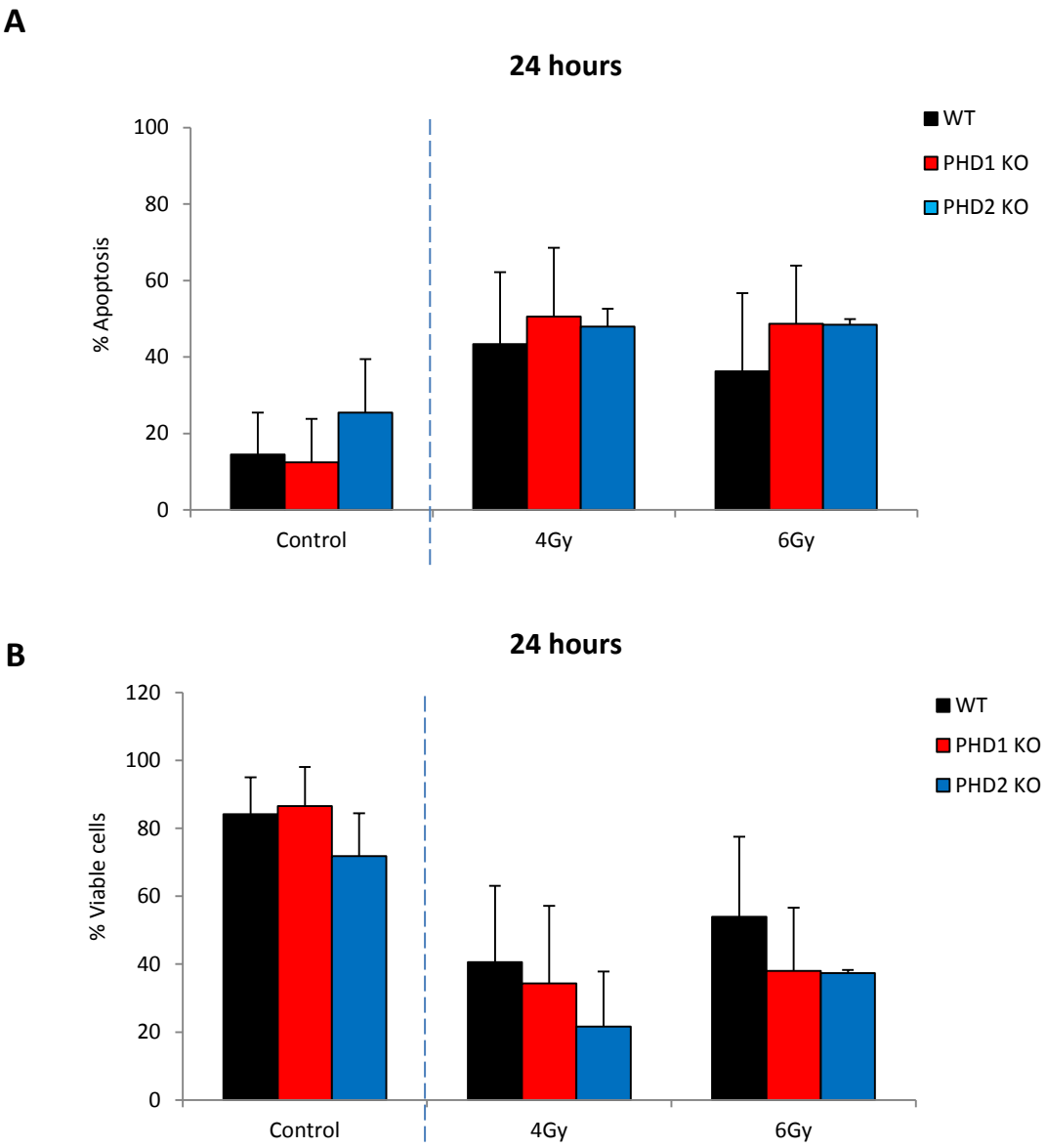


Figure 3.5.8. Apoptotic frequencies of pre-LSC cell lines following radiation treatment.
Cells were subject to radiation treatment. Annexin binding was used to assess the frequency of apoptotic cells and viable cells. A. Data shown represent the change in apoptotic frequencies following treatment relative to apoptotic frequencies of control cells that did not undergo treatment. B. Data shown represent total viability of cells based on Annexin V^- cells. Data shown represent mean frequencies of apoptotic and viable cells. (WT n=3, PHD1 KO n=4, PHD2 KO n=1).

Next, the effect of radiation treatment on mitochondrial function was assessed. Differences in mitochondria can be subtle and so a lesser dose of radiation was chosen for this experiment. As mitochondrial dysfunction is often the cause of apoptosis, mitochondrial frequencies were analysed at an earlier time point than Annexin V binding frequencies, to give a more accurate measure of mitochondrial damage. Frequencies of functional and dysfunctional mitochondria were analysed by flow cytometry as previously described and used to determine the level of damage sustained by radiation. Following dosing with 2Gy radiation, WT pre-LSCs had a significantly higher frequency of functional mitochondria compared to both PHD1 KO and PHD2 KO pre-LSCs, with PHD1 KO pre-LSCs showing the most significant decrease in functional mitochondria (Fig. 3.5.9). Statistical analysis showed that both PHD1 KO and PHD2 KO had significantly lower frequencies of functional mitochondria than WT pre-LSCs. PHD1 KO and PHD2 KO pre-LSCs also had significantly higher frequencies of dysfunctional mitochondria compared to WT pre-LSCs. These data imply that pre-LSCs lacking PHD1 or PHD2, have a greater response to irradiation and suffer more severe mitochondrial damage.

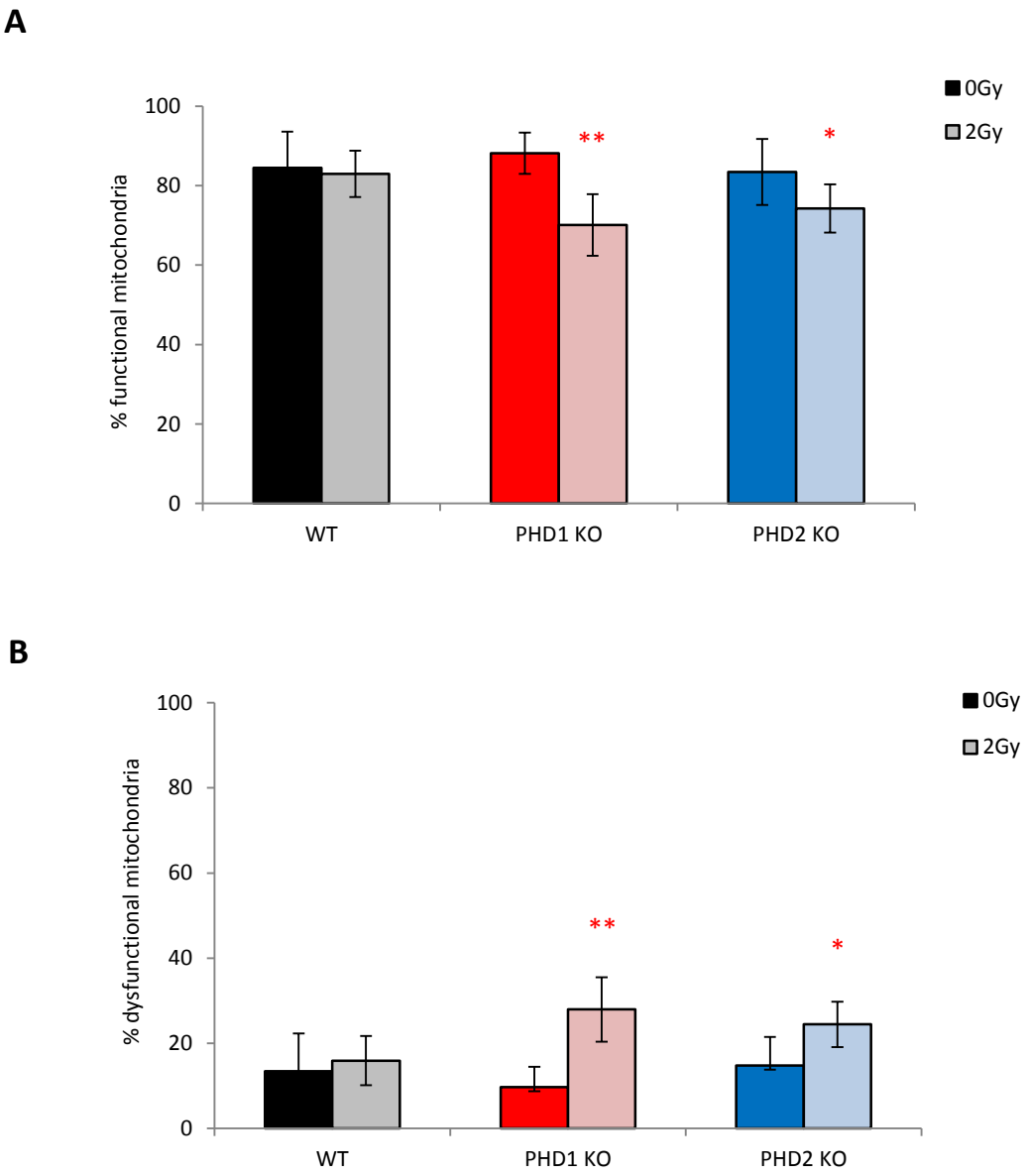


Figure 3.5.9. Mitochondrial function of pre-LSC cell lines following irradiation.

Cells were irradiated and the frequency of functional mitochondria analysed by flow cytometry. A. Graph shows frequency of functional mitochondria. B. Graph shows frequency of dysfunctional mitochondria. Data shown represent mean frequencies of functional and dysfunctional mitochondria. (WT n=4, PHD1 KO n=4, PHD2 KO n=3).

Radiation treatment is known to cause double strand breaks in DNA (Podhorecka et al, 2010). Phosphorylation of H2AX (γH2AX) is commonly used to identify DNA damage within a cell (Podhorecka et al, 2010; Takacova et al, 2012). The frequency of DNA damage can be compared between groups to determine if a particular genotype is more susceptible to DNA damage following radiation. As this assay was used to detect the effect of the treatment rather than the response, it is likely that the high doses of radiation used to measure apoptosis, may be too high to detect any subtle differences. Therefore, cells were subjected to lower doses of radiation (1 and 2Gy) in order to assess DNA damage. As this is an initial response to irradiation, $\gamma H2AX^{+}$ frequencies were analysed 1 hour following dosing. At 1 hour, γH2AX expression was significantly higher in cells lacking PHD1 compared to WT cell. However, frequencies were comparable between WT and PHD2 KO cells (Fig. 3.5.10). This suggests that cells lacking PHD1 suffer more DNA damage or may be more susceptible to damage than WT cells, following radiation.

Following initial DNA damage, cells attempt to repair the damage. Retention of γH2AX at later time points may indicate that cells have failed to repair their damaged DNA and so γH2AX expression can be used to indicate which cells are likely die following the insult. Additionally, a reduction of γH2AX may suggest that the DNA damage has been repaired or that damaged cells have undergone apoptosis. As apoptotic frequencies were analysed after 24 hours, γH2AX frequencies were also determined at this time point. At 24 hours, the expression of γH2AX was reduced in all cells treated with 1 or 2Gy radiation, suggesting that by this time point, DNA damage had been repaired. It is also likely that cells with irreparable damage had undergone apoptosis (Fig. 3.5.10). The expression of γH2AX was similar in WT and PHD2 KO cells, suggesting that the DNA damage response of these cells is comparable. However, PHD1 KO cells had a lower retention of γH2AX following dosing with 1Gy which proved to be statistically significant. The relevance of this is unclear.

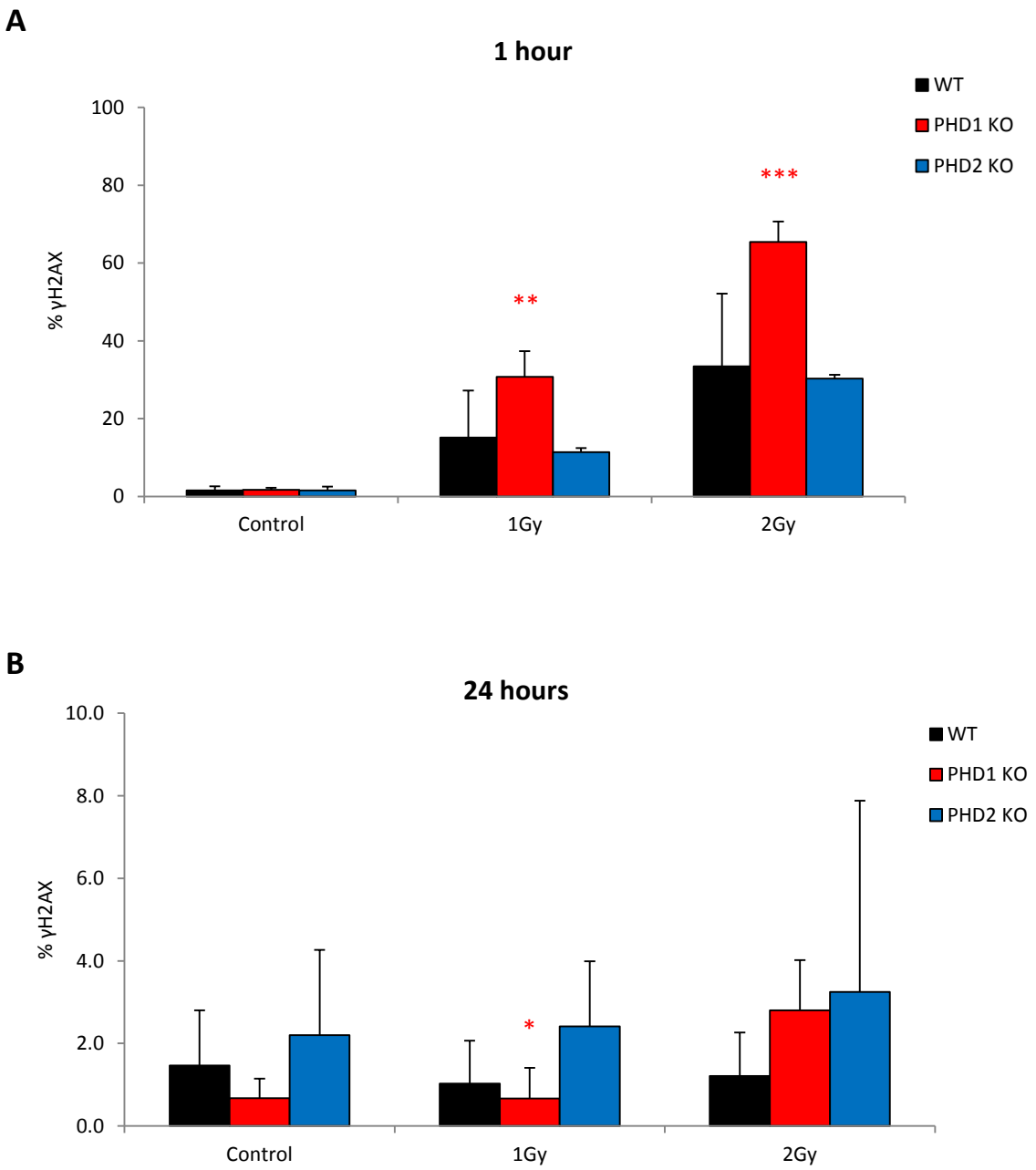


Figure 3.5.10. DNA damage of pre-LSCs following radiation treatment.

Pre-LSC cell lines were subject to low doses of radiation treatment to assess DNA damage and to look for more subtle differences. Frequency of DNA damage was assessed based on the frequency of cells positive for γH2AX expression which was analysed by flow cytometry. A. γH2AX expression was assessed 1 hour following irradiation. B. γH2AX expression was also assessed 24 hours following irradiation. Data shown are mean frequencies of $H2AX^{+}$ cells. Student t-test was performed to determine if PHD KO genotypes have significantly different frequencies compared to WT Pre-LSCs. (WT n=4, PHD1 KO n=4, PHD2 KO n=3) *p<0.05, **p<0.005, ***p<0.0005.

Next the cell cycle frequencies of pre-LSCs were analysed according to Ki67 expression and DAPI incorporation. At 24 hours following irradiation, the frequencies of cells in G0 were increased across all genotypes, suggesting that radiation caused a reduction in cycling cells (Fig. 3.5.11). According to the literature, gamma radiation is also known to cause cells to arrest at the G2 checkpoint, which is thought to convey radiation resistance in some cancer cells (Gogineni et al, 2011; Anastasov et al, 2012). In accordance with this, all cells showed an increase in G2/S/M phases which increased in correlation with the dose of radiation. Cells also showed a reduction in G1, which also correlated with irradiation dose. Ki67 analysis showed that all cells responded to the radiation treatment by arresting in G0 and G2 phases and by a reduction in proliferation. However, no differences were seen between WT and PHD KO pre-LSCs in cell cycle frequencies suggesting that cell cycle response to radiation was unaffected by the absence of PHD genes.

Overall, these data imply that pre-LSCs lacking PHD1 or PHD2 may respond differently to radiation treatment *in vitro*. The frequency of dysfunctional mitochondria significantly increased in pre-LSCs lacking PHD1 and PHD2 suggesting that cells incurred more mitochondrial damage in response to radiation. Annexin V data indicated that although cells lacking PHD isoforms showed marginally higher levels of apoptosis, this difference was not significant. The increased levels of γH2AX seen in PHD1 KO cells suggested that these cells were more sensitive to damage. However, this effect was not seen in PHD2 KO cells and it is possible that although PHD1 and PHD2 appeared to have similar phenotypes in some aspects of this study, they also have unique functions and so may be responding differently.

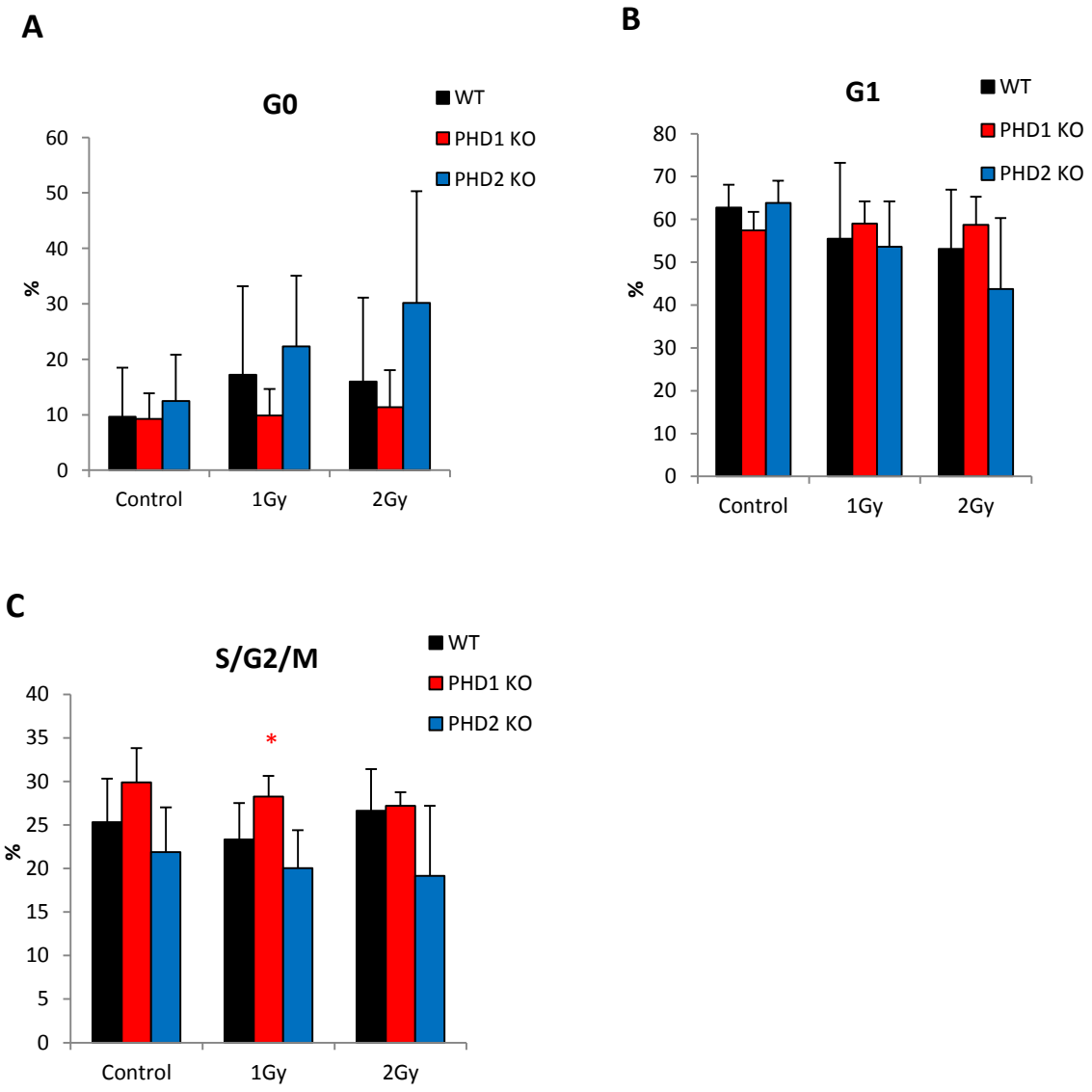


Figure 3.5.11. Cell cycle frequencies of pre-LSCs following radiation treatment.

Cells were irradiated and cell cycle frequencies assessed based on Ki67/DAPI staining which was analysed by flow cytometry. Cell frequencies in G0 (A), G1 (B) and G2/S/M phases (C) for WT, PHD1 KO and PHD2 KO pre-LSC cell lines 24 hours following treatment. Data shown are means frequencies of cells in individual phases of the cell cycle. Student t-test was performed to determine if PHD KO genotypes have significantly different frequencies compared to WT Pre-LSCs. (WT n=4, PHD1 KO n=3, PHD2 KO n=3). *$p<0.05$.

3.5.4 Conclusion

Overall, cells lacking PHD1 or PHD2 appeared to have a higher frequency of cell death in response to common leukaemia treatments, that is, chemotherapy and radiation, compared to WT cells. Taken together with the observation that pre-LSCs lacking PHD had an impaired ability to generate leukaemia *in vivo*, these data indicate that the eradication of PHD isoforms may help to increase the effectiveness of treatments for leukaemia.

4 Discussion

4.1 An Experimental Model to Study Leukaemogenesis

This project aimed to identify a role for PHD isoforms in the transformation and function of AML stem cells, by conducting a study that incorporated both *in vitro* and *in vivo* techniques. This study aimed to optimise an experimental model which would allow the generation of pre-LSCs for *in vitro* study, which were proven capable of forming leukaemia *in vivo*. The results of this study show that the model has multiple benefits and has allowed several questions to be answered concerning the biological properties, leukaemic potential and response to chemotherapy of pre-LSCs. HSPCs transduced with Meis1 and Hoxa9 retroviruses, were seen to successfully generate colonies in methylcellulose cultures. Furthermore, cells which were serially replated continued to produce colonies, displaying enhanced self renewal, beyond the capacity of non-transduced HSPCs, characteristic of transformed cells. The successful transformation of these cells allowed immortalised cell lines to be generated which could be used to explore additional biological functions by performing a variety of biochemical assays. In addition, transplantation of cells generated *in vitro* were capable of forming leukaemia in recipient mice with a short latency, demonstrating that HSPCs were transformed *in vitro* to pre-LSCs, which, in an *in vivo* setting, gave rise to LSCs with leukaemic potential. Although the leukaemic potential of these cells did indicate the successful transformation of cells by Meis1 and Hoxa9, it would be important to provide evidence of Meis1 and Hoxa9 over-expression. Confirmation of the increased expression of these genes through qPCR or the presence of protein products by Western blotting, would help to validate the successful transformation of pre-LSCs. Final optimisation of this assay would allow further work to be conducted to unravel the role of hypoxia signalling in leukaemia.

4.2 The Role of PHD in the Self Renewal and Transformation of HSPCs

The experimental model developed in this study was used to estimate the self renewal capacity of HSPCs transduced with Meis1 and Hoxa9, as well as to investigate the

transformation of cells to generate pre-LSCs. This study analysed the ability of cells to produce colonies following serial replating. HSPCs extracted from mice lacking individual PHD isoforms, as well as several mice lacking various combinations of PHD isoforms, were compared to WT HSPCs to determine if the absence of PHD genes affected the self renewal capacity. No differences were observed in any of the genotypes. Successful transformation of HSPCs to pre-LSCs was based on the ability of cells to produce colonies after several rounds of replating. All cells were able to generate colonies suggesting that PHD ablation did not affect the transformation of HSPCs to pre-LSCs. However, it would be important to ensure that WT and PHD KO cells had equal Meis1 and Hoxa9 expression as differential expression of these ocogenes may have contributed to any differences in the cells' ability to transform. . An additional assay was performed to estimate the frequency of self renewing cells. In line with previous results, no differences in self renewal frequencies were observed. Data presented within this thesis suggests that PHDs do not have a role in maintaining self renewal capacity or in the transformation of leukaemic cells.

4.3 The Role of PHD in the Biological Functions of Pre-LSCs

Several biological properties of pre-LSCs were analysed during this study in order to identify any dysregulated pathways which may indicate any non-hypoxic functions of PHD that are affected in pre-LSCs. PHD has previously been linked to the regulation of several biological processes, including cell cycle progression, apoptosis and metabolism. However, the cell and cancer-specific properties of PHD make it difficult to speculate if PHD has a role in any of these processes in a leukaemic context. Ki67 analysis was used to determine the frequency of cells in individual cell cycle phases, which appeared comparable between all genotypes. However, PHD1 KO and PHD2 KO cells both showed a decrease in cell growth compared to WT cells. It is interesting that this difference was not seen at every time point which may have been related to the density of the cells in culture hence this assay requires further optimisation. These results have indicated that PHD may be involved in the regulation of pre-LSC cell growth, however, more work would need to be done to clarify this. In order to explore this hypothesis further, it would be

important to determine the proliferation rate of the cells. BrdU incorporation is a useful assay that is often used to assess proliferation of cells. This would allow the proliferation of cells to be analysed over time to determine if the difference seen in the cell growth of PHD KO cells is attributable to enhanced proliferation.

Apoptotic pathways are often dysregulated in cancers, with resistance to apoptosis being a common characteristic of cancer cells. Furthermore, PHD isoforms have been associated with the regulation of apoptosis (Lee et al, 2005; Natarajan et al, 2006). Data presented in this thesis revealed that pre-LSCs lacking PHD2 and generated after several rounds of re-plating *in vitro* had a higher frequency of apoptosis. However, it is possible that this was an artefact of *in vitro* culture due to the requirement of environmental cues from the bone marrow, which were not being received by cells in culture and that WT and PHD KO cells responded to this differently. The apoptotic status of PHD KO versus WT HSPCs was also assessed in cells isolated directly from the mice Unlike the phenotype seen in transformed PHD2 KO pre-LSCs compared to WT pre-LSCs, no difference was seen in the apoptotic frequencies of non-transformed PHD KO HSPCs compared to WT counterparts directly isolated from bone marrow However, it may be that this assay requires further optimisation. Following extraction from the bone marrow, cells are washed and RBCs excluded to enrich for the WBC population. It is possible that this process is also selecting healthy cells over dead or dying cells that are lost during purification. This would give a skewed result that would not include apoptotic cells, making it impossible to confirm an accurate reading of apoptotic frequency. Histologic analysis of bone marrow sections may provide a more accurate account of apoptosis *in vivo* and this method is commonly used to measure cell death *in vivo*.

Due to the observation of increased apoptosis in PHD2 KO pre-LSCs, along with the frequent association of PHD with metabolic regulation (Kaelin & Ratcliffe, 2008), mitochondrial function was assessed. No differences were observed in transformed pre-LSCs or HSPCs isolated directly from the bone marrow, suggesting that increased apoptosis was not attributed to mitochondrial dysfunction in the absence of PHD.

4.4 The Regulation of HIF Proteins by PHD Isoforms

PHDs are well known for the role they play in the regulation of HIF. However, the exact roles of each isoform are largely undetermined and it is not known if isoforms are capable of compensating for the loss of another. Considering this, the stabilisation of HIF proteins in WT pre-LSCs and pre-LSCs lacking PHD isoforms was assessed. As PHD is known to facilitate the degradation of HIF, it was expected that the ablation of PHD would affect the regulation of hypoxia signalling and that HIF proteins may be stabilised. HIF-1α and HIF-2α were undetectable in WT, PHD1 KO and PHD2 KO pre-LSCs suggesting that the ablation of single isoforms of PHD is not sufficient to stabilise HIF. Interestingly, HIF-1α and HIF-2α were stabilised in cells lacking both PHD1 and PHD2. These results give some insight into the individual roles of PHDs in the regulation of HIF and suggest that PHD1 and PHD2 may be able to compensate for each other. Compensatory functions between isoforms may result in altered expression levels of functional isoforms. Therefore, it would be important to assess the expression levels of individual PHD isoforms in WT and PHD KO cells to determine if the expression of particular isoforms is upregulated in response to the ablation of another. Western blotting would show the expression levels of PHD isoforms which could be compared between genotypes. Further analysis would also be required to validate the regulation of HIF. Gene expression assays could investigate the expression of HIF target genes to confirm the stabilisation of HIF.

4.5 The Role of PHD in Leukaemic Development

The penetrance of leukaemia in recipient mice following transplantation of pre-LSCs indicated that PHD may play a role in promoting leukaemic development. Pre-LSCs lacking PHD1, PHD2 or both PHD1 and PHD2, all showed a lower penetrance of disease compared to WT pre-LSCs, with PHD2 KO having the lowest frequency of leukaemia. Furthermore, survival rate of the mice was significantly increased by PHD ablation. These data suggest that PHDs play a critical role in leukaemia development but it will be essential to repeat this experiment and to ensure its reproducibility in a larger study. It is also possible that this effect is specific for Meis1/Hoxa9 induced leukaemia. It will be

important to utilise other AML models to identify if a similar effect is seen in other models, such as MML-AF9 or Flt3-ITD.

The response of pre-LSCs to hypoxic stress was assessed to investigate if the phenotype seen *in vivo* was attributed to a difference in cellular response to the hypoxic conditions of the bone marrow. No differences were seen in hypoxic responses suggesting that the phenotype is a difference in leukaemogenicity rather than a response to transplantation.

HIF-1α has been seen to play an essential role in leukaemia. As previously discussed, HIF-1α and HIF-2α were stabilised in cells lacking both PHD1 and PHD2, but were not stabilised in cells lacking single isoforms. However, the expression of HIF *in vivo* has not been assessed. It is possible that although HIFs are not stabilised by single PHD deletion *in vitro*, they are stabilised *in vivo* through the ablation of PHD and that the diminished leukaemic progression seen in PHD KO cells is attributed to HIF stabilisation. It will be important to assess if HIF is expressed in LSCs *in vivo* and if the reduced leukaemic potential seen in PHD KO cells is dependent on HIF stabilisation. However, HIF is quickly degraded in normoxic conditions meaning that an ex vivo approach would not be appropriate. It would be necessary to detect HIF expression through *in vivo* imaging techniques, such as those developed by Nombela-Arietta et al, in which they combined real time imaging of HSCs within the bone marrow with HIF staining to identify HIF-positive HSCs *in vivo* (Nombela-Arietta et al, 2013). It would also be interesting to extend this study to include a model in which both HIF and PHD are inhibited, to investigate if the effect of PHD ablation is abrogated by inhibiting HIF. The availability of HIF KO mice would allow a double KO mouse model to be generated by cross breeding HIF KO and PHD KO mice. This would achieve various KO combinations and would require a substantial study to assess the phenotype of each genotype. However, this would be essential to investigate if PHDs' role in leukaemic development is dependent on HIF. In addition, HIF-1α has been reported to have an essential role in leukaemia so inhibition of HIF-1α, as well as PHD, may result in a more pronounced phenotype.

Analysis of the apoptotic frequencies of leukaemic cells harvested directly from the bone marrow of leukaemic mice, showed no difference in apoptosis between WT and PHD KO cells. However, this analysis was done under *in vitro* conditions and as discussed,

an analysis of apoptosis *in vivo* would be more accurate and the possibility that PHD2 KO leukaemic cells are apoptotic *in vivo* cannot be ruled out. Apoptotic cells are frequently present in other cancers (Gregory & Pound, 2010). Studies have shown that cells undergoing apoptosis can have a large impact on the microenvironment and that they may be involved in the recruitment of tumour-promoting macrophages, which are known to play a role in tumour progression in other cancers through the secretion of pro-tumourigenic factors (Gregory, 2011). The regulation of immune cells by PHD remains largely unexplored. However, a recent publication has suggested that PHD2 plays a role in the immune response to tumours, in which, ablation of PHD2 in lineage cells resulted in reduced tumour growth in a xenograft model, which was attributed to a higher level of necrosis (Mamlouk & Wielockx, 2013). This study, along with the observations of a pro-apoptotic role resulting from the loss ofPHD2 in pre-LSCs, would make it interesting to investigate if the recruitment of tumour-promoting macrophages is affected in leukaemia in the absence of PHD, possibly explaining the reduced leukaemic penetrance seen in PHD2 KO cells.

4.6 The Response to Chemotherapy in Absence of PHD

Results of this study suggest that pre-LSCs lacking PHDs have a greater response to chemotherapy. Pre-LSCs treated with AraC and doxorubicin showed a higher level of apoptosis and a greater reduction in viability in the absence of PHDs. In addition, cell growth was also reduced in PHD KO pre-LSC. These results suggest that PHD inhibition increases the cells response to chemotherapy and that PHD may play a protective role in pre-LSCs. This finding is also in line with studies by Oliveira et al which proposed a protective role for PHD. Oliveira et al showed that ablation of PHD2 increased the response of solid tumours to chemotherapy which resulted in a reduction of tumour mass. In the study by Leitte de Oliveira et al, cells were also assessed for their response to radiation in the absence of PHD. Pre-LSCs lacking PHD showed a higher frequency of mitochondrial dysfunction, suggesting that ablation of PHD may cause cells to incur more cellular damage. It would be interesting to extend this study to assess the response to chemotherapy *in vivo*. The treatment of leukaemic mice with chemotherapy would allow

the investigation into the role of PHD in chemotherapy response, within a pathophysiological model which would more accurately reflect the therapeutic implications of this finding. Chemotherapy response is often assessed using methylcellulose based assays similar to those adapted in this study. Repeating this assay using a methylcellulose based method would validate the results achieved so far. Furthermore, colony production quantification will determine if self renewal of cells is affected. Cell cycle arrest is a common response to chemotherapy and this would also help to further clarify the response to chemotherapy. Ki67 analysis can show the frequencies of cells in individual cell cycle phases, allowing a difference in cell cycle arrest to be determined.

4.7 Gene Expression

Overall these results do suggest that PHD plays a role in the development of leukaemia and the response to chemotherapy. However, this study has not identified the mechanism by which PHD acts. This report has already discussed the importance of investigating the dependence on HIF, however, PHDs are known to interact and regulate many other proteins in a HIF-independent manner, and it would be of equal importance to identify any dysregulation in non-hypoxia pathways that may contribute to the phenotype seen in PHD KO pre-LSC. As PHD2 KO pre-LSC have shown a difference in apoptosis, investigating the expression of apoptotic genes would be interesting. Furthermore, PHD has been linked to the regulation of apoptosis in other studies. The cell specific functions of each PHD isoform makes it difficult to pinpoint which PHD-regulated pathway may be affected so large scale gene expression analysis, such as microarray. would be extremely advantageous to this study. Performing microarray analysis on WT and PHD KO pre-LSC would allow a large scale comparison of the gene expression profiles in pre-LSCs that might indicate a difference in how these cells develop. Furthermore, microarray analysis of LSCs taken from leukaemic mice would identify differences in the gene expression of leukaemic cells in the absence of PHD.

4.8 Therapeutic Implications

The effects seen in cells lacking PHD may be an indication that inhibition of PHD in AML, through treatment with PHD inhibitors, may increase the response to chemotherapy. This may suggest that PHD inhibition may be a novel therapeutic strategy for AML treatment. However, the sensitivity of normal HSCs following PHD inhibition would first need to be assessed in order to ensure a 'therapeutic window' where LSCs would respond efficiently to chemotherapy without normal HSCs being adversely affected. This study has provided some insight into the biological functions and sensitivity of pre-LSCs lacking PHD in *in vitro* conditions, however, it does not explore the properties of normal HSCs or the effect of PHD ablation on their function, generating uncertainty concerning the side effects of this therapeutic strategy. It would be important to extend this study in order to investigate the properties of HSCs lacking PHD isoforms. Serial bone marrow transplantation assays as described by Takubo et al, would be useful to explore the self renewal capacity of HSCs *in vivo* as well as their reconstitution ability. Takubo et al showed that HSCs lacking HIF-1α exhaust during serial transplantation, suggesting that HIF-1α is essential for the maintenance of HSCs. Repeating this assay using HSCs lacking individual isoforms of PHD would indicate if PHD has a role in HSC maintenance. As previously discussed, studies exploring the role of PHD in HSCs have shown contradictory results and it would be important to validate this role before any therapeutic strategies were developed. Unpublished studies by Guitart et al have suggested that PHD2 does not play an essential role in HSC maintenance and that PHD2 ablation did not affect the self renewal capacity or reconstitution ability of HSCs during serial transplantation. If these results can be validated, this would suggest that PHD inhibitors may in fact be a viable therapeutic option as HSCs would remain unaffected by PHD inhibition, while this treatment may cause LSCs to become more sensitive to chemotherapy.

References

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